Development of capillary electrophoresis-based assays for nucleoside and nucleotide metabolizing enzymes

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Contents

Abbreviations

A-134974	(N^7-[(1´R,2´S,3´R,4´S)-2´,3´-dihydroxy-4´-aminocyclopentyl]-amino-5-iodopyrrolopyrimidine)
ABT-702	4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-*d*] pyrimidine)
ACV	acyclovir
ACVMP	acyclovir monophosphate
ADA	adenosine deaminase
ADME	Absorption, distribution, metabolism, excretion
Ado, ADO	adenosine
ADP	adenosine-5'-diphosphate
AK	adenosine kinase
AMP	adenosine-5'-monophosphate
Ap_4A	diadenosinetetraphosphate
ARL67156	N^6-Diethyl-β,γ-dibromomethylene-ATP
AR(s)	adenosine receptor(s)
ATP	adenosine-5'-triphosphate
AU	absorption units
AUC	area under the curve
AZT	azidothymidine
BVDU	(*E*-5-(2-bromovinyl))-2'-deoxyuridine
cAMP	cyclic adenosine-3',5'-monophosphate
CE	capillary electrophoresis
CHO	Chinese hamster ovary
CITP	transient capillary isotachophoresis
CMC	critical micelle concentration
CZE	capillary zone electrophoresis
DAD	diode array detector
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid

dT	thymidine
dTMP	thymidine monophosphate
E-ALP	ecto-alkaline phosphatase
EC	enzyme code
EDTA	ethlylenediamine tetraacetic acid
EMMA	electrophoretically mediated microanalysis
E-NTPDase	ecto-nucleoside 5‘-triphosphate diphosphohydrolase
EOF	electroosmotic flow
FC	fused silica capillary
GCV	ganciclovir
GMP	guanosine-5’-monophosphate
Hepes	N-[2-hydroxyethyl]piperazine-N’-2-ethansulfonic acid
HPLC	high performance liquid chromatography
HSV 1	herpes simplex virus type 1
I.D.	internal diameter
I.S.	internal standard
kD	kilodalton
K_M	Michaelis-Menten constant
LIF	laser-induced fluorescence
LOD	limit of detection
LOQ	limit of quantification
MECC	micellar electrokinetic capillary chromatography
(m, µ, n) M; µm	(milli-, micro-, nano-) molar; micro meter
NC	neutral capillary
NG108-15	neuroblastoma x gliomahybrid cell line
5’-NT	5’-nucleotidase
NTPDase	nucleoside triphosphate diphosphohydrolase
O.D.	outer diameter
p.s.i	pound per square inch; 1 p.s.i. = 0,069 bar = 6,895 kPa
PC	personal computer
PCV	penciclovir

P_i	inorganic phosphate
PPADS	pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
PSB-298	8-[4-(2-hydroxyethylamino)-2-oxoethoxy)phenyl]-1-propylxanthine
RB2	Reactive blue 2
RSD	relative standard deviation (%)
SAR	structure-activity relationship
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TEMED	tetramethylethylenediamine
TK 1	cytosolic thymidine kinase
TK 2	mitochondrial thymidine kinase
TK	thymidine kinase
TMP	thymidine -5'-monophosphate
Tris	tris(hydroxymethyl)aminomethane
UDP	uridine-5'-diphosphate
UMP	uridine-5'-monophosphate
UTP	uridine-5'-triphosphate
UV	ultraviolet
5-IT	5'-iodotubercidin

Summary

Aim of study

The aim of the present work was to develop and optimise capillary electrophoresis-based methods for the assaying of nucleoside and nucleotide metabolizing enzymes.

More specifically the aim included:

- Developing and optimizing a micellar electrokinetic capillary chromatographic method for the separation of nucleotides and nucleosides.
- Developing, optimizing and validating a capillary zone electrophoresis method for the separation of nucleotides and their enzymatic degradation products and application of the method to study nucleotide metabolism.
- Developing and validating an electrophoretically mediated microanalysis with partial-filling technique for the in-line monitoring of adenosine kinase reactions.
- Developing, optimizing and validating at-capillary-inlet enzymatic microreaction methods for the characterization of ecto-nucleoside triphosphate diphosphohydrolases and automation of the analytical process.
- Developing and validating an at-capillary-inlet reaction method to study the extracellular nucleotide metabolism of living cells.

Analysis of nucleotides and nucleosides by micellar electrokinetic capillary chromatography

A method for the analysis of nucleotides (AMP, ADP, ATP and UMP) and the nucleoside adenosine using capillary electrophoresis has been developed. A fused-silica capillary, 100 mM SDS, 20 mM phosphate buffer, pH 7.4, was used, which produced reproducible separations of all nucleotides and the nucleoside within 17 min. Linear relationships between peak areas and sample concentrations, an average minimum detectable concentration in the range of 3 to 6 µM, and an average minimum detectable quantity in the range of 2 to 4 µM was obtained. The developed method allowed the qualitative and quantitative analysis of nucleotides and nucleosides in enzymatic reactions.

Adenosine kinase assay

Adenosine kinase catalyzes the transfer of phosphate from nucleoside triphosphates to adenosine. The development of specific adenosine kinase inhibitors represents a promising strategy for the rational development of clinically useful drugs.

In the present study, a fast and convenient capillary electrophoresis method was developed for the screening of adenosine kinase inhibitors and substrates. Efficient separation conditions were achieved using a 20 mM sodium phosphate buffer (pH 7.5 or pH 8.5) and a constant current of 95 μA. Normal polarity mode was applied for the separations. The samples were injected by pressure and detected by their UV absorbance at 260 nm. The validity of the newly developed method was confirmed by the determination of K_i values of standard antagonists. Dose response curves and calculated K_i values were in excellent agreement with data obtained by the standard radioactive assay.

Based on the off-line method, an in-line CE method was developed by a methodology known as electrophoretically mediated microanalysis (EMMA). A single plug of assay buffer containing adenosine kinase enzyme was injected by hydrodynamic injection into the capillary, followed by a pulg of assay buffer containing substrate with or without inhibitor. These two plugs were sandwiched by two assay buffer plugs. Then the reaction was started by the application of 5 kV separation voltage (negative polarity) for 0.20 min. After the reaction, a constant current of –60 μA with reverse polarity was applied to separate the reaction products.

By in-capillary reaction, the scale of the enzymatic reaction was radically reduced to the nano-scale (nL) as compared to off-line analysis of the reaction carried out in a microcentrifuge tube. Moreover, since the capillary is used as a reaction vessel, all the assay steps (mixing, reaction, separation, and quantitation) were combined in a fully automated nanoscale activity assay. The K_i values for standard antagonists obtained by EMMA were in good agreement with those obtained using the off-line CE assay.

Thymidine kinase assay

Thymidine kinase (TK) is a key enzyme in the salvage pathway of nucleotide metabolism catalyzing the transfer of the terminal phosphate group of ATP to the 5'-hydroxyl group of thymidine and thus yielding thymidine monophosphate. Herpes simplex virus type 1 thymidine kinase exhibits an extensive substrate promiscuity, accepting a variety of different nucleobases and sugar moieties, in contrast to other TKs. This substrate diversity is the crucial molecular basis of selective antiviral therapy. HSV-1 thymidine kinase in not required for the

efficient replication of viruses in dividing cells but may be important in reactivation of virus from the latent state. A strategy for the control of herpetic recurrences would be to interfere with the reactivation process by applying inhibitors of the virus-encoded TK.

A simple and rapid capillary electrophoresis method was developed for the determination of substrates of HSV-1 TK. The method was optimized with two different types of capillaries, fused-silica and a neutral polyacrylamide-coated one. In case of the fused-silica capillary the separations were carried out using 20 mM sodium phosphate as a running buffer and samples were hydrodynamically injected. Due to sample adsorption and low sensitivity with the fused-silica capillary, a method was developed using a polyacrylamide-coated capillary with electrokinetic injection. With this method, good separation was achieved using a short capillary of 20 cm effective length, and 50 mM phosphate as a running buffer, within less than 7 min.

This CE method is also very useful for the investigation of novel potential inhibitors of HSV-1 TK. Determination of K_m and K_{cat} values by the new capillary electrophoresis method has multiple advantages such as no need for expensive radiolabeled substrates and a minimal use of reagents.

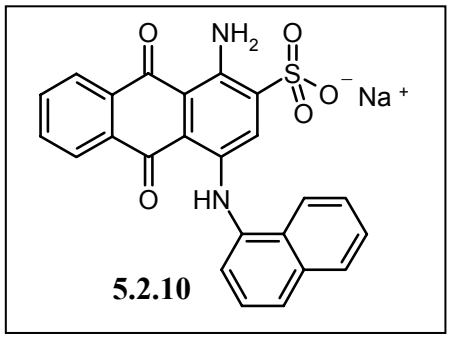
Ectonucleotidase assay

Nucleotides, such as ATP, ADP, UTP and UDP act as physiological agonists at so-called P2 (nucleotide) membrane receptors. The nucleotides are quickly degraded by an arsenal of ecto-enzymes to limit the nucleotides' effects. Inhibitors of nucleotide degradation would act as site- and event-specific indirect P2 receptor agonists by increasing the concentration of the nucleotides.

The ecto-nucleoside triphosphate diphosphohydrolases (EC 3.6.1.5) represent a major family of ecto-nucleotidases. They catalyze the sequential hydrolysis of the γ- and β-phosphate residues of nucleoside tri- and diphosphates, producing the corresponding nucleoside di- and/or monophosphate derivatives.

A CE method for the characterization of recombinant NTPDases and for assaying NTPDase inhibitors has been developed performing the enzymatic reaction within the capillary. After hydrodynamic injection of plugs of substrate solution with or without inhibitor, followed by a plug of enzyme, and subsequent injection of another plug of substrate solution with or without inhibitor, the reaction took place for 5 min at the capillary inlet. The method employing a polyacrylamide-coated capillary and reverse polarity mode provided baseline resolution of substrates and products within a short separation time of less than 7 min. A 50 mM phosphate

buffer (pH 6.5) was used for the separations and the products were detected by their UV absorbance at 210 nm. The Michaelis-Menten constants (K_m) for the recombinant rat NTPDases1-3 obtained with this method were consistent with previously reported data obtained by other methods. The new method was fast and accurate, it required only tiny amounts of material (nanoliter scale), no sample pretreatment and can be fully automated. With this newly developed in-capillary electrophoresis method we tested a series of suramin and reactive blue 2 analogs as potential inhibitors. Compound **5.2.10** was identified as a selective inhibitor of NTPDase3 with a K_i value of 1.5 µM, while being inactive at NTPDase1 and 2. This compound is the first selective NTPDase3 inhibitor described to date and may be a useful pharmacological tool.



5.2.10

It is noteworthy that all of the potent inhibitors identified in the present study share two common structural features: multiple negative charges (sulfonates) and hydrophobic fused rings suggesting that these are key determinants for NTPDase inhibition. Analogs lacking these features were inactive.

Extracellular nucleotide metabolism of living cells

In our previous studies, we established CE assays using membrane preparations as a source of enzymes, or isolated enzymes, respectively to search for inhibitors and investigate substrates. We wanted to push the method even further and test whether enzymatic reactions could be monitored on living cells injected into the capillary.

Thus, we developed a new CE method to study the metabolism of extracellular nucleotides using intact neuroblastoma x glioma NG108-15 cells. During this study, plugs of assay buffer containing intact cells were injected and in between two plugs, a single plug of assay buffer containing nucleotide was injected by hydrodynamic injection into the capillary and the mixture was allowed to react for different time intervals. Then, a constant current of –60 µA with reverse polarity was applied to separate the reaction products. Subsequently, after

electrophoretic separation, the concentrations of nucleoside tri-, di-, and monophosphates were determined.

The method worked well. However, some problems related to the stability of the cells inside the capillary at longer incubation times of 30 minutes or more were observed. Also the rate of degradation of nucleotides was different in different batches of the cells. However, the method was successful to study the metabolism of nucleotides. The method can be further improved for high-throughput screening of inhibitors of nucleotide metabolism in living cells, on which a combination of degrading enzymes are active.

Ester hydrolysis assay

A CE-based micellar electrokinetic chromatography method was developed for the determination of the chemical and enzymatic stability of 5'-deoxy-5'-methylthioadenosine-2',3'-diester a novel natural product isolated from ascidians, which exhibited affinity for A_1 and A_3 adenosine receptors. A constant current (95 μA) was applied, and a sodium tetraborate buffer containing 100 mM SDS, pH 8.5 was used for the separations. The compound was stable by heating at 99 °C for 10 min and in the presence of rat brain cortex after incubation at 37 °C for 90 min, while it showed slow hydrolysis by incubation with carboxylesterase.

Purity determination of an A_{2B} receptor antagonist

A micellar electrokinetic chromatographic method was developed for determining the purity of a newly synthesized antagonist radioligand for A_{2B} adenosine receptors [^{3}H]PSB-298. A fused-silica capillary of 30 cm of effective length and a positive voltage of 10 kV in 150 mM Tris-HCl buffer containing 100 mM SDS, pH 9.1, was used for the separations. The CE method was validated with respect to linearity, limit of quantitation, limit of detection and accuracy.

Conclusions

The present work demonstrated that capillary electrophoresis could be used to study enzyme kinetics, and to search for inhibitors and substrates of the enzymes, specifically of nucleotide- and nucleoside-metabolizing enzymes. It requires short analysis times and only tiny amounts (nanoliter) of samples. Compared with HPLC and spectrophotometric methods, capillary electrophoresis is an alternative and simple method for the determination of nucleosides and nucleotides in complex biological samples. It has been proven that CE indeed could and should further be developed into a routine method for on-line monitoring of enzymatic

processes. Moreover, this nano-scale analysis technique can be used in the areas of inhibitor screening and drug development.

Chapter 1. Capillary electrophoresis

Capillary electrophoresis (CE) was evolved from electrophoresis, a method for separating charged molecules. In CE, a sample is separated into its individual components as it migrates through a capillary (Figure 1.1.). Due to its sensitivity and versatility, CE has moved to the fore-

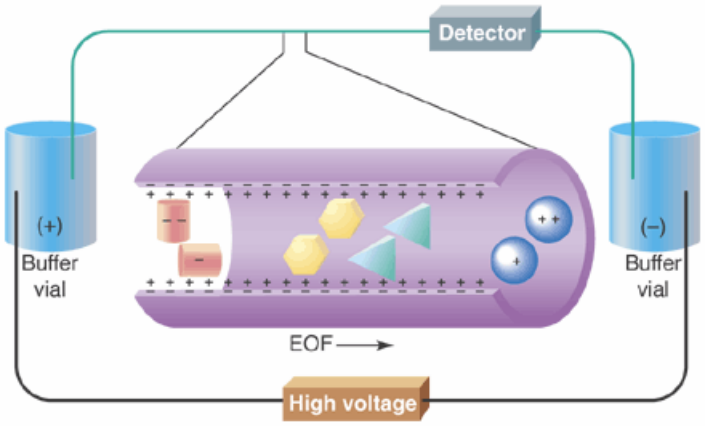


Figure 1.1. *Illustration of the electroosmotic flow in a capillary. An open-ended capillary extends between two buffer reservoirs, across which a high voltage is applied.*

front of analytical methodology. Its applicability is enhanced by the development of facile and rapid methods, including the automation of processes, high resolution, short separation times, and a very small amount of analyte sample requirement [1].

Electrophoresis is based on the migration of analytes within an electrolyte solution under the influence of an electric field. The propelling forces of the electric field and the retarding effects of the viscosity of the solution govern the movement of ions in solution. The mobility of an ion under the influence of an electric field can be summarized in the form:

$$\mu e = q / 6\pi\eta\ rv$$

The values μe, q, r, v and η are electrophoretic mobility (μe), charge (q), radius (r), velocity (v) of ion and viscosity of solution (η), respectively. The electric field is given as a function of the applied voltage and the capillary length (in volts/cm). As seen from the equation the mobility of

an ion is dependent upon its size and charge; analytes with different charge-to-mass ratios move at different velocities under the same conditions[2]. In order to get a complete separation in CE, high separation efficiency is required. There are several parameters (Table 1.1) which are responsible for the separation efficiency, diffusion, adsorption at the capillary wall, the length of the injected plug, and, more important, electrodispersive effects due to the ionic strength within the sample zone and joule heating[3].

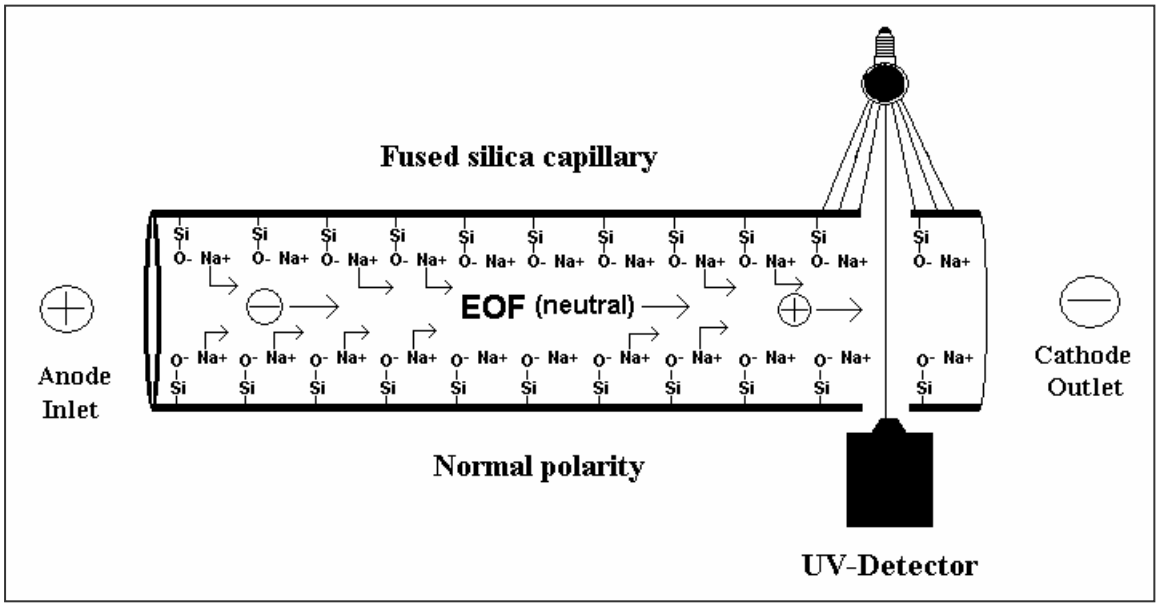
Table 1.1. *Factors affecting separation efficiency (plate numbers, N) in CE and means to reduce their contribution*

Factor	**Means to decrease its contribution**
Diffusion	increasing applied potential, increasing viscosity, decreasing temperature, increasing hydrodynamic size of the analyte
Joule heating	active cooling, lowering the applied potential, increasing area-to-volume ratio of capillary, increasing viscosity of the background electrolyte, reducing the ionic strength of the solution
Electrophoretic dispersion	lowering analyte/electrolyte concentration ratio
Analyte-wall interactions	optimizing the electrolyte pH or ionic strength, dynamic or permanent capillary coating
Injection plug length	decreasing the injection plug length
Detection width	decreasing detection width

1.1. Electroosmotic flow

Electroosmotic flow (EOF) is the most striking feature and a fundamental constituent of CE. Separation is usually performed in 10 to 100 cm long capillaries made from fused silica. The negatively charged silanol groups present in this material give rise to a surface negative charge or zeta (ζ) potential, which causes the flow of electrolyte from the anode (where the sample is applied) to the cathode (where the sample is analyzed). This flow of electrolyte through the capillary is called electroosmotic flow. EOF drives positively charged, neutral, and negatively charged analytes through the capillary to the detector. A second force, electrophoresis, which drives cations toward the cathode and anions toward the anode, enhances EOF for cations and opposes EOF for anions, resulting in their further separation (Figure 1.2.). The magnitude of EOF is highly dependent on the pH of the running buffer; below pH 4, the ionization is small and the

EOF flow rate is not significant, at this pH the capillary wall loses its charge. At pH ~9 the silanol groups on the inner wall of the silica capillary are ionized, giving the wall a negative



Cations	$\mu_{obs\,(+)}$	=	$\mu_{EOF} + \mu_{e(+)}$
Neutral species	μ_{EOF}	=	mobility of neutral species
Anions	$\mu_{obs\,(-)}$	=	$\mu_{EOF} + \mu_{e(-)}$

Figure 1.2. *Effect of EOF on electrophoretic mobility*

charge and resulting in an EOF. Some important parameters, which are used to control the EOF experimentally, are listed in table 1.2. In HPLC the pump systems give a laminar flow, by the slower velocity of the solution at the column walls as compared to the centre. Laminar flow gives broad peaks as the solution travels along the column. Whereas in CE sharp peaks are produced due to the EOF. Due to their chemical nature, the charged molecules may interact with the ionic capillary wall through several different mechanisms such as ionic interactions, electrostatic interactions, van der Waals interactions, and hydrogen bonding. If these interactions are strong, they can adversely affect resolution and EOF. In order to minimize or eliminate surface charge, it is possible to chemically modify or coat the inner surface of capillaries[2,3].

Table 1.2. *Methods to control electroosmotic mobility*

Variable	Result	Comment
Ionic strength or buffer concentration	Reducing the double-layer thickness	• High ionic strength generates high currents and Joule heating • Low ionic strengths may result in sample adsorption • May distort peak shape if conductivity different from sample conductivity • Limits sample stacking if reduced
Organic modifier	Changing the viscosity of the buffer	• Complex changes, effects need to be determined experimentally • May alter selectivity
Surfactant	Adsorbs to capillary wall via hydrophobic and/or ionic interactions	• Anionic surfactants can increase EOF • Cationic can reverse or decrease EOF • Can significantly alter selectivity
Multivalent ions	Change the surface charge density	• Can reverse the EOF direction • Cations can potentially bind to the analyte and change its net charge and electrophoretic mobility
Covalent coating	Chemical bonding to capillary wall	• Many modifications possible • Stability often problematic
Buffer pH	EOF decreased at low pH, increased at high pH	• Best method to change EOF • May change charge or structure of solute
Neutral hydrophilic polymer	Adsorbs to capillary wall via hydrophobic interactions	• Decreases EOF by shielding surface charge and increasing viscosity
Temperature	Changes viscosity, surface charge density and thickness of double layer	• Often useful as temperature is controlled instrumentally

1.2. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) is the most common mode; here the capillary is filled with background electrolyte that consists of an aqueous buffer solution. The separations are based on the relative migration of the negatively charged analytes to the positively charged anode and the attraction of the ions in the buffer for the negatively charged cathode. The overall mobility of the

electroosmotic flow must be greater than that of the analytes, or the analyte cannot be detected. The selectivity of analyte separations by CZE is based on buffer pH and composition. Buffer pH is important as it determines the ionic charge on each analyte species[2]. Transient capillary isotachophoresis (CITP)-capillary zone electrophoresis (CZE) in the presence of electroosmotic flow (EOF) can be used to increase sensitivity. Using CITP-CZE products of the adenosine deaminase reaction, inosine and hypoxanthine, were detected with a limit of detection as low as 28 nM[4]. The proteolytic activity of elastases from different sources was investigated with CZE[5]. The Michaelis constant of the irreversible deamination of adenosine to inosine by adenosine deaminase, using capillary zone electrophoresis employing electrophoretically mediated microanalysis (EMMA), was investigated[6]. CZE was used for the detection and assay of protein kinase and phosphatase activities in complex biological mixtures. By means of the capillary zone electrophoresis methodology, the phosphorylated and dephosphorylated forms of the peptide Kemptide, a 46-amino-acid fragment from protein phosphatase inhibitor-1 and a peptide fragment corresponding to the RII subunit of cAMP-dependent protein kinase (PKA), were resolved. It was found that CZE might prove to be extremely useful for the analysis of peptides that are phosphorylated at multiple sites in vivo[7]. In one review article the theory and methodological developments of sample stacking developed for charged analytes in CZE and also in electrokinetic chromatography have been described[8]. Furthermore, on-line concentration methods for charged species, namely sample self-stacking, acetonitrile stacking, sweeping, cation selective exhaustive injection sweeping, and use of a pH junction, have been discussed[8].

1.3. Micellar electrokinetic capillary chromatography

If separation in CE is not possible by simply varying the buffer pH, or if neutral compounds are to be analyzed, then micellar electrokinetic capillary chromatography (MECC) is a powerful tool for separation. Here high separation efficiency of CE can be combined with a partition chromatography[3]. Partition chromatography in its simplest form consists of a stationary phase and a mobile phase where the separation of molecules is based on different affinities of the analytes for the two phases. When surfactants are added to a buffer in concentrations above their critical micellar concentration (CMC), micelles are formed. Micelles are ordered aggregates of surfactants with structures that hide their hydrophobic tails from water and orient their polar head groups toward the water. The micelles in MECC are the equivalent of the stationary phase in

HPLC. Because micelles are not stationary, they are subject to both EOF and electromotive forces. Separations are based on the partitioning of molecules between the differently migrating micellar and running buffer phases[3]. A number of pharmaceuticals (e.g. acetaminophen (paracetamol), salicylic acid, sulfamethoxazole, theophylline, tolbutamide and trimethoprim) have been determined in human plasma by micellar electrokinetic chromatography (MECC), without sample pre-treatment, using underivatized fused-silica capillaries[9]. Electrophoretically mediated microanalysis (EMMA) combined with micellar electrokinetic capillary chromatography (MECC) was used to perform an on-capillary enzymatic reaction of bovine plasma amine oxidase with benzylamine as substrate[10]. MECC, employing reversed electroosmotic flow by cationic surfactant and reversed-polarity mode was used to study the in situ measurement of the kinetics of ATP metabolic transformation by MgATPase in the fast-twitch gastrocnemius muscle[11]. Micellar electrokinetic chromatography with laser-induced fluorescence (MECC-LIF) detection has been used to determine the phosphorylation activity of the substrate peptide kemptide using cAMP-dependent protein kinase (PKA). Kemptide and phosphorylated kemptide could be reacted with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) as a fluorophor. It was found that the MECC-LIF is very useful for protein kinase assays and can be applied to the testing of inhibitors[12]. The metabolism of extracellular nucleotides (ATP, ADP, AMP, UTP, UDP, and UMP) in NG108-15 cells, a neuroblastoma x glioma hybrid cell line, was studied by means of capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) in the presence of ecto-nucleotidases and ectophosphatases in a previous Ph.D. thesis in our group[13].

1.4. Coating of the fused-silica capillary surface

The capillary wall in CE may be coated to overcome nonreproducible EOF due to the adsorption of analytes to the capillary wall. The EOF can be suppressed or controlled at a certain pH, and analyte-wall interactions can be reduced or eliminated, by coating the active sites on the inner surface of the fused-silica capillary. The active sites comprise inert siloxane bridges, hydrogen bonding sites and ionizable vicinal, geminal and isolated silanol groups[14]. The coating of the capillary wall reduces or eliminates analyte-wall interactions, by changing the electroosmotic flow to effect more rapid separations, improved reproducibility, or resolution of difficult

separation problems. Dynamic and permanent coatings of the fused-silica capillary inner surface have been studied extensively[14-17].

1.4.1. Dynamic coating

In dynamic wall coating, additives are added to the background electrolyte to quench analyte adsorption on the capillary wall, in addition to the rinsing of the capillary with a solution containing a coating agent that is either a polymer or a small molecular-mass compound. There are three main classes of modifiers added to the background electrolyte: (i) amines or oligoamines; (ii) neutral polymers, and (iii) nonionic and zwitterionic surfactants[15]. The main advantages of dynamic coating are the simplicity and the renewable character. The dynamic coating is also easily removed from the capillary wall by rinsing. Some additives are only useful in a specific pH range, due to their pH-dependent dissociation. But on the other hand, covalently bonded capillaries still exhibit longer lifetimes and require less maintenance than dynamically coated capillaries[17].

1.4.2. Permanent coating

A permanent wall coating is an attractive way to eliminate EOF and wall-analyte interaction in the separation capillary. Based on the separation performance, acrylamide-coated capillaries are superior to any other type of coating and cannot be prepared by adsorptive methods. Preparation of a permanent wall coating consists of three steps: capillary pre-treatment, introduction of double bonds to the capillary wall, and binding of a polymer to this intermediate layer[17]. Various types of coating layers can be generated either by silanization of the SiOH as anchor groups for the fixation of polar, neutral or ionic small and larger oligomers or by multipoint adsorption of neutral and charged polymers that can be cross-linked[14]. At present, different permanently coated CE capillaries with reproducible properties have become available, with either hydrophobic (alkylpolysiloxane) or hydrophilic (poly(ethyleneglycol) and poly(vinylalcohol) coatings and also charged coatings that are fixed by covalent bonding or by strong adsorption and cross-linking of polymeric layers[14].

Chapter 2. Development of capillary electrophoresis methods for high-resolution separation and quantitation of nucleosides and nucleotides

2.1. Separation and quantitation of adenosine, AMP, ADP, ATP and UMP by capillary electrophoresis using micellar electrokinetic capillary chromatography

2.1.1. Introduction

Extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP) are important signalling molecules that mediate diverse biological effects via cell-surface receptors termed purine receptors. There are two main families of purine receptors, adenosine or P1 receptors, and P2 receptors, recognizing nucleotides, such as ATP, ADP, UTP, and UDP. P1 receptors have been further subdivided, according to convergent molecular, biochemical, and pharmacological evidence into four subtypes, A_1, A_{2A}, A_{2B}, and A_3, all of which couple to G proteins. Based on differences in molecular structure and signal transduction mechanisms, P2 receptors divide naturally into two families of ligand-gated ion channels and G protein-coupled receptors termed P2X and P2Y receptors, respectively; to date seven mammalian P2X receptors ($P2X_{1-7}$) and eight mammalian P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) have been cloned, characterized, and accepted as valid members of the P2 receptor family. Extracellular purines and pyrimidines have important and diverse effects on many biological processes including smooth muscle contraction, neurotransmission, exocrine and endocrine secretion, the immune response, inflammation, platelet aggregation, pain, and modulation of cardiac function[18].

The pharmacological characterization of P2 purinoceptors is hampered due to the absence of selective and potent antagonists. Morever, ATP, as well as other nucleotide agonists for the P2 receptors, are subject to degradation by ecto-nucleotidases. Breakdown of ATP is the first step towards complete dephosphorylation of ATP yielding adenosine[19]. Consequently, a selective ``ecto-ATPase`` inhibitor would be an important pharmacological tool to study structure-activity relationships of ATP and its analogs at P2 purinoceptors[20]. The effect of ATP in tissues such as rabbit ear artery[21] and guinea-pig vas deferens[22] is enhanced in the presence of an inhibitor of

ecto-ATPase, ecto-ATPase may serve a physiological role of terminating the effect of released ATP and thereby limit stimulation of nucleotide receptors[23].

Enzymes are excellent drug targets as they have blockable catalytic sites and are governed by a number of well understood properties. These properties allow mechanistic enzymologists and medicinal chemists to rationally design and synthesize inhibitors of enzymes. In some cases, an enzyme may be a member of a family of related enzymes, e.g NTPDases (several isoforms are known), each with similar but not identical structure and function. Within enzyme families, a specific isoform or a group of isoforms may be responsible for disease while other family members may be associated with normal cell function. In such cases it is important to design isoform-selective enzyme inhibitors.

Capillary electrophoresis is a useful tool for the analysis of nucleotides. Due to easy quantitation of separated peaks and the possibility of automation, CE continues to play a more and more important role in analytical biochemistry[24-27]. The kinetic study of metabolic processes is of prime importance in biological sciences and biomedicine. The adenine nucleotide adenosine 5'-triphosphate (ATP) is the primary source of cellular energy and essential for the maintenance of cellular energy homeostasis. The measurement and kinetic analysis of the tissue metabolites associated with the cellular energetic state have a special significance in biology[11,28,29]. Several enzymes catalyze reactions involving nucleosides and/or nucleotides as substrates, and the resulting products (also nucleotides) have been characterized by capillary electrophoresis[6,13,30-32]. Nucleotides are easily analyzed by CE methods because they are negatively charged in a pH range from 2-12. Both the structures and the net charges on the molecule play a part in the separation of the compound. Above pH 6, each phosphate group adds an additional negative charge to the nucleotide; thus a nucleoside triphosphate such as adenosine triphosphate, ATP, has a higher charge than its mono- or diphosphorylated forms[2]. MECC was originally developed to separate neutral compounds by CE, it has also been found useful in the separation of charged compounds. The addition of a surfactant, sodium dodecyl sulphate (SDS), to the buffer creates micelles within the buffer, which act as a chromatographic stationary phase. MECC has previously been used in the separation of nucleotides and nucleosides by CE[13].

The aim of this project was to develop a simple and robust method for the separation of nucleosides, nucleosides mono-, di- and triphosphates, which would be used to study enzymatic reactions.

2.1.2. Experimental

2.1.2.1. Materials and methods

2.1.2.1.1. Capillary electrophoresis instrumentation

High-performance capillary electrophoresis was performed on a P/PACE System 5500 (Beckman Coulter Instruments, Fullerton, CA, USA) equipped with a photodiode array (PDA) detection system. The instrument was controlled by P/ACE Station software (Beckman instruments). The runs were performed under the following conditions: T = 25 ^{0}C, λ = 210 nm, V = 10 kV, 20 mM potassium hydrogen phosphate, 100 mM SDS, pH 7.4. The electrophoretic separations were carried out using an eCAP fused silica capillary (75.0 μm diameter (I.D.) x 375 μm outside diameter (O.D.), 37.0 cm length (30 cm to the detector). The capillary was washed with 0.1 N NaOH for 2 min, deionized water for 1 min, and 20 mM potassium hydrogen phosphate, 100 mM SDS, pH 7.4 for 1 min before each injection. Injections were made by applying a slight pressure to the sample solution for 5 s delivering approximately 5 nL and finally the buffer to be used for the subsequent separations.

2.1.2.1.2. Chemicals

Adenosine-5'-triphosphate (A 2383), adenosine-5'-diphosphate (A 2754), adenosine-5'-monophosphate (A 1752), adenosine (A 9251), uridine-5'-monophosphate (U 6375) and sodium dodecyl sulphate (SDS) were from Sigma (Taufkirchen, Germany). Dipotassium hydrogen phosphate for the CE-buffer was obtained from Fluka (Neu-Ulm, Germany).

2.1.2.1.3. Preparation of standard solutions

The nucleotides and the nucleoside were dissolved in deionized water to obtain 10 mM stock solutions and were further diluted to get 1.0 mM stock solutions. The 1.0 mM stock solutions were further diluted for the standard calibration curves. For validating the method of the ATP, ADP, and adenosine determination, 20 μM of AMP was used as an internal standard. For the AMP determination, 20 μM of UMP was used as an internal standard.

2.2. Results and discussion

2.2.1. Separation of nucleotides

High-performance liquid chromatography has been used to quantify nucleotides and nucleosides details can be found in a review article[33], but it has its limitations. The columns are expensive,

and large amounts of mobile phase are needed for the analysis. The sample volume required is relatively large, often between 10-100 µl. Column equilibration in the ion exchange and ion-pairing modes can be time-consuming. While CE analyses are generally faster than comparable HPLC analyses, the solvents used are inexpensive, and smaller quantities of both buffer and sample are required. It is a flexible technique with several separation and detection modes. CE has been used extensively for the determination of nucleotides and nucleosides[2,11,13,24,25,27-30,34-38]. The selectivities achieved in CE separations are mainly determined by the differences in electrophoretic mobilities of solutes, which are mainly influenced by buffer composition and buffer pH. The resolution in CE separations is also significantly affected by electroosmotic flow, which refers to the bulk buffer flow caused by the electrical double layer on the capillary inner surface and the applied voltage[25]. Phosphate buffer has been used as a separation buffer due to its good buffer capacity. It gives better peak efficiency and higher resolution than other buffers for acidic compounds. A separation buffer of pH 7.4 was chosen because the nucleotides were relatively stable at a low pH, but readily hydrolyzed at a high pH value. At this pH value the nucleotides were negatively charged and moved from the cathode to the anode. Alkaline buffers provide a stable EOF and if the capillary is already preconditioned in an alkaline environment, capillary equilibration time after NaOH rinse is short[2]. In a fused silica capillary a strong EOF exists with direction from the anode to the cathode. MECC with SDS was used to control the EOF and enhance the separation of the ribonucleotides along with the separation of neutral nucleoside (adenosine) and charged nucleotides simultaneously. Our goal was to determine the nucleotides and nucleosides after enzymatic reactions in a short time and to improve the detection limits.

2.2.2. Development of a capillary electrophoresis method for the determination of nucleotides

2.2.2.1. Method validation

The method for the determination of nucleotides was validated with respect to specificity, linearity, and limits of quantitation, detection and precision. A known concentration of UMP was used as an internal standard (I.S.) to quantify AMP. AMP was used as an internal standard for the validation of the determination of adenosine, ATP and ADP. The use of internal standard has been generally accepted to be crucial for reproducibility in CE to compensate for injection errors

and minor fluctuations of the migration times. AMP and UMP were selected as I.S. due to their structural similarities with the other nucleotides. In this case charged analytes move towards the cathodic side with the EOF, and separation is achieved on the basis of size and charge of the analytes. Adenosine being neutral in charge was separated from the EOF by the addition of SDS. After that, AMP followed by ADP, and at last ATP came out of the capillary, in the order of increasing charge (AMP < ADP < ATP) and molecular size (AMP < ADP < ATP).

2.2.2.2. Limit of detection and quantitation

For the determination of linearity and the limit of detection and quantitation, calibration curves of standard solutions were obtained from 7 to 8 different concentrations of adenosine, AMP and ATP (2.0, 5.0, 10.0, 15.0, 20.0, 30.0, 40.0 and 50.0 μM) and UMP and ADP (5.0, 10.0, 15.0, 20.0, 30.0, 40.0 and 50.0 μM). The limit of detection (LOD) calculated at a signal-to-noise ratio equal to 3 was found to be between 3.69 and 2.16 μM for the various nucleotides and adenosine (see Table 2.1). Good linearity was found in the range from the compounds' lowest measured concentration within the linear range to the highest concentration at 40 μM or 50 μM. The limit of quantitation (LOQ) calculated at a signal-to-noise ratio equal to 10 was found in the range of 3.8 to 6.1 μM. (see Table 2.1).

Table 2.1. Method validation parameters for nucleotide and nucleoside determination

Compound	**AMP**	**ADP**	**ATP**	**UMP**	**ADO**
Limit of detection (μM) ± SD	3.30 ± 0.60	3.69 ± 0.66	3.70 ± 0.70	2.16 ± 0.38	2.30 ± 0.48
Limit of quantification (μM) ± SD	4.94 ± 0.60	5.88 ± 0.66	6.06 ± 0.70	3.77 ± 0.38	3.50 ± 0.48
% recovery rate ± SD	98.5 ± 0.59	99.5 ± 0.63	99.2 ± 0.68	99.3 ± 0.30	98.7 ± 0.30
RSD (%) of recovery rate	3.85	3.00	3.58	1.40	1.40
R^2 of recovery rate	0.999	0.999	0.999	0.999	0.999
Coefficient of correlation; R^2	0.998	0.999	0.982	0.998	0.999
Mean value of migration time (min) ± SD (n= 6)	7.61 ± 0.07	10.79 ± 0.18	17.14 ± 0.21	8.10 ± 0.03	4.73 ± 0.03
RSD of migration time (%)	0.92	1.67	1.23	0.37	0.63

2.2.2.3. Precision of migration time

The precision of the assay was investigated with respect to repeatability. Each standard was run 6 times. Standard deviation and variation coefficients of migration times were determined. There was less difference in migration times of adenosine, AMP and UMP while relatively more difference was observed in migration times of ATP and ADP. The relative standard deviation of migration times for AMP and UMP and adenosine was low, 0.92, 0.37 and 0.63 %, respectively, while it was only slightly higher for ADP and ATP, 1.67 and 1.23 %, respectively. The results are summarized in Table 2.1.

2.2.2.4. Linearity of calibration curve and recovery

To determine the linearity of calibration curve and recovery rates, standard solutions of 2.0 to 50.0 μM concentrations of nucleotides were run, and linear relationships with regression coefficients (R^2) of 0.999 were found by plotting corrected peak areas against concentrations for all nucleotides. The found concentrations were calculated from the obtained linear regression curves and plotted against the concentrations originally present in the standard solutions to obtain the recovery equations. Recovery rates were found to be high 98.5-99.5% for all nucleotides (Table 2.1).

2.3. Summary

An improved method for the analysis of nucleotides (AMP, ADP, ATP and UMP) and the nucleoside adenosine using micellar capillary electrophoresis was developed. All these analytes were separated in a fused-silica capillary (30 cm to the detector, I.D. 75 μm) and normal polarity mode at constant voltage of 10 kV in a 20 mM phosphate, 100 mM SDS, buffer (pH 7.4), which produced reproducible separations. The peaks of the analytes were detected by their respective UV absorbances at 210 nm. In the presence of phosphate buffer, the migration order of nucleoside and nucleotides based on charge-to-molecular mass ratios is adenosine followed by AMP (monophosphate), followed by ADP (diphosphate) and finally ATP (triphosphate). Linear relationships between peak areas and sample concentrations were found at the calibration range. This method allowed reproducible and reliable qualitative and quantitative analysis of AMP, ADP, ATP and UMP and adenosine. Uhrova et al. performed the separation of eleven nucleotides on a fused silica capillary using 20 mM phosphate-borate buffer (pH 8.0-9.0). The detection limits achieved for the nucleotides ranged between 0.62 and 3.8 μg/mL. The analysis time was about 14-16 min[24]. Huang et al. used a polyacrylamide-coated capillary and a Tris-HCl phosphate-mixed buffer for the separation and quantitation of 14 ribonucleotides in 50 min with 5.4 μM minimum detectable concentration and 0.08 pmol minimum detectable quantity[25]. O'Neill et al. developed a method for the separation and quantitation of nucleotides in cell extracts using Tris-HCl and phosphate buffer, (pH 5-6), and a coated fused silica capillary[37]. They performed the separation of 15 nucleotides within 45 min and minimum detctable concentration of 2-8 μM. In the present study baseline separation was achieved for the nucleoside and nucleotides in less than 18 min with high separation efficiencies. The compounds can be detected with linear response over the concentration range of 2 μM to 50 μM, and detection limits from 2 μM to less than 4 μM. The developed separation method for nucleotides and nucleosides can now be applied to biological studies such as enzymatic assays.

Chapter 3. Capillary electrophoresis assays of adenosine kinase

3.1. Introduction

Adenosine kinase (AK; EC 2.7.120) is the key cytosolic enzyme that catalyzes the phosphorylation of adenosine (ADO) to AMP and is one of two enzymes responsible for ADO metabolism. ADO deaminase (ADA, adenosine aminohydrolase, EC 3.5.4.4) also contributes to ADO conversion, but AK appears to predominate under physiologic conditions[39,40]. AK has been purified from a variety of tissues from different species, including humans[41,42] and the AK gene has been cloned from rat and human tissue[43-46]. ATP is generally considered to be the preferred phosphate source for the reaction catalysed by AK. Magnesium is also required for AK activity and the true AK co-substrate is the $MgATP^{2-}$ complex[45]. AK was found to contain two binding sites, a catalytic site with high affinity for ADO and a low affinity regulatory site that may be the $MgATP^{2-}$ binding site. The predominant ADO-specific transport system operates as a non-concentrative, bi-directional, facilitated diffusion transport[47]. The AK enzyme is a monomer with a molecular weight in the range of 38-56 kDa, whose structure consists of one large α/β domain with nine β-strands, eight α-helices, and one small α/β-domain with five β-strands and two α-helices. The active site is formed along the edge of the β-sheet in the large domain while the small domain acts as a lid to cover the upper face of the active site (Figure 3.1.1). One adenosine molecule is located in a site that matches the ribose site in ribokinase and probably represents the binding site for the nucleoside undergoing phosphorylation. As seen in Figure 3.1.1, a Mg^{2+} ion binding site is observed in a trough between the two adenosine sites. The structure of the active site is consistent with the observed substrate specificity. The active-site model suggests that Asp300 is an important catalytic residue involved in the deprotonation of the 5'-hydroxyl during the phosphate transfer. The second adenosine site probably represents the ATP/ADP binding site[48].

The endogenous purine nucleoside adenosine (ADO) functions as an extracellular signalling molecule by interacting with specific extracellular receptors (P1 receptors). The prototypic endogenous inhibitory neuromodulator adenosine (ADO) is one component of a purinergic cascade (Figure 3.1.2) that results from the metabolic inactivation of ATP. The availability of these purines (ADO, AMP, ADP, and ATP) is under metabolic control in the extracellular milieu and each has distinct, receptor-mediated activities. There is abundant evidence that ADO affects a

variety of physiological processes, all potentially therapeutically useful[47,49,50]. The search for compounds that modulate ADO receptor function as potential therapeutic entities has focused-

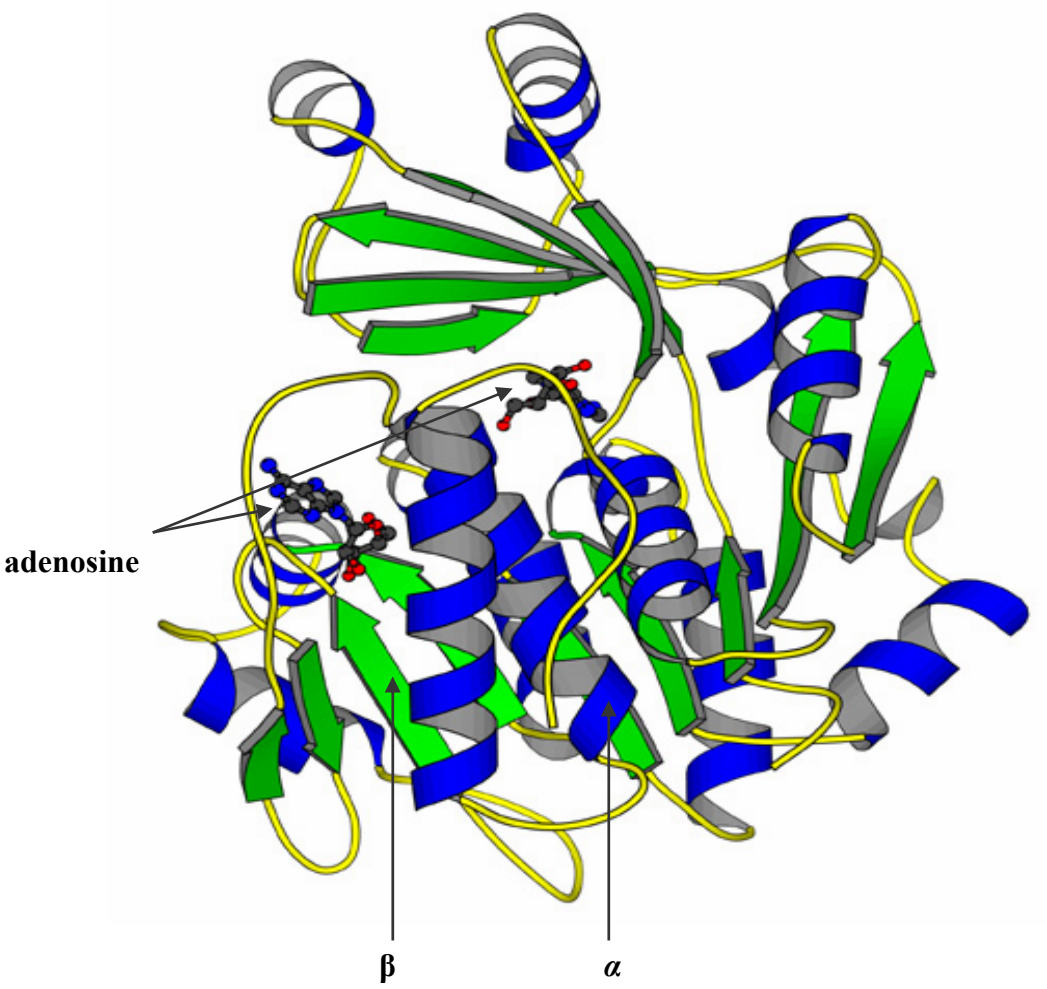


Figure 3.1.1. *Crystal structure of human adenosine kinase bound with two molecules of adenosine.*

largely on direct-acting ADO receptor agonists and antagonists. ADO receptor agonists are effective in animal models, but their therapeutic utility has been limited by side effects, in particular hypotension, renal anti-diuresis and bradycardia, a result of their non-tissue specific interaction with ADO receptor subtypes throughout the body[49]. Adenosine kinase inhibitors are of considerable interest as novel site- and event-specific indirect adenosine receptors agonists and more powerful in situations where more adenosine is released. They exhibit potent antinociceptive, anticonvulsive and antiinflammatory activity without the limiting cardiovascular

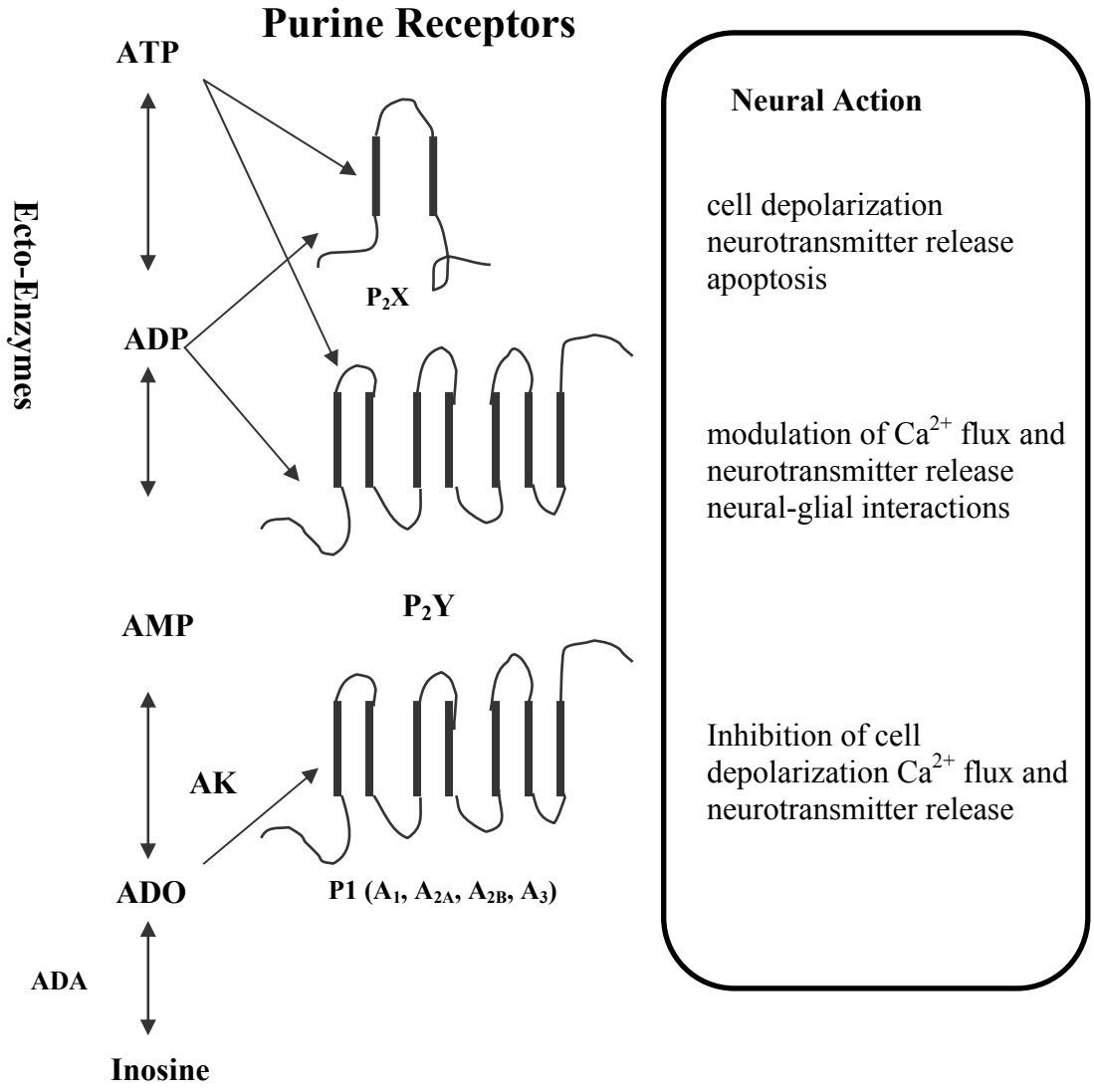


Figure 3.1.2. *The purinergic cascade. ATP, ADP, AMP and, ADO are released from nerves or cells into the extracellular space. These purines are readily metabolized by enzymes that are located both intra- and extracellularly. The phosphorylation of ADO to AMP is mediated primarily by the action of AK.*

side effects exhibited by direct adenosine receptor agonists[51,52]. Furthermore, the adenosine kinase of the protozoa *Toxoplasma gondii*, which is pathogenic in immunocompromised individuals such as patients suffering from AIDS, has been identified as a novel potential chemotherapeutic drug target[53,54]. Because inhibition of AK can significantly increase levels of

endogenous ADO, the development of specific AK inhibitors represents a promising strategy for the rational development of clinically useful purinergic drugs[47].

ADO has a half-life in the order of seconds in physiological fluids, and its beneficial actions are, therefore, restricted to the tissue and cellular site where it is released. Reuptake of ADO into the cell, followed by its intracellular metabolism is responsible for the rapid disappearance of ADO from the extracellular space (Figure 3.1.3.). Since ADO has been shown to effectively limit pathophysiological processes resulting from tissue trauma and injury, the identification of ligands that mimic or potentiate the beneficial actions of ADO has been the subject of substantial drug discovery efforts[50,49,39].

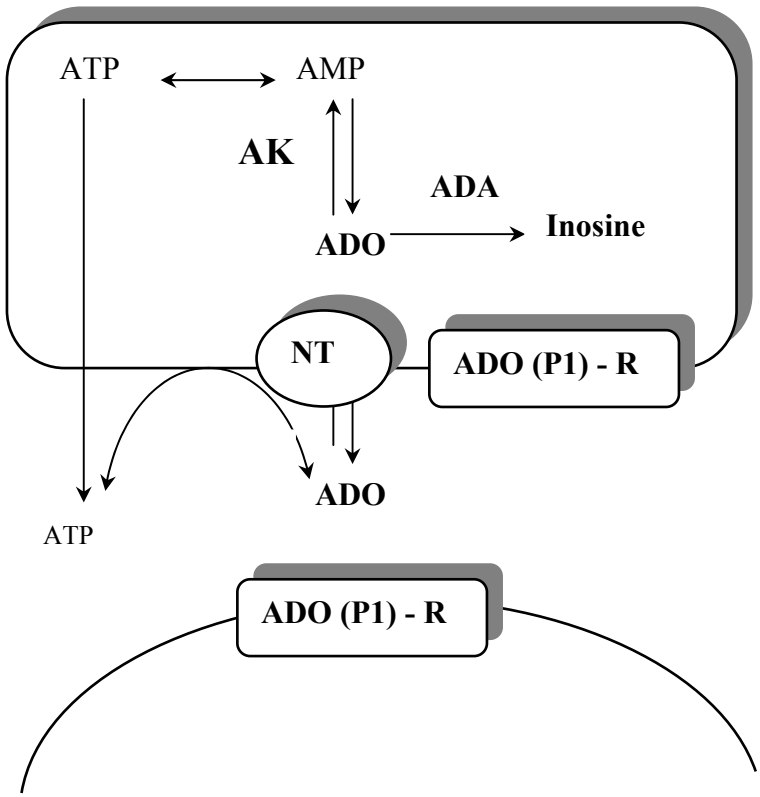


Figure 3.1.3. *ADO is released from cells to function as a local homeostatic modulator of cellular function by activating specific extracellular P1 receptors. Reuptake of adenosine (ADO), and its intracellular metabolism by adenosine kinase (AK), are key factors regulating local extracellular ADO levels at P1 receptors sites. AK inhibitors potentiate the local concentrations and, therefore, the actions of endogenously released extracellular ADO. Adenosine deaminase (ADA), nucleotidase (NT).*

3.1.1. Structures and activities of adenosine kinase inhibitors

AK phosphorylates ADO utilizing ATP as a phosphate donor, which might suggest that AK inhibitors could be designed as ATP mimics. However, due to their anionic nature, with multiple charged atoms, nucleoside triphosphate analogs are likely to have very poor ADME (Absorption, Distribution, Metabolism, and Excretion) properties, and therefore have not received serious consideration. To date, AK inhibitors have been designed to bind to the substrate ADO site, and these inhibitors have been demonstrated to competitively displace ADO. Many of these molecules are small, and have at most a single charge[47]. Novel nucleoside and nonnucleoside inhibitors of AK have recently been reported that show a high degree of selectivity for the AK enzyme and have *in vivo* effects consistent with the augmentation of the actions of endogenous ADO in animal models of pain, inflammation, and seizure activity[55,56].

3.1.2. Nucleoside inhibitors of adenosine kinase

Miller and coworkers have tested 119 different nucleosides for their ability to inhibit the purified AK from rabbit liver[44]. Tubercidin and analogs with a pyrrolo[2,3-*d*]pyrimidine ring structure have binding affinity for AK (Figure 3.1.4). Although 5-Iodotubercidin (**5-IT**) is a competitive inhibitor of the binding of ADO to AK, it is a alternative substrate for the enzyme by virtue of having a hydroxy group at the 5'-position[57,58]. Other closely related nucleosides, 5'-deoxy-5-iodotubercidin (**5'*d*-5IT**) and 5'-Amino-5'-deoxy[58] analogues of 5-bromo- (**A-285601**) and 5-iodotubercidin (**A-134974**) were found to be the most potent AK inhibitors reported to date (IC_{50} < 0.001 μM) with anticonvulsant activity in the rat maximal electric shock (MES) induced seizure assay[57]. 5-Iodotubercidin has the potential to undergo in vivo 5'-O-phosphorylation and therefore produce cytotoxicity; therefore, to eliminate toxicity l-lyxofuranosyl analogues of tubercidin had benn synthesized[59]. The lead compound was **GP790** (IC_{50} 0.47 nM)[59]. Similarly substitution of the tubercidin molecule with aromatic rings at the N^4- and the C^5-positions yield N^4-arylamino-5-arylpyrrolo[2,3-*d*]pyrimidine analogs, a modification which not only retains AK inhibitor potency but also improves in vivo activity[60]. Combining the beneficial effects of having the 5'-amino group and the 5-iodo group yielding 4-amino-5-iodo-7-(5'-deoxy-5'-amino-β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine (**3.1**) and 4-amino-1-(5'-amino-5'-deoxy-1-β-D-ribofuranosyl)-3-bromo-pyrazol[3,4-*d*] pyrimidine (**GP 515**) (Figure 3.1.4) resulted in potent and selective AK inhibitors with *in vivo* activity as antiinflammatory agents[61,62]. The most potent AK inhibitor A-134974 (IC_{50} = 60 pM) was obtained by removing the 5'-methylene group to give the

4'-aminocarbocyclic analog of 5-IT. This compound, A-134974, has shown antihyperalgesic activity in rats[53]. Recently, 6,8-disubstituted purine nucleosides, e.g. **3.4** were found as adenosine kinase inhibitors. Among the series of 6,8-bisarylamine-N,N'-diylpurine nucleosides such as 8-aniline(N-yl-6-indolin-N-yl-9-(β-D-ribofuranosyl)purine (**3.4**) (Figure 3.1.4) was found to be a potent AK inhibitor with an IC_{50} value of 0.019 μM[63].

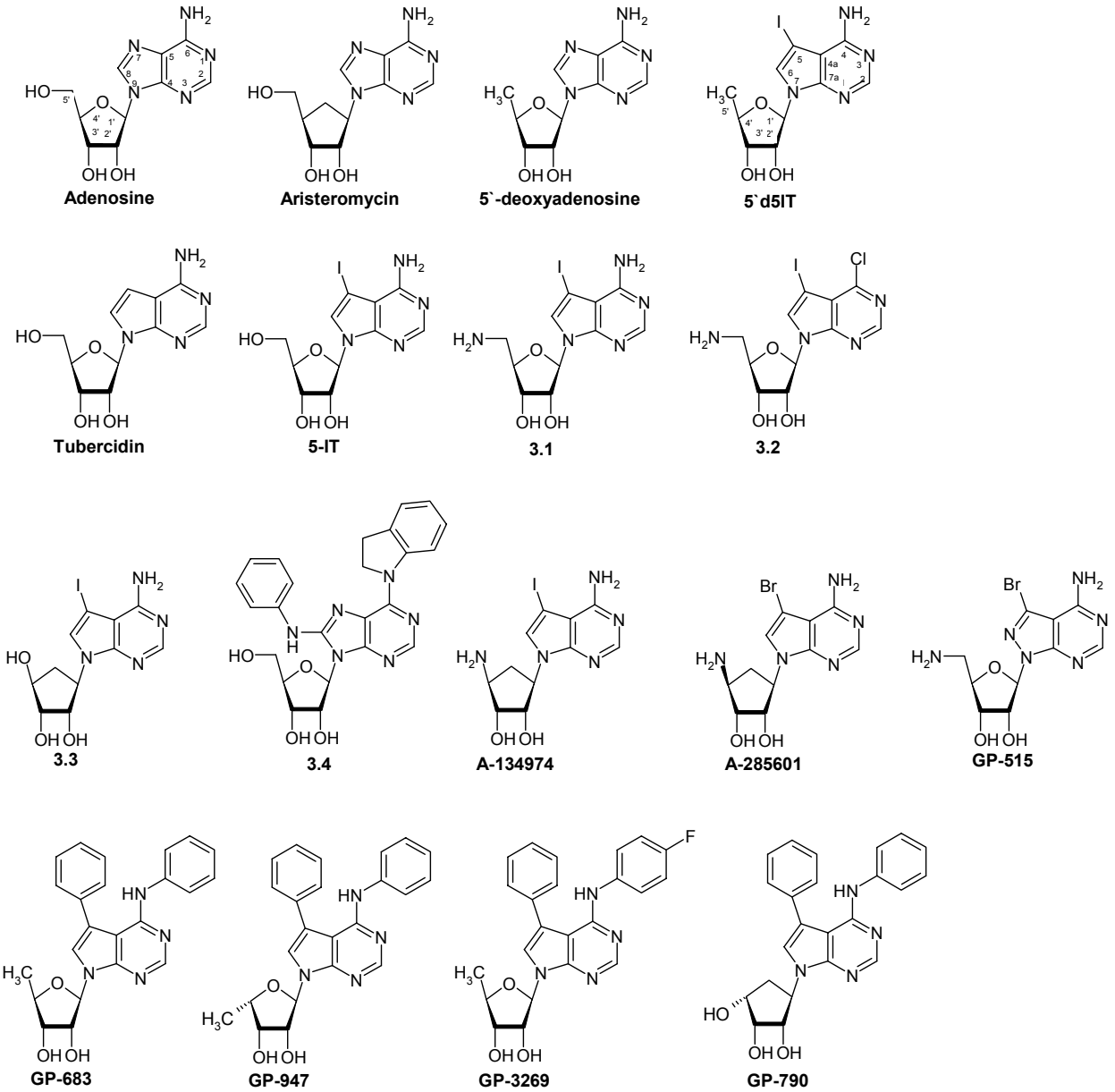


Figure 3.1.4. *Structures of nucleoside adenosine kinase inhibitors*[39,47,50]

3.1.3. Non-nucleoside inhibitors of adenosine kinase

There are a number of non-nucleoside AK inhibitors that have been disclosed, many of them obtained through modification of compound **3.5** (see Figure 3.1.5) that had been discovered by high throughput screening[64]. The more recently generated AK inhibitors were also found through a high throughput screening approach and are generally more potent than the natural product 5-bromopyrrolopyrrolidine, the first compound recognized in this class. By optimizing this series, the pyridopyrimidine ABT-702 was produced exhibiting exceptional potency (ED_{50} = 0.7 μmol/kg) in the mouse against thermal hyperalgesia[55,64]. The 5-position of the pyridopyrimidine ring in ABT-702 has also been modified, and in the case of the 5-benzyl-substituted compound **3.7**, the *in vitro* potency was increased 12-fold over that of ABT-702, but without an increase in the *in vivo* potency. Solubility was not improved with most of the 5- modified analogs, with the exception of the highly soluble **3.9**, which reduced hyperalgesia but was inactive after oral administration[65]. A different type of modification, the incorporation of a nitrogen atom at the 5-position of the pyridopyrimidine ring in **3.12** increased water solubility while retaining the *in vitro* potency as well as *in vivo* activity in a rat inflammatory hyperalgesia model[66]. Novel non-nucleoside AK inhibitors based on 4-amino-6-alkynylpyrimidines, 4-Amino-5-[(2-chlorobenzyl)methylamino]-6-[6-(4-morpholinyl)-3-pyridinylethynyl]pyrimidine **3.13** (IC_{50} = 15 nM), 4-Amino-5-(benzylmethylaminomethyl)-6-[6-(4-morpholinyl)-3- pyridinylethynyl]pyrimidine **3.14** (IC_{50} = 2 nM) as well as similar analogs in the series were prepared, for which high potency was demonstrated *in vitro*, and they effectively reduced pain and inflammation in animal models[40]. The more recently described 2-pyridyloxazolo-pyrimidine derivative **3.15** was synthesized by high throughput derivatization of the oxazolo-pyrimidine resulting in a ≤ 10 nanomolar IC_{50} value[67]. Recently by the modification of existing pyridopyrimidines pyrazolopyrimidine **3.16** was produced with an IC_{50} value of 7.5 nM[68].

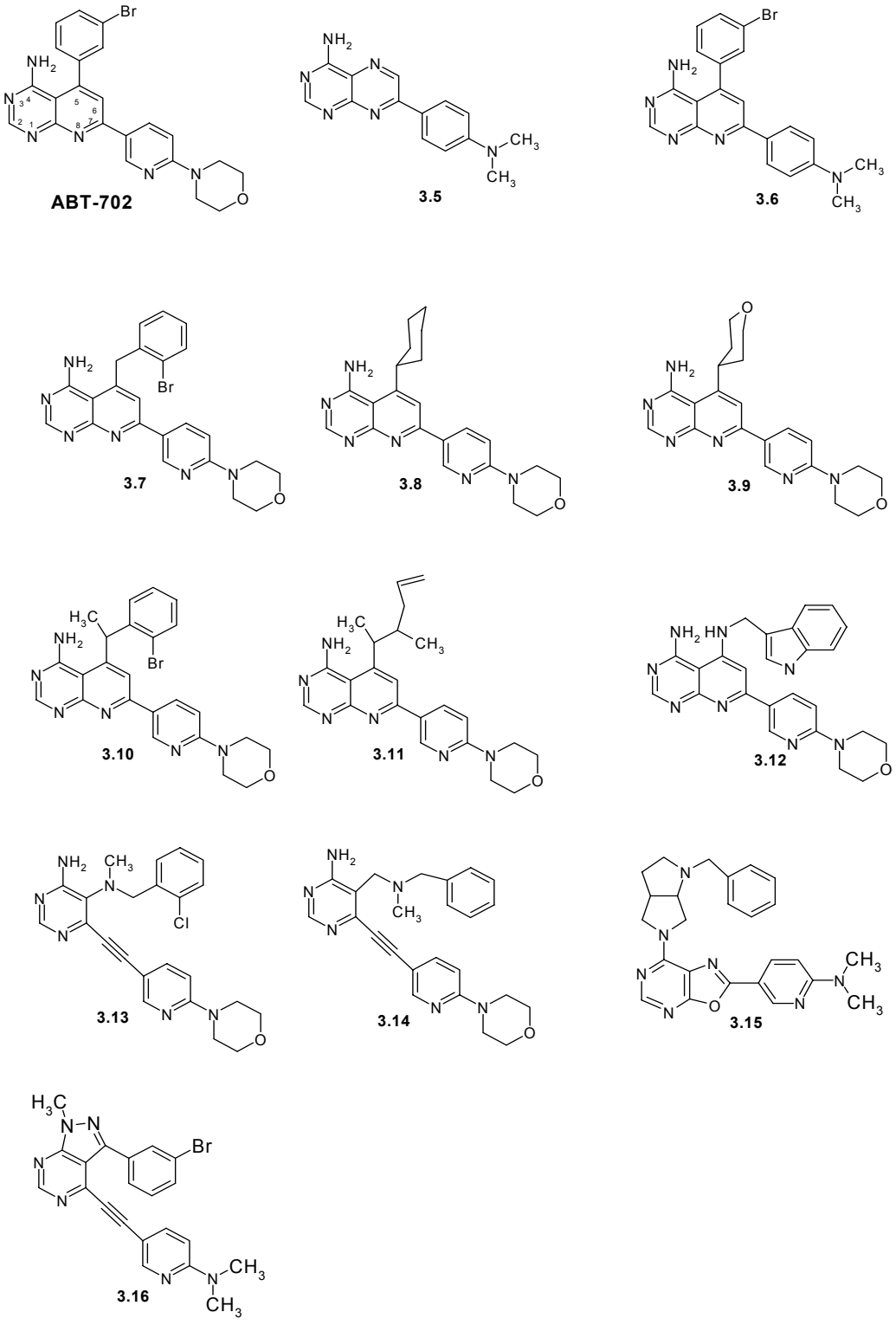


Figure 3.1.5. *Structures of non-nucleoside adenosine kinase inhibitors*

Structure-activity relationships of nucleoside inhibitors of adenosine kinase

Structure-activity relationships have been established for both substrates and inhibitors of AK, the majority of which have nucleoside-like structures[50] (Figure 3.1.4). The initial reports of the structure-activity relationships for nucleoside-like compounds as inhibitors and substrates of AK were reported by Miller et al[44]. For nucleoside-like compounds trans-hydroxy groups with respect to the base portion are necessary for substrate or inhibitor activity[42]. Substitutions, deletion, replacement or inversion of the hydroxyl groups at the 2'- or 3'- positions eliminates ligand recognition by the enzyme[39,47,50]. Replacing the ribofuranosyl ring oxygen with a methylene group (aristeromycin) results in AK inhibitory actvity[39]. Removal or substitution of the 5'-hydroxyl phosphorylation site would be expected to eliminate substrate activity. But substitution of the 5'-hydroxyl group gives potent activity as inhibitors, such as in 5'-deoxyadenosine. A particularly interesting observation is that additional substitution of an amino group at the 5'-position enhances potency still further as shown by 5'-amino-5'-deoxyadenosine (IC_{50} = 9 nM). Inversion of the stereochemical configuration of the 5'-hydroxymethylene group results in a potent AK inhibitor[39]. The 1-aza and the 3-aza nitrogen atoms in the adenine ring are necessary, and their replacement leads to a loss of activity[49]. Replacement of the N_7 nitrogen of the adenine ring with carbon leads to tubercidin, which shows reduced activity compared with the parent adenosine. However, very potent pyrrolopyrimidine analogs can be created by appending lipophilic aryl or halogen moieties on the C5 of the heterocyclic ring of tubercidin (e.g. 5'-deoxy-5-iodotubercidin, IC_{50} = 1 nM). A related synthetic analog (**5-IT**, 17 nM) (Figure 3.1.4) was prepared[69]. Substitution at C_6 (e.g. methoxy, mercapto) retains activity while substitution at C_2 eliminates actvity[47]. Potency can further be enhanced by substitution of lipophilic moieties at the 4- and 5- position of tubercidin by combining this modification with the aforementioned favorable 5'-amino group to give a family of AK inhibitors, most prominently **GP-515** and **GP-683**, along with the related α-lyxofuranosyl analog **GP-947** (see Figure 3.1.4)[39]. One of the most potent *in vitro* inhibitor is compound **3.2**, illustrating the beneficial effect of combining the potency-enhancing 5'-amino moiety and the iodotubercidin structure. Henderson and coworkers[70] found many of the purine nucleoside analogs as inhibitors of AK, but all the pyrimidine nucleosides tested were inactive. Among the inhibitors of AK, 5-IT (Figure 3.1.4) showed activity in the micromolar range[70]. Replacement of the ribose ring with a cyclopentane carbocyclic ring gives more potent inhibitors[71]. Replacement of the adenine ring of noraristeromycin with a 5-iodopyrrolidine gave the even more potent compound **3.3** (IC_{50} = 13

nM) and combining the 4'-amino-truncated carbocyclic moiety with a heterocyclic ring gave very high potency (**A-134974**, IC_{50} = 0.06 nM) (Figure 3.1.4)[39].

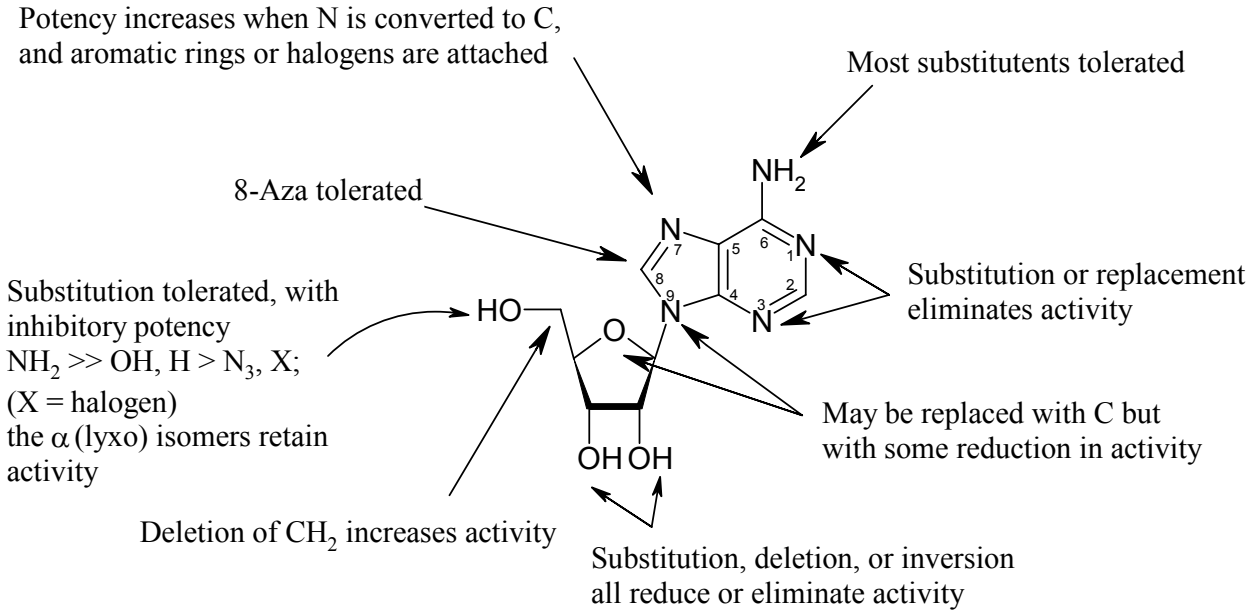


Figure 3.1.6. *General structure-activity relationships for nucleoside-analogs as AK inhibitors.*

A novel family of non-nucleoside inhibitors of AK has been recently reported[71] (Figure 3.1.5)[66,72,73] that show a high degree of selectivity and have in vivo effects consistent with the augmentation of the actions of endogenous ADO in animal models of pain, inflammation and seizure activity[55,56]. Most of the compounds were obtained through modification of a compound (**3.5**) which was discovered by high throughput screening[64]. A number of analogs were examined, and it was found that replacement of the 5-aza atom with a carbon, when coupled with lipophilic substitution at the 5-position, gave compounds potent *in vitro*, e.g. compound **3.6** (IC_{50} = 5 nM). To improve the water solubility of 7-dimethylaminophenyl-substituted compounds, the 7-phenyl moiety of compound **3.6** was substituted with a 7-morpholine (or replaced by a 2-pyridyl) moiety as seen in ABT-702 which increased water solubility as well as antinociceptive actions *in vivo*[55,56]. An examination of the SAR of many analogs showed that compounds bearing a wide variety of lipophilic C_5-substituents displayed potent in vitro inhibitory activity; compounds **3.7** and **3.10** have subnanomolar potency to inhibit AK. Compounds ABT-702 and **3.8** exhibited in a carageenan-induced thermal hyperalgesia assay ED_{50} values of 5-10 μmol/kg in rate[47,56].

3.2. Development of off-line and in-line capillary electrophoresis methods for the screening and characterization of adenosine kinase inhibitors and substrates

3.2.1. Project

Adenosine kinase (AK; EC 2.7.120) catalyzes the phosphorylation of adenosine to AMP[74] (see Figure 3.2.1).

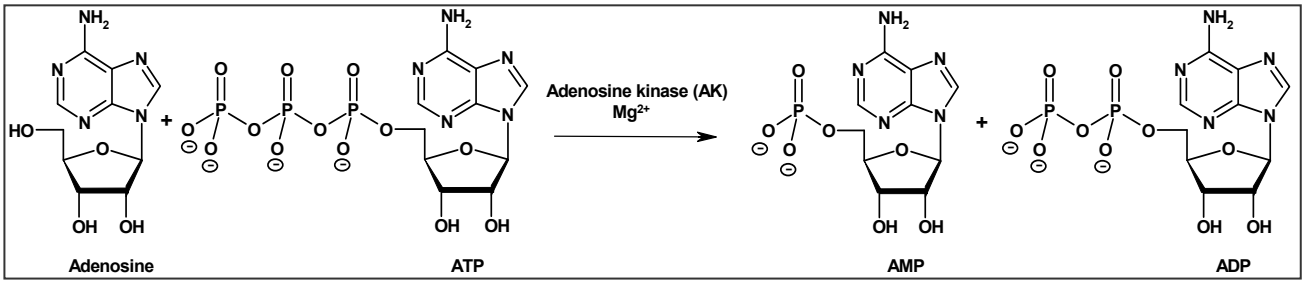


Figure 3.2.1. *Reaction catalyzed by adenosine kinase*

Several methods including radioisotopic[69], thin-layer chromatographic (TLC)[75] and HPLC-UV methods[44,46], have been devised to monitor the adenosine kinase reaction and associated kinetics, but all of the currently known methods have serious drawbacks. The radioisotopic method requires the use of radioactive materials, which is neither economic nor environmentally friendly. It is limited to radiolabeled substrates (e.g. [^{3}H]adenosine or [^{14}C]adenosine); alternative substrates that are not available in radiolabeled form cannot be investigated. Therefore, the radioactive method is useful for testing inhibitors, but less suitable for investigating substrates. On the other hand, TLC is not suitable for the determination of minute enzymatic activity in complex biological medium, because it shows low resolution, is time-consuming and not sensitive enough. HPLC is also used for enzyme assays; however, it suffers from the requirement of large quantities of solvents, relatively high prices for columns, and frequently long retention times.

Capillary electrophoresis (CE) has recently emerged as a powerful tool for enzyme analysis[5,76,77], including the determination of Michaelis-Menten constants[78-81], and for enzyme inhibition assays[79-83] exhibiting a number of advantages over conventional methods. These include rapid separation, the ability to simultaneously discriminate between and identify multiple components

of similar structure with high resolution[1], ultra-low sample volume requirements, and high throughput by automation. CE is particularly useful for investigating enzymatic reactions involving charged substrates or products, e.g. for the monitoring of phosphorylation or dephosphorylation reactions. Phosphorylated and nonphosphorylated products and substrates have significantly different electrophoretic mobility, and therefore can readily be separated and quantified[84].

Electrophoretically mediated microanalysis (EMMA), first described by Bao and Regnier[85] has been successfully used for in-line enzyme assays[86]. In this technique the capillary is used as a microbioreactor as well as for the separation of substrate and products. There are two major types of EMMA methods. In the first continuous format[85], the capillary is filled with an appropriate substrate solution and upon injection of a zone of enzyme; the product will continuously form during the electrophoretic mixing of enzyme and substrate. One drawback of this method is that the separation buffer has to allow the enzymatic reaction to proceed.

In the second technique[87], substrate and enzyme are introduced into the capillary as distinct plugs. Upon application of an electric potential, these zones mix with each other due to differences in their electrophoretic mobilities. The reaction proceeds during the mixing process. The resultant product is transported to the detector separately from enzyme and educts under the influence of an applied voltage, where they are individually detected. Recently, Van Dyck et al.[10] introduced the combination of the EMMA methodology with a partial filling technique. Using this method part of the capillary is filled with the buffer optimized for the enzymatic reaction whereas the rest of the capillary is filled with the background electrolyte optimal for the separation of substrate and product. EMMA with partially filled capillary has been used for the inhibition studies of haloalkane dehalogenase[86] and for the determination of Michaelis-Menten constants of bovine plasma amine oxidase and haloalkane dehalogenase[10,87].

CE enzymatic assays can be categorized into three formats depending on the step when the enzymatic reaction takes place: pre-column, on-column and post-column. In the first pre-column CE-enzymatic assay format, the substrate is mixed with enzyme and the mixture is incubated for a specified amount of time. The products of the enzymatic reaction are then injected into the CE column, electrophoretically separated, and quantified. In the second type of CE-enzymatic assays all the steps of the assay take place within the capillary (including mixing, separation and detection). EMMA consists of three steps; during the first (pre-reaction step) the zones of the enzyme and the substrate are brought into contact by hydrodynamic injection inside the capillary.

During the second step the enzymatic reaction is allowed to occur in the presence or absence of an electric field. During the third (post-reaction) step the product is electrophoretically transported to a detector.

In the present study, methods for analyzing the enzymatic reactions of adenosine kinase have been developed. These methods allow to measure substrates and potential inhibitors for adenosine kinase within a few minutes with high accuracy and reproducibility by means of capillary electrophoresis. These are suitable for the high-throughput screening of test compounds. CE-based adenosine kinase studies are extremely fast and require only very small amounts of sample. This is of particular advantage when the enzyme, substrate or test compounds (potential substrates or inhibitors) are very expensive.

3.2.2. Materials and methods

3.2.2.1. Reagents and chemicals

N′-linked 5′-AMP-Sepharose 4B was purchased from Amersham Pharmacia Biotech (Steinheim, Germany). Sodium acetate and phenylmethylsulfonyl fluoride (PMSF) were obtained from Fluka Biochemika (Neu-Ulm, Germany). CM-cellulose, DEAE-sephacel, adenosine, adenosine-5′-triphosphate, magnesium chloride hexahydrate, adenosine-5′-triphosphate, adenosine, magnesium chloride hexahydrate, adenosine-5′-triphosphate, thymidine-5′-monophosphate, sucrose, ethlylenediamine tetraacetic acid (EDTA) disodium salt, tris(hydroxymethyl)aminomethane (Trizma Base), ABT-702 (4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-*d*]pyrimidine), A-134974 (N^7-[(1′R,2′S,3′R,4′S)-2′,3′-dihydroxy-4′-aminocyclopentyl]-4-amino-5-iodopyrrolopyrimidine) and 5-iodotubercidin were from Sigma (Taufkirchen, Germany). Fresh bovine liver was obtained from a local slaughterhouse in Cologne, Germany. [2,8-^{3}H]Adenosine (33.10 Ci/mmol) was obtained from Perkin-Elmer Life Sciences, Rodgau-Jügesheim, Germany. 2-Substituted adenosine derivatives **3.2.2–3.2.8** (see Table 3.1) were synthesized in analogy to published procedures[51].

3.2.2.2. Adenosine kinase purification

Adenosine kinase was purified from bovine liver by a modification of the procedure described by Anders and Fox[41]. All procedures were carried out at 4 °C, unless otherwise noted. Bovine liver (140 g) was freed of fat and connective tissue and cut into small pieces, placed in a small beaker and homogenized with an Ultra Turrax homogenizer at full speed for one minute in the isolation

buffer (280 ml, 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.1 and 0.1 mM of the proteinase inhibitor phenylmethylsulfonyl fluoride). The homogenate was transferred to a Teflon glass homogenizer and further homogenized for 30 s, then transferred to centrifuge tubes and centrifuged at 30,000 g for 1 hr at 4 °C. The clear supernatant was decanted and brought to pH 6.0 with 1 N HCl. It was then mixed with an equal volume of settled CM-cellulose previously equilibrated with 10 mM sodium acetate buffer pH 6.0. After shaking the mixture for 90 min, the sample was filtered through a Büchner funnel, and washed once with 10 mM sodium acetate, pH 6.0. It was mixed with a volume of settled DEAE-sephacel equal to the initial volume of bovine liver supernatant previously equilibrated with 10 mM sodium acetate, pH 6.0. The mixture was stirred for 2.5 hr, then filtered, and washed as before. The filtrate was centrifuged again at 30,000 g for 30 min at 4 °C to remove the suspended particles. The supernatant was loaded on a 5'-AMP-Sepharose 4B column (XK 2.6 × 30 cm; 30 ml), which was pre-equilibrated with 10 mM sodium acetate buffer pH 6.0 and eluted at a flow rate of 4.0 ml/min.

The column was washed with the same buffer (150 ml of sodium acetate buffer, pH 6.0) to remove non-specifically bound protein, and then, in turn, with 100 ml of 10 mM Tris-HCl buffer pH 7.4 containing 1M KCl, 68 ml of 0.1 M Tris-HCl buffer pH 7.4 containing 5 mM ATP and finally with 90 ml of 0.1 M Tris-HCl buffer pH 7.4 containing 5 mM ATP-Mg and 5 mM adenosine. Adenosine kinase was eluted with the final wash, at a flow rate of 4.0 ml/min, with collection of 5 ml fractions. The final preparation was concentrated with Centriplus YM-10 (Amicon) centrifugal filter devices. Adenosine kinase was freed from salts, adenosine and ATP by dialysis for 24 hours in 10 mM Tris-HCl buffer pH 7.4. Capillary electrophoresis analysis of adenosine kinase was done to confirm that adenosine kinase was free from adenosine and ATP. Protein concentration of the finally purified enzyme was determined by the method of Lowry et al.[88] Using bovine serum albumin as a standard. The enzyme was stored at –85 °C in 0.5 µg/ml bovine serum albumin and 10 mM Tris-HCl buffer pH 7.4. The K_m and specific activity of purified adenosine kinase preparation were found to be 1.03 µM and 3.1 mM AMP/min/mg protein, respectively as determined by standard methods[89].

3.2.2.3. Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the method of Lämmli[90] with 10% cross-linked gel. Electrophoresis was done in 0.025 M Tris-HCl and 0.192 M glycine

buffer, pH 8.8 under denaturating conditions (addition of 1% SDS). Bromophenol blue was used as the tracking dye, while staining was done with Coomassie Blue.

3.2.2.4. Determination of K_m and V_{max} values of adenosine kinase by radioactive method

The standard radiochemical assay was performed as described earlier with modifications[89] in a final volume of 100 µl containing 20 mM Tris-HCl, 0.2 mM $MgCl_2$, 5 mM K_2HPO_4, 1 mM ATP and 10 µl of different concentrations of [^{3}H]adenosine (0.1, 0.5, 1.0, 2.0, 2.5, 5.0, 10.0, 20.0, 25.0 and 50.0 µM). The reaction was initiated by the addition of 10 µl of suitably diluted adenosine kinase enzyme (39 ng of protein). Incubation was performed for 5 minutes at 37 °C and then the reaction was stopped by the addition of 1.0 ml of cold 0.1 M aqueous lanthanum chloride solution in order to precipitate the radiolabeled AMP formed through the enzymatic reaction. The sample was kept on ice for at least 3 hours, and then filtered through GF/C glass fiber filters using a cell harvester (Brandel, purchased through Adi Hassel, München, Germany) in order to separate the precipitated labeled [^{3}H]AMP from soluble [^{3}H]adenosine. The filter papers were washed twice with 20-25 ml of cold water and then transferred to scintillation vials. Three ml of scintillation cocktail Ultima Gold® (Canberra Packard, Dreieich, Germany) was added to each sample vial and radioactivity was counted in a scintillation counter (Tri-Carb 2100 TR, Canberra Packard).

3.2.2.5. Effects of Mg^{2+} on the activity of adenosine kinase

Effects of different concentrations of Mg^{2+} (10 to 300 µM) on the enzyme activity were monitored. The reaction mixture contained 1 mM ATP, 200 µM adenosine, 64 mM Tris-HCl buffer, pH 7.5, and different concentrations of $MgCl_2$. The reaction was started by adding 10 µl of suitably diluted adenosine kinase and the mixture (final volume 100 µl) was incubated at 37 °C for 10 min, then stopped by heating at 99 °C for 5 minutes. The AMP formed was measured in duplicate by capillary electrophoresis. Each analysis was repeated three times in two separate experiments.

3.2.2.6. Effects of phosphate on the activity of adenosine kinase

Effects of different concentrations of phosphate were investigated by adding 0, 1, 5 and 25 mM of dipotassium hydrogen phosphate to the reaction mixture which had the following composition: 80 µM adenosine, 20 mM Tris-HCl buffer, pH 7.5, 1.0 mM ATP, 0.2 mM $MgCl_2$. The reaction

was started by adding 10 µl of adenosine kinase (0.078 µg). The reaction was carried out at 37 °C for 35 min and was stopped by heating at 99 °C for 2 minutes. An aliquot (50 µl) of the reaction mixture was added to a small CE vial containing 20 µl of UMP solution with final a concentration of 20 µM as an internal standard and 30 µl of deionized water.

3.2.2.7. Adenosine kinase inhibition assay by radioactive method

The standard radiochemical inhibition assay was performed as previously described[89] with minor modifications. In a final volume of 100 µl containing 20 mM Tris-HCl, 0.2 mM $MgCl_2$, 5 mM K_2HPO_4, 1 mM ATP, 1 µM [^{3}H]adenosine and different concentrations of the inhibitor, the reaction was initiated by the addition of 10 µl of adenosine kinase enzyme (ca. 39 ng of protein). Incubation was performed for 5 minutes at 37 °C and the reaction was stopped by the addition of 1.0 ml of cold 0.1 M aqueous lanthanum chloride solution in order to precipitate the radiolabeled AMP formed. The sample was kept on ice for at least 3 hours, and then filtered through GF/C glass fiber filters using a cell harvester (Brandel). The filter papers were washed twice with 20-25 ml of cold water and then transferred to scintillation vials. Three ml of scintillation cocktail Ultima Gold® (Canberra Packard, Dreieich, Germany) was added to each sample vial and radioactivity was counted in a scintillation counter (Tri-Carb 2100 TR, Canberra Packard).

3.2.3. Capillary electrophoresis method

3.2.3.1. Off-capillary enzymatic reaction: Method A

CE separations were carried out using a P/PACE system (Beckman Coulter Instruments, Fullerton, CA, USA) equipped with a DAD detection system. The electrophoretic separations were carried out using an eCAP fused-silica capillary [40 cm (30 cm effective length) × 75 µm internal diameter (I.D) × 375 µm outside diameter (O.D) obtained from Beckman Coulter]. On-line UV detection was performed in the range of 190-350 nm. The runs were performed under the following conditions: T = 25 ^{0}C, λ_{max} = 260 nm, current = 95 µA, running buffer 20 mM sodium phosphate buffer, pH 7.5. The capillary was washed with 0.1 N NaOH for 2 min, deionized water for 1 min, and running buffer for 1 min before each injection. Injections were made by applying 0.1 psi of pressure to the sample solution for 25 s, and 20 mM sodium phosphate buffer, pH 7.5, or pH 8.5, respectively, were used for the separations in adenosine kinase inhibition assays, while 100 mM SDS, 30 mM borate buffer, pH 9.5 was used for substrate assays.

The CE instrument was fully controlled through a personal computer, which operated with the analysis software 32 KARAT obtained from Beckman Coulter. The evaluation of the electropherograms was done using the same software. The temperature was kept constant at 25 °C in the capillary as well as in the sample storing unit.

3.2.3.2. In-capillary adenosine kinase inhibition studies by EMMA with partial filling technique: Method B

The electrophoretic separations were carried out using an eCAP polyacrylamide-coated fused-silica capillary [(30 cm (20 cm effective length) x 50 µm internal diameter (I.D.) x 360 µm outside diameter (O.D.), obtained from CS-Chromatographie (Langerwehe, Germany)]. 96-Well plates were obtained from Beckman Coulter. The separation was performed using an applied current of -60 µA. Analytes were detected using direct UV absorbance at 210 nm, it was used to get high sensitivity as compared to 260 nm. The capillary was conditioned by rinsing with water for 2 min and subsequently with buffer (phosphate 50 mM, pH 6.5) for 1 min. Sample injections were made at the cathodic side of the capillary. The capillary temperature was kept constant at 37 °C. The temperature of the sample storing unit was adjusted to 10 °C.

3.2.3.3. Preparation of standard solutions of AMP and nucleosides for method validation

Nucleosides (2-substituted adenosine derivatives) were dissolved in DMSO to obtain 10 mM stock solutions and AMP was dissolved in enzyme assay buffer (20 mM Tris-HCl, 0.2 mM $MgCl_2$, 5 mM K_2HPO_4 and appropriately diluted enzyme). AMP and nucleosides were further diluted in enzyme assay buffer to get 1.0 mM stock solutions. The 1 mM stock solutions were further diluted for the standard calibration curves. For validating the method of AMP and nucleoside determination, 20 µM of TMP was used as an internal standard. A fused silica capillary was used for the separation and quantitation of AMP and the nucleosides according to method A.

3.2.4. Biological assays

3.2.4.1. Off-capillary adenosine kinase substrate assay: Method A

Adenosine kinase substrate specificity assays were performed similarly as adenosine kinase inhibition assays, but different nucleosides were used instead of adenosine. The assays were carried out at 37 °C in a final volume of 100 µl. The reaction mixture contained 20 mM Tris-HCl,

0.2 mM $MgCl_2$, 5 mM K_2HPO_4, 1 mM ATP and 200 µM of test compound (nucleoside) in 1 % DMSO and 20 µM of TMP was used as an internal standard. The separation conditions were 100 mM SDS, 30 mM borate buffer, and pH 9.5. Calculating the percent phosphorylation of substrates requires a calibration curve to convert the product peak areas of the reaction to the product concentration. Due to the unavailability of isolated reaction products, we could not obtain a product calibration curve directly. However, the percent phosphorylation of substrates were calculated using the calibration curves of nucleosides. Because the phosphate group has not significant difference in UV absorption as compared to the substrates.

3.2.4.2. Adenosine kinase inhibition assay by capillary electrophoresis, off-line enzymatic reaction: Method A

Enzyme inhibition assays were carried out at 37 °C in a final volume of 100 µl. The reaction mixture contained 20 mM Tris-HCl, 0.2 mM $MgCl_2$, 5 mM K_2HPO_4, 1 mM ATP and 100 µM adenosine without internal standard. A-134974 was again tested by using 20 µM UMP as an internal standard, whereas the other assay conditions were the same as without internal standard. 10 µl of different concentrations of adenosine kinase inhibitors or test compounds were added and the reaction was initiated by the addition of 10 µl of the appropriately diluted enzyme. The mixture (final volume 100 µl) was incubated for 15 min and terminated by heating at 99 °C for 2 min. The reaction mixture was then transferred to mini CE vials (aliquot of 50 µl) and injected into the CE instrument under the conditions described above. Inhibition of adenosine kinase was tested over a range of 7 to 8 concentrations of antagonist spanning 3 orders of magnitude to determine K_i values of standard compounds, while new test compounds were screened at 30 and 3 µM concentrations, and in some cases also at 100 µM. Each analysis was repeated three times in two separate experiments. The Cheng-Prusoff equation[91] and a K_D value of 1 µM for adenosine was used to calculate the K_i values from the IC_{50} values, determined by the non-linear curve fitting program PRISM (GraphPad, San Diego, California, USA).

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{Adenosine}]}{K_m\,(\text{Adenosine})}}$$

3.2.4.3. Development of a high throughput screening method on 96-well plates using EMMA: Method B

Before use, a new capillary was washed with deionized water for 10 minutes. Prior to analysis, the capillary was conditioned by a wash cycle at 40 p.s.i (1 p.s.i = 6894.76 Pa) starting with deionized water for 2 min, followed by a 1 min rinse with CE running buffer (K_2HPO_4, 50 mM, pH 6.5) for 1 min. After each analysis the capillary was rinsed in the same way with CE running buffer followed by deionized water for 2 min and 1 min, respectively. After each injection, the capillary ends (and electrodes) were dipped into water to prevent carry over of sample.

3.2.4.4. Automation of analytical process

Due to the 96-well plate, pressure injection was not used, because it has no lids/stoppers and it is not possible to use pressure for injection of sample to the capillary. Therefore vacuum injection was used to inject the substrate, inhibitor and enzyme assay buffer in to the capillary.

The automation cycle consists of (i) washing with water for 2 min (40 p.s.i) (ii) equilibration with the CE running buffer dipotassium hydrogen phosphate 50 mM, pH 6.5 for 1 minute (40 p.s.i), (iii) injection of a plug of solution containing 20 mM Tris-HCl, and 0.2 mM $MgCl_2$, pH 7.4 (iv) a plug of suitably diluted enzyme (v) injection of a plug of solution containing 20 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$, 1 mM ATP, 100 µM adenosine, 20 µM UMP as an internal standard and various concentrations of inhibitor in the same solution. (vi) injection of a plug of solution containing 20 mM Tris-HCl, and 0.2 mM $MgCl_2$, pH 7.4. The reaction was initiated by the application of 5 kV separation voltage (negative polarity) for 0.20 min to let the two plugs interpenetrate. The voltage was turned off for 5 min (zero-potential amplification) and again turned on at constant current –60 µA, to elute the products formed. The capillary was washed with deionized water 40 psi for 2 minutes and 50 mM phosphate buffer pH 6.5 as a background electrolyte for 1 minute before each run.

The enzyme and substrate along with inhibitor were placed in the sample storage unit and its temperature was kept constant at 10 °C. At this temperature, enzyme activity remained unaffected for at least 24 h, therefore, there was no loss of enzymatic activity during the required time for collecting a series of data. Substrate and buffers used in the enzyme reaction were kept at 10 °C in the 96-well plate at the autosampler reservoir.

3.3. Results and discussion

Extracellular levels of adenosine can be increased by blocking the phosphorylation of adenosine to AMP by adenosine kinase[92]. Adenosine kinase inhibitors have been shown to be potent analgesic[72,73] antinociceptive and antiinflammatory agents in animal models[39]. Substrates for adenosine kinase of the protozoa *Toxoplasma gondii*, which is pathogenic in immunocompromised individuals such as patients suffering from AIDS can be used for the treatment of toxoplasmosis. Subversive substrates of *Toxoplasma gondii*, but not the human, adenosine kinase are preferentially metabolized to their monophosphorylated forms and become selectively toxic to the parasites but not their host, such as 6-benzylthioinosine[53,93]. Similarly 8-aminoadenosine is a potential therapeutic agent for the treatment of multiple myeloma, for 8-aminoadenosine is phosphorylated to its triphosphate form, which is cytotoxic for multiple myeloma[94].

In the present study, a capillary electrophoresis method was developed and validated for the separation and quantification of AMP and base-modified AMP derivatives in biological samples, and subsequently a rapid, convenient method for adenosine kinase assays was developed. Adenosine kinase isolated from bovine liver was used in inhibition and substrate assays.

3.3.1. Development of a CE-based adenosine kinase assay Method A: Off-line enzymatic reaction

3.3.1.1. Method validation

Capillary electrophoresis based methods for the quantitative determination of nucleosides and nucleotides[25,34-38] and for monitoring their metabolism[30] were previously developed in a number of laboratories including ours. Our previously developed methods were not suitable for the determination of the current series of alternative substrates for adenosine kinase because of the substituents at the 2-position of the nucleosides, which increased the migration times of most of the derivatives with respect to adenosine. Therefore, we optimized the separation buffer concentration and pH, which decreased the migration time. For the nucleosides a 30 mM borate buffer in the presence of 100 mM SDS, pH 9.5, was found suitable for the substrate assay. This buffer decreased the migration time and improved the resolutions of the peaks. For the quantitative determination of the reaction products of adenosine kinase, a fused-silica capillary was used. In this case the products of the enzymatic reaction (nucleotides: AMP, ADP and excess of ATP along with adenosine) move towards the cathodic side. In case of inhibition assays, for

high resolution and fine peak shape 20 mM potassium phosphate buffer, pH 7.4, was used for AMP method validation, later on, UMP was used as an internal standard to quantify the enzymatic product (AMP), and the method was further improved by increasing the pH of the running buffer from 7.4 to 8.5, which increased the negative charge on the capillary surface and hence decreased the migration times.

The method for the determination of nucleosides was validated with respect to specificity, linearity, and limits of quantitation, detection and precision. A known concentration of TMP was used as an internal standard (I.S.) for nucleosides because UMP as an internal standard co-migrated with ADP when borate buffer was used for the separation. Therefore, TMP was employed as an internal standard for substrate assays.

3.3.1.2. Limit of detection, quantitation and precision of migration time

The determination of K_i values of adenosine kinase inhibitors was achieved by measuring the corrected peak area of AMP formed during the enzymatic reaction, while the percentage of phosphorylation of nucleosides was calculated by measuring the corrected peak area of nucleotides. For substrate assays we did not have the phosphorylated products as standards to generate calibration curves for the measurement of phosphorylated product of the enzymatic reaction. Therefore, we determined the calibration curves using the nucleosides and from calibration curves of nucleosides the amount of nucleotides were determined because phosphate groups neither have any effect on the maximum absorption wavelength nor the molar absorption coefficient.

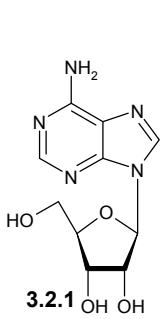
For the determination of linearity and the limit of detection and quantitation, calibration curves of standard solutions were obtained from 5 to 6 different concentrations of AMP (2.0, 5.0, 10.0, 20.0, 30.0, and 40.0 μM) and nucleosides (2.0, 5.0, 10.0, 20.0, and 40.0 μM). The limit of detection (LOD) calculated at a signal-to-noise ratio equal to 3 was found to be between 4.7 and 1.4 μM for the various nucleosides, and for AMP it was 1.6 μM (see Table 3.1). Good linearity was found in the range from the compounds' lowest measured concentration within the linear range to the highest concentration at 40 μM. The limit of quantitation (LOQ) calculated at a signal-to-noise ratio equal to 10 was found in the range of 5.2 to 15.3 μM (see Table 3.1). The precision of the migration time was investigated with respect to repeatability. Each standard was run 5 times. Standard deviation and variation coefficients of migration times were determined. There was less difference in migration times of fast migrating nucleosides while relatively more

difference was observed in migration times for slower migrating nucleosides. The relative standard deviation of migration times for AMP and compounds **3.2.4** and **3.2.6** was low, 1.7, 1.3, and 1.2 %, respectively, while it was only slightly higher for compounds **3.2.2**, **3.2.3** and **3.2.8**, being 3.5, 2.9, and 3.0 %, respectively. The results are summarized in Table 3.1.

3.3.1.3. Linearity of calibration curve and recovery

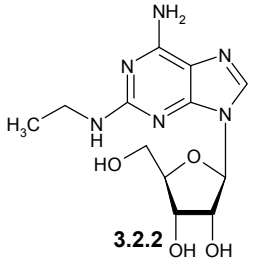
To determine the linearity of calibration curve and recovery rates, standard solutions of 2.0, 5.0, 10.0, 20.0, and 40.0 μM concentrations of AMP and nucleosides were run, and linear relationships with regression coefficients (R^2) in the range of 0.994 to 0.999 were found by plotting corrected peak areas against concentrations for AMP and all nucleosides. The found concentrations were calculated from the obtained linear regression curves and plotted against the concentrations originally present in the standard solutions to obtain the recovery equations. Recovery rates were found to be high, 98.8-99.9% for AMP and all nucleosides (Table 3.1).

Table 3.1. *Method validation parameters for AMP and adenosine derivatives (nucleosides) using 20 µM TMP as an internal standard for nucleosides and 20 µM UMP for AMP.*



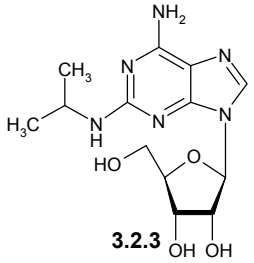
3.2.1

Adenosine



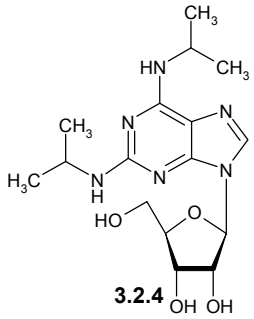
3.2.2

2-Ethylamino-adenosine



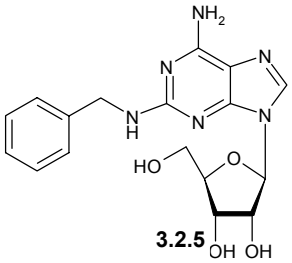
3.2.3

2-Isopropylamino-adenosine



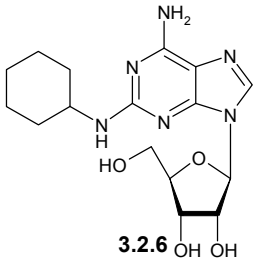
3.2.4

2-Isopropylamino-N^6-isopropyl-adenosine



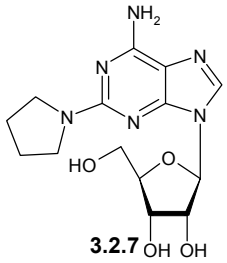
3.2.5

2-Benzylamino-adenosine



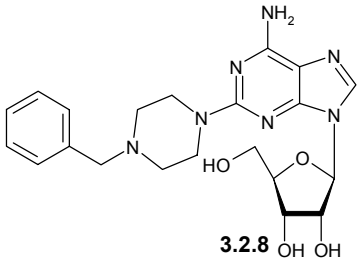
3.2.6

2-Cyclohexylamino-adenosine



3.2.7

2-(Pyrrolidin-1-yl)-adenosine



3.2.8

2-(4-Benzylpiperazin-1-yl)-adenosine

Compound	**AMP**	**3.2.2**	**3.2.3**	**3.2.4**	**3.2.5**	**3.2.6**	**3.2.7**	**3.2.8**
Limit of detection (µM) ± SD	1.6 ± 0.2	3.4 ± 0.2	2.4 ± 0.2	4.7 ± 0.2	4.1 ± 0.2	1.7 ± 0.1	1.8 ± 0.2	1.4 ± 0.1
Limit of quantification (µM) ± SD	5.9 ± 0.4	13.0 ± 0.3	9.1 ± 0.4	7.3 ± 0.3	15.3 ± 0.5	6.4 ± 0.2	6.7 ± 0.3	5.2 ± 0.2
% Recovery rate ± SD	99.3 ± 0.52	98.2 ± 0.51	99.1 ± 0.54	97.3 ± 0.42	96.7 ± 0.64	95.6 ± 0.60	98.5 ± 0.31	99.5 ± 0.21

RSD (%) of Recovery rate	3.11	7.2	5.2	8.5	8.1	8.5	5.4	5.5
R^2 of Recovery rate	0.998	0.974	0.997	0.988	0.991	0.992	0.998	0.999
Coefficient of correlation; R^2	0.999	0.990	0.998	0.994	0.995	0.996	0.999	0.999
Mean value of migration time (min) ± SD (n= 6)	9.50 ± 0.14	8.60 ± 0.30	10.4 ± 0.3	7.60 ± 0.1	19.73 ± 0.34	6.90 ± 0.08	5.68 ± 0.08	12.16 ± 0.36
RSD of migration time (%)	1.5	3.5	2.9	1.3	1.7	1.2	1.4	3.0

3.3.2. Purification and characterization of adenosine kinase from bovine liver

Adenosine kinase was purified from bovine liver using ion exchange and affinity chromatography, and subsequent concentration was achieved with Amicon centrifugal filter devices. During ion exchange chromatography, positively and negatively charged contaminating proteins bind to CM-cellulose and DEAE-cellulose, respectively, while adenosine kinase does not bind under these conditions. Ion exchange chromatography removed most of the contaminating proteins. Subsequent affinity chromatography played the crucial role in purifying adenosine kinase by binding the enzyme to the 5-AMP-sepharose column material whereas the other proteins were eluted. Adenosine kinase was finally eluted with a buffer containing 5 mM ATP-Mg and 5 mM adenosine. The molecular weight of the enzyme was estimated as 38 kDa by SDS-polyacrylamide gel electrophoresis. This value was similar to published values for the enzyme from bovine liver[89], human liver[95], human placenta[41], rat brain[96], Leishmania donovani[46] and mycobacterium tuberculosis[97], but slightly different from that found in bovine adrenal medulla (40 kDa), determined by the same method.

K_m and V_{max} values of the isolated enzyme were determined by the standard radioactive method using constant concentrations of 0.2 mM $MgCl_2$ and 1 mM ATP. The K_m and V_{max} values of the adenosine kinase preparation were found to be 1.03 μM and 3.1 mM AMP/min/mg protein, respectively, at pH 7.4 (Figure 3.2.2). The K_m value for adenosine, determined in this study was very similar to the literature value for adenosine kinase from the same source[89].

The activity of adenosine kinase as a function of various concentrations of $MgCl_2$ had been examined in previous studies[69,45,95]. Mg^{2+} was found as a co-substrate of this enzyme. In our studies, adenosine kinase from bovine liver showed maximal activity at a concentration of 0.2 mM of $MgCl_2$. After increasing the concentration of $MgCl_2$ above this limit, there was no further increase in the activity of adenosine kinase, whereas lower concentrations of $MgCl_2$ had reduced enzymatic activity (Figure 3.2.3). Adenosine kinase did not show any activity in the absence of magnesium, it showed an absolute requirement for magnesium. The dependence of the AK activity on Mg^{2+} and ATP suggests that the ATP-Mg complex is the true substrate for the enzyme. In a previous study[98], an excess of free Mg^{2+} and ATP^{4-} both have been shown to produce strong inhibition of AK. We did not find any inhibition by Mg^{2+} up to concentrations of 0.3 mM. Higher concentrations of Mg^{2+} were not tried due to separation problems by CE.

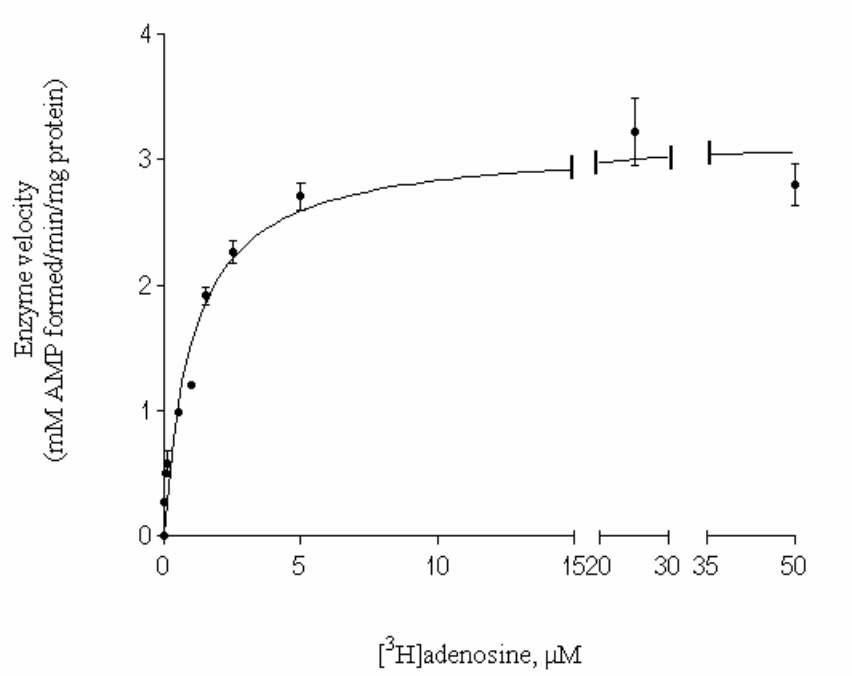


Figure 3.2.2. *Plot of initial adenosine concentration with respect to the reaction velocity (mM of AMP produced in one minute per mg of enzyme) for the determination of K_m and V_{max} values by the standard radioactive method. Enzyme activity was determined at pH 7.5 using 1 mM ATP, 0.2 mM $MgCl_2$ and various concentrations of [^{3}H]adenosine. K_m was 1.03 µM, V_{max} was 3.1 mM AMP/min/mg protein.*

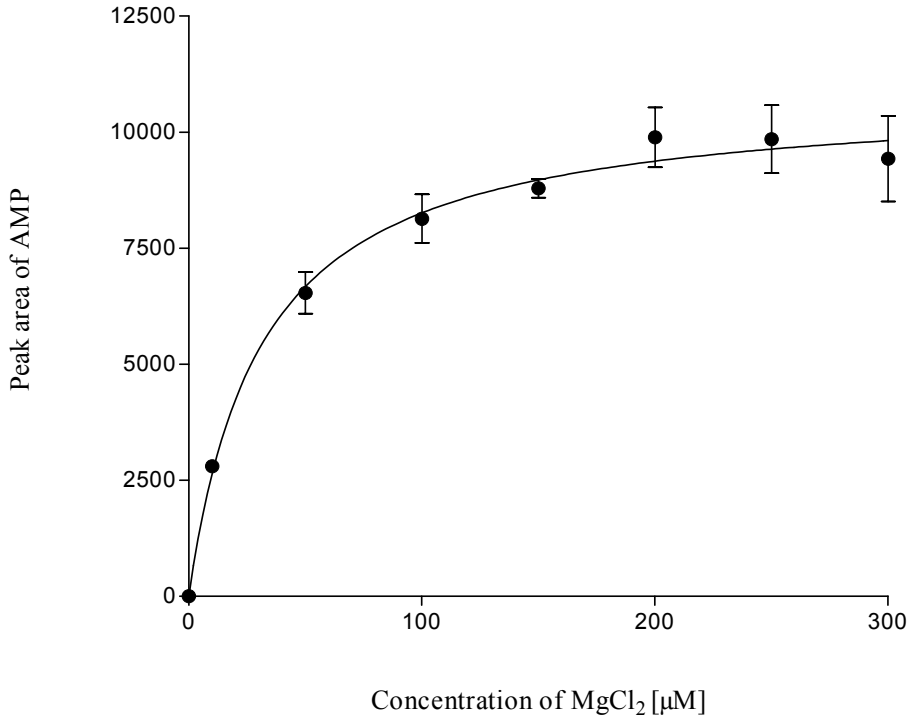


Figure 3.2.3. *Effects of different $MgCl_2$ concentrations on the activity of bovine liver adenosine kinase at a fixed concentration of ATP, determined by capillary electrophoresis. K_m was determined at pH 7.5 using 1 mM ATP, 200 µM adenosine and various concentrations of $MgCl_2$. The separation conditions were 20 mM phosphate buffer, pH 7.41, a fused silica capillary, 40 cm length (30 cm to the detector), 75 µm I.D.; 95 µA; 25 °C; detection at 260 nm; pressure injection.*

Previous studies showed that the catalytic activity of adenosine kinase could be greately affected by the presence of inorganic phosphate[74]. Thus, we investigated the effects of increasing phosphate concentrations (from 1 to 25 mM) at pH 7.5 on adenosine kinase from bovine liver. We did not find any change in the activity of adenosine kinase with increased concentrations of phosphate (data not shown). In previous reports[69,74,89] it was found that phosphate markedly influenced the activity of adenosine kinase from Chinese hamster ovary (CHO) cells, bovine liver, yeast and Leishmania donovani. It was suggested that inorganic phosphate enhanced the reaction rate along with an improved affinity of the enzyme for adenosine by formation or stabilization of an enzyme-adenosine-ATP transition state complex. The catalytic activity of *Escherichia coli* recombinant and bovine liver AK was almost completely dependent on the presence of inorganic phosphate at pH 6.2. However, at pH 7.4, the activity of AK was not completely dependent on the presence of inorganic phosphate, but V_{max} was doubled in the presence of 20 mM inorganic phosphate[74]. Similar studies were conducted with AK from various rat tissues[99], which did not show any effect of inorganic phosphate on K_m and V_{max} at pH 6.0 and pH 7.4 in the presence of various concentrations of Mg^{2+} and ATP. We used 80 µM of adenosine to investigate the effects of phosphate on bovine liver adenosine kinase, a concentration of adenosine much higher than its K_m value. It is possible that at lower concentrations of adenosine, phosphate would show an effect.

3.3.3. Investigation of alternative substrates

A series of nucleosides (2-substituted adenosine derivatives, Table 3.1) were tested as potential substrates of bovine liver adenosine kinase. The nucleosides were used at a concentration of 200 µM in the presence of 0.2 mM $MgCl_2$ and 1 mM ATP. Several of these adenosine derivatives were phosphorylated to the corresponding monophosphates by the enzyme (Table 3.2). All of the 2-substituted 2-aminoadenosine derivatives were poorer substrates of adenosine kinase than the natural substrate adenosine.

A pyrrolidinyl- and an isopropylamino-substitution were best tolerated by adenosine kinase; while smaller (ethylamino) or larger 2-substituents (benzylamino, cyclohexylamino, 4-benzylpiperazinyl) resulted in a lower degree of phosphorylation. The best substrate of the present small series of 2-substituted adenosine derivatives was a 2,N^6-disubstituted adenosine derivative, 2-isopropylamino-N^6-isopropyladenosine **3.2.4**, exhibiting 81% of phosphorylation in comparison with adenosine (set at 100%, see Figure 3.2.5). The phosphorylation of adenosine analogs, including 2-chloroadenosine, N^6-cyclohexyladenosine, and N^6-*R*-phenylisopropyladenosine had been studied; it was observed that these adenosine derivatives are phosphorylated and thus would likely be incorporated into nucleic acids[100].

With this newly developed method it is easy to determine the phosphorylated products (nucleotides). Electrophoretic mobility of the nucleoside analogs changed after phosphorylation by adenosine kinase and product peaks appeared in the electropherogram with similar migration time as that of AMP.

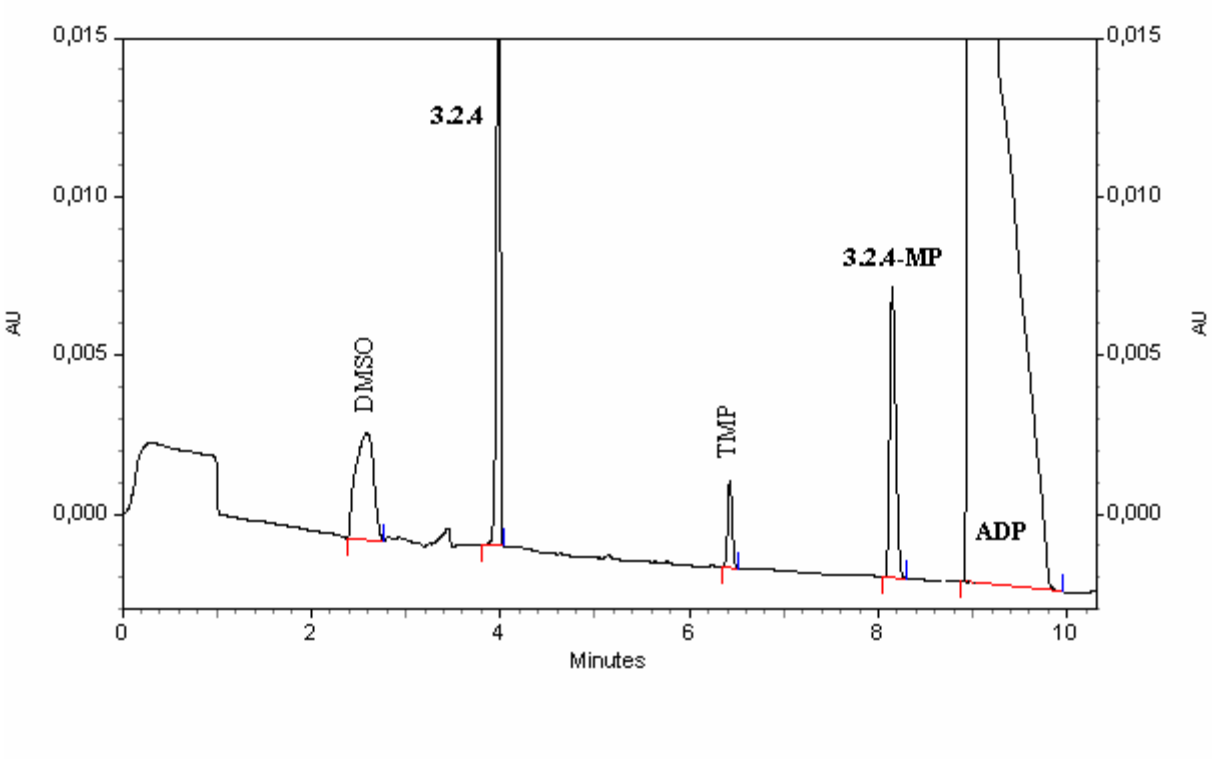


Figure 3.2.5. *Typical electropherogram of AK enzyme substrate assay with 20 µM TMP as an internal standard in enzyme assay buffer. Concentration of adenosine derivative* ***3.2.4*** *was 200 µM in 1 % DMSO, incubation time 10 min, concentration of AK 0.078 µg. The separation conditions were 100 mM SDS, 30 mM borate buffer, pH 9.5, fused silica capillary, 40 cm length*

*(30 cm to the detector), 75 µm I.D.; 95 µA; 25 °C; detection at 260 nm; pressure injection (**3.2.4** = 2-Isopropylamino-N^6-isopropyadenosine, **3.2.4-MP** = 2-Isopropylamino-N^6-isopropyadenosine-5'-monophosphate).*

Table 3.2. *Percent phosphorylation of 2-substituted 2-aminoadenosine derivatives by adenosine kinase in comparison with the physiological substrate*

No.	Compound	Phosphorylation (%)[a] (n = 3)
1	Adenosine	100 ± 2
2	2-Ethylaminoadenosine	3 ± 1
3	2-Isopropylaminoadenosine	4 ± 0
4	2-Isopropylamino-N^6-isopropyladenosine	81 ± 3
5	2-Benzylaminoadenosine	4 ± 0
6	2-Cyclohexylaminoadenosine	3 ± 0
7	2-(Pyrrolidin-1-yl)adenosine	53 ± 2
8	2-(4-Benzylpiperazin-1-yl)adenosine	3 ± 0

[a] Percentage of phosphorylated product obtained with respect to the natural substrate adenosine set at 100%. Quantitative determination was achieved by standard calibration curves of corresponding nucleosides. For reaction conditions see Figure 3.2.5.

3.3.4. Investigation of adenosine kinase inhibitors by off-capillary method

Inhibition of adenosine kinase was determined by a range of concentrations of inhibitors, in the presence of a fixed amount of adenosine (100 μM). The K_i values of the standard inhibitors tested in this study were very close to the K_i values obtained by the standard radiometric method (Table 3.3).

ABT-702 is a non-nucleoside adenosine kinase inhibitor, 5-iodotubericidin (5-IT) and A-134974 are adenosine analogs (for structures see Table 3.3). ABT-702 is an orally active antinociceptive agent and more potent than 5-IT[55]. A-134974 was the most potent compound among all adenosine kinase inhibitors tested. Using bovine liver adenosine kinase and the newly developed

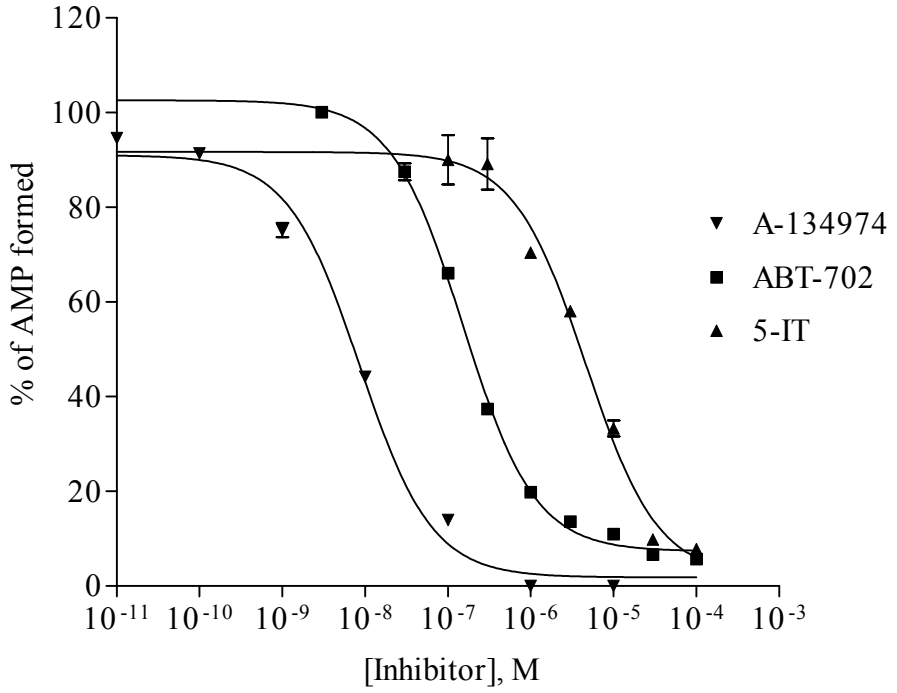
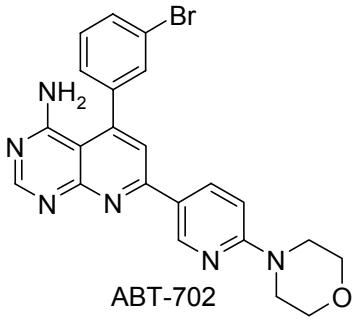


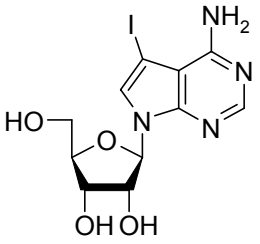
Figure 3.2.6. *Concentration-dependent inhibition of AK by the standard inhibitors A-134974 (▼), ABT-702 (■) and 5-iodotubercidin (▲) determined by incubation of the reaction mixture outside the capillary by capillary electrophoresis at pH 7.5 using 1 mM ATP, 0.2 mM $MgCl_2$ and various concentrations of inhibitor. The separation conditions were 20 mM phosphate, pH 7.41, fused silica capillary, 40 cm length (30 cm to the detector), 75 μm I.D.; 95 μA; 25 °C; detection at 260 nm; pressure injection.*

CE method, concentration-dependent inhibition of adenosine kinase by the standard inhibitors was observed (Figure 3.2.6). All three standard inhibitors were tested without internal standard, but later on A-134974 was also tested in the presence of an internal standard. The use of internal standard did not result in significantly different K_i value, it only let to a moderate improvement of the relative standard deviation (see Table 3.3).

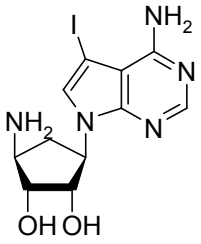
Table 3.3. *Comparison of K_i values for standard AK inhibitors obtained with the new capillary electrophoresis (CE) methods and with the classical radioactive method. All values are means ± S.E.M of three separate experiments.*



ABT-702



5-IT



A-134974

Inhibitor	***K_i [nM] ± SEM***			
	CE method bovine liver AK	**Radioactive method bovine liver AK**	**Radioactive method human AK**	**Radioactive method rat brain cytosolic AK**
ABT-702	1.4 ± 0.04[a] 1.7 ± 0.07[b]	3.40 ± 0.03	3.0 ± 0.8[55]	1.7 ± 0.5[55]
5-Iodotubercidin (5-IT)	30.6 ± 1.7[a]	29.0 ± 6.1	40[61], 26 ± 6[101]	9.3 ± 1.5[55]
A-134974	0.090 ± 0.006[a,d] 0.097 ± 0.004[a,e] 0.060 ± 0.031[b]	0.070 ± 0.006	0.06 ± 0.07[53]	N.D[c]

a) Off-line method

b) EMMA

c) N.D = not determined

d) Without I.S.

e) With I.S.

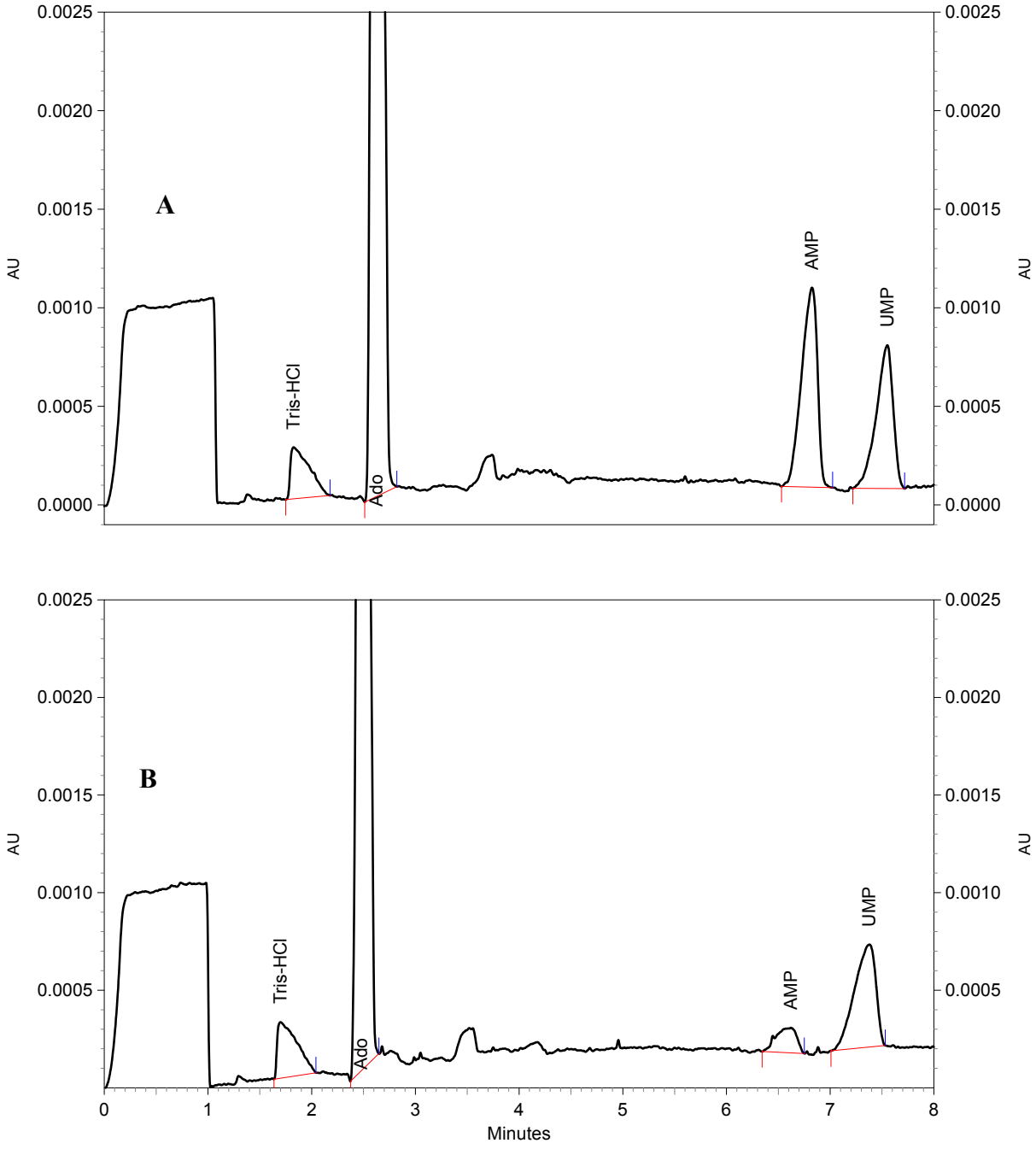


Figure 3.2.7 A*. Typical electropherogram of AK enzyme control assay (without inhibitor). Concentration of adenosine was 100 µM and 20 µM UMP was used as an internal standard. The separation conditions were 20 mM phosphate, pH 7.5, fused silica capillary, 40 cm length (30 cm*

to the detector), 75 µm I.D.; 95 µA; 25 °C; detection at 260 nm; pressure injection; normal polarity.

***B**. Typical electropherogram of adenosine kinase enzyme inhibition assay. Concentration of A-134974 was 1.0 µM, same separation conditions as **A**. The concentration of the inhibitor A-134974 was below the detection limit of CE, therefore, the peak for A-134974 did not appear in the electropherogram.*

A typical electropherogram from the adenosine kinase control assay is shown in Figure 3.2.7A in which 100 µM of adenosine was used without inhibitor. The percentage conversion of substrate into product was in the range of 15-20 %. It showed a larger peak of AMP, while the second electropherogram of a typical adenosine kinase inhibition experiment is shown in Figure 3.2.7B, in which 1.0 µM of A-134974 was present. In that electropherogram the peak size for AMP is significantly smaller compared to the control assay. The product peak (AMP) eluted within less than 8 minutes using a fused silica capillary and sodium phosphate buffer, pH 7.5, while the neutral molecule adenosine migrated with the EOF or slightly later depending on the conditioning of the capillary. In the normal polarity mode AMP comes frist than the UMP. Later on, the method was made even faster by increasing the pH value of the sodium phosphate buffer from 7.5 to 8.5. With an increase in pH of the running buffer, the surface of the silica capillary is deprotonated to a larger extent leading to a decreased migration time of the nucleotides.

3.3.5. Electrophoretically mediated microanalysis with the partial-filling technique

In the EMMA method, the capillary was first filled with a given background electrolyte. Subsequently, plugs of Tris-HCl buffer, pH 7.5, enzyme solution, substrate solution, Tris-HCl buffer, pH 7.5, and of the background electrolyte were injected into the capillary. For schematic illustration of the combination of EMMA with a partial filling technique see Figure 3.2.8. The slowly moving enzyme was injected first followed by the faster moving substrate. Small buffer plugs were injected before the enzyme and after the substrate to shield the enzymatic reaction from the background electrolyte. These buffer plugs contained Mg^{2+} ions which are necessary for the enzymatic reaction to proceed. Initially, the classical EMMA arrangement was tried out without partially filling the capillary but the enzymatic product was too little to be measured. Upon the application of the 5 kV potential, the two plugs will interpenetrate and after 0.20 min

the voltage was turned off to allow the reaction to continue in the absence of an electric field. This zero-potential amplification was necessary to accumulate enough product to detect it spectrophotometrically. Internal standard (I.S.) was used to compensate for minor variability in injection volumes, because nanoliter volume plugs of substrate and inhibitor were used. In the EMMA method a neutral capillary was used to prevent the adsorption of enzyme onto the

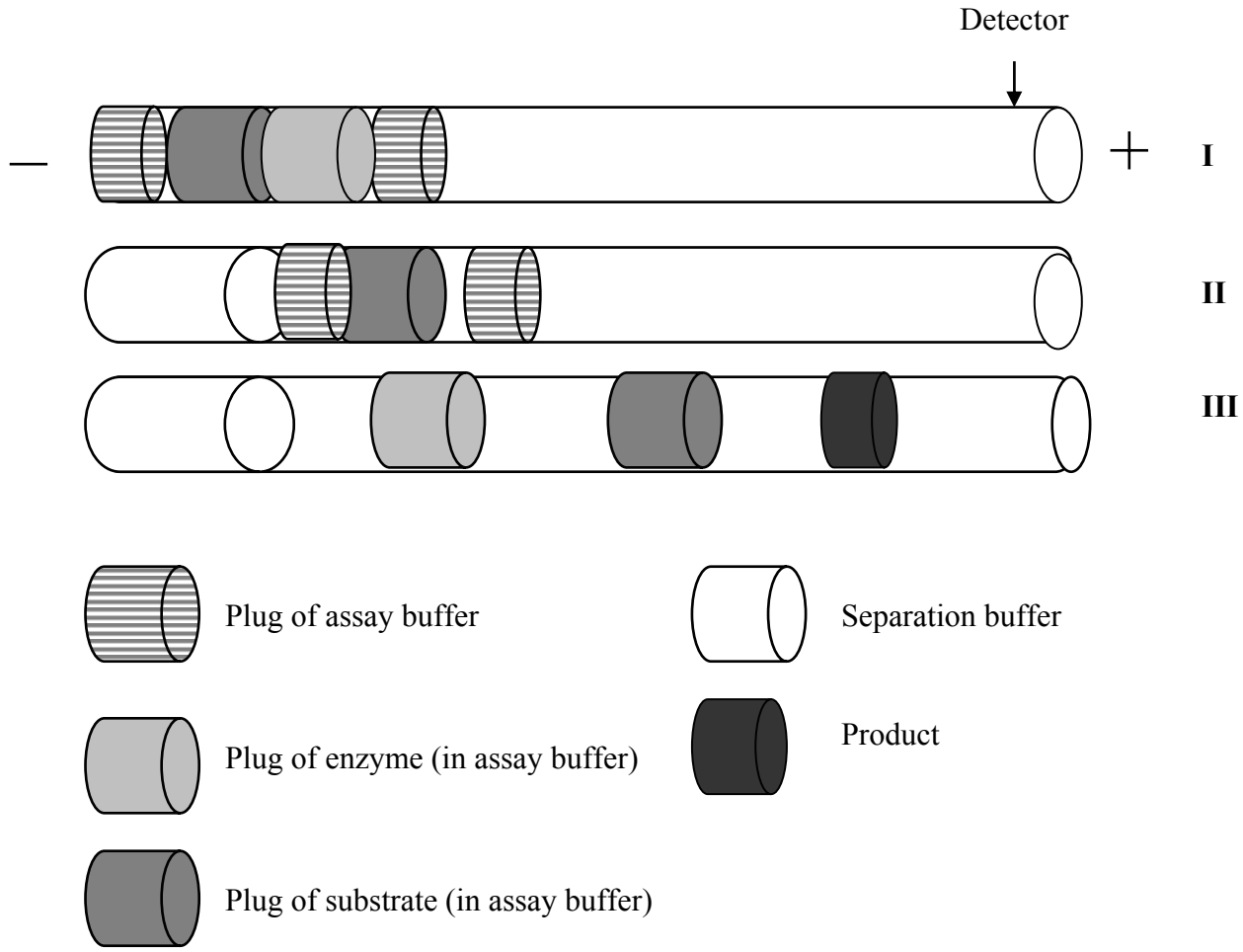


Figure 3.2.8. *Schematic illustration of the different steps of the in-capillary reaction assay. (I) a plug of assay buffer followed by a plug of enzyme and a plug of substrate, and again a plug of assay buffer are introduced hydrodynamically into the capillary; (II) the voltage is turned on and the fast migrating substrate plug penetrates the slow migrating enzyme plug. When the two plugs have merged, the voltage is turned off (zero-potential amplification) and incubation starts; (III) product is generated during the reaction and the compounds are swept towards the detector upon the subsequent application of the voltage.*

capillary wall because in a fused silica capillary the proteins can adsorb onto the capillary surface, which may lead to an increase in migration time and to current breakdown. A typical electropherogram of the in-capillary enzymatic reaction, using 50 mM phosphate buffer, pH 6.5 as a background electrolyte is shown in Figure 3.2.9. By using a coated capillary and reverse polarity mode the elution order of the nucleotides becomes reversed. UMP comes first, then AMP. The standard adenosine kinase inhibitors ABT-702 and A-134974 were tested by this method, and K_i values of the inhibitors were found to be very close to the K_i values obtained by the standard radiometric method and the off-capillary enzymatic reaction method (Table 3.3).

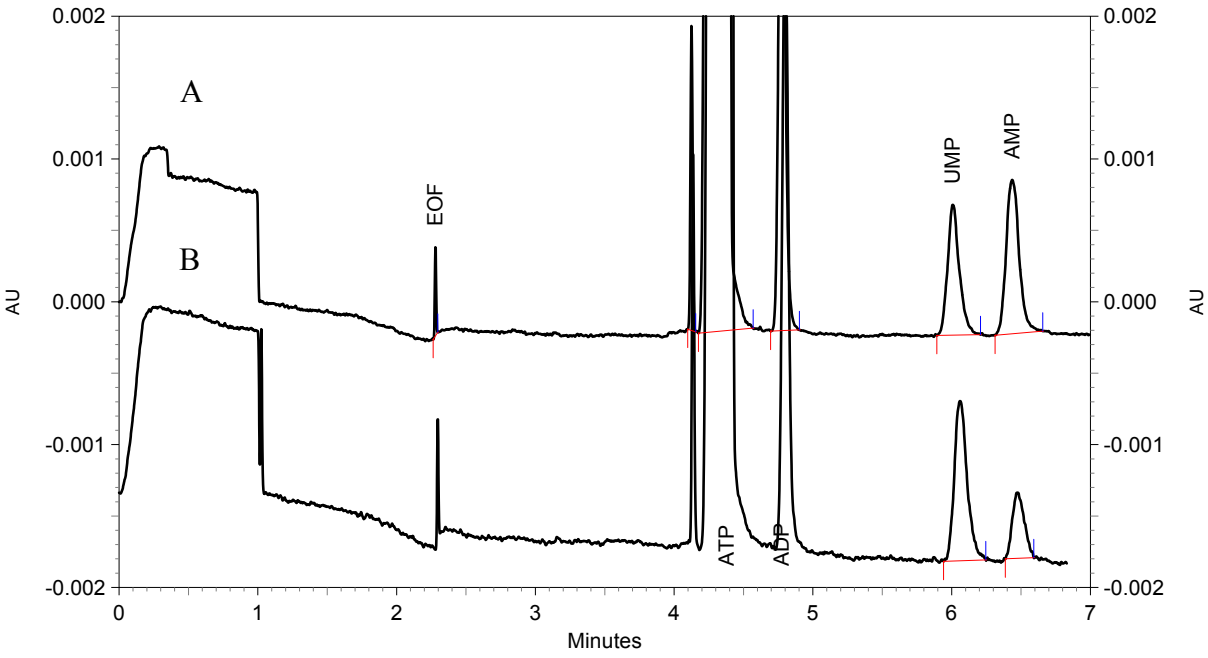


Figure 3.2.9 A. *Typical electropherogram of AK enzyme control assay (EMMA). Concentration of adenosine was 100 µM and 20 µM UMP was used as an internal standard. The separation conditions were 50 mM phosphate, pH 6.5, neutral capillary, 30 cm length (20 cm to the detector), 50 µm I.D.; -60 µA; detection at 210 nm; vacuum injection; reverse polarity.*

B. *Typical electropherogram of AK enzyme inhibition assay. Concentration of A-134974 was 1.0 µM, same separation conditions as* ***A****.*

3.4. Summary

A fast and convenient capillary electrophoresis (CE) assay was developed for the screening of adenosine kinase inhibitors and substrates. The method employing a fused-silica capillary and normal polarity mode provided good resolution of substrates and products of the enzymatic reaction and a short analysis time of less than 15 min. After stopping the enzymatic reaction by heating, the samples were injected by pressure and detected by their UV absorbance at 260 nm. Constant current (95 μA) was applied, and a phosphate buffer (pH 7.5 or pH 8.5) was used for the separations. Adenosine kinase isolated from bovine liver was used for these experiments. Dose response curves and calculated K_i values for standard antagonists obtained by CE were in excellent agreement with data obtained by the standard radioactive assay. In contrast to the radiometric assay, the CE assay can easily be used for investigating adenosine kinase substrates as well. A 2,N^6-disubstituted adenosine derivative, 2-isopropylamino-N^6-isopropyladenosine, was found to be a good substrate (81 % of phosphorylation with respect to adenosine = 100 %), while 2-(ar)alkyl-substituted 2-aminoadenosine derivatives without an additional N^6-substituent were poorer substrates, e.g. 2-ethylamino-, 2-benzylamino-, and 2-cyclohexylaminoadenosine showed only less than 5 % phosphorylation with respect to adenosine (100 %). Capillary electrophoresis-based enzyme assays can be used for high throughput screening of potential adenosine kinase inhibitors and substrates. They may prove to be useful not only for adenosine kinase, but for kinase assays in general.

Based on the off-line assay, an in-line adenosine kinase inhibition assay was developed by a methodology known as electrophoretically mediated microanalysis (EMMA). All the different steps (i.e. mixing, incubation, separation and in-line quantitation) are combined in the capillary, which is used as a microreactor for the enzymatic reaction. In EMMA after hydrodynamic injection of a plug of solution containing 20 mM Tris-HCl, and 0.2 mM $MgCl_2$, pH 7.4, a plug of suitably diluted enzyme was injected, followed by a plug of solution containing 20 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$, 1 mM ATP, 100 μM adenosine, and 20 μM UMP as an internal standard along with various concentrations of inhibitor in the same solution. The reaction was initiated by the application of 5 kV separation voltage (negative polarity) for 0.20 min to let the two plugs interpenetrate. The voltage was then turned off for 5 min (zero-potential amplification) and again turned on at a constant current of –60 μA, to elute the products formed. The method employing a polyacrylamide-coated capillary of 20 cm effective length and reverse polarity mode provided good resolution of substrates and products within a short separation time of less than 7 min. A 50

mM phosphate buffer (pH 6.5) was used for the separations and the products were detected by their UV absorbance at 210 nm. By in-capillary reaction, the scale of the enzymatic reaction was radically reduced to the nL scale as compared to off-line analysis of the reaction carried out in a microcentrifuge tube. Moreover, since the capillary is used as a reaction vessel, all the assay steps (mixing, reaction, separation, and quantitation) are combined in a fully automated nanoscale activity assay. This process was carried out automatically using a temperature-controlled autosampler in order to eliminate routine handling and to speed-up the process. The K_i values for standard antagonists obtained by EMMA were in good agreement with those obtained using an off-line CE assay.

Chapter 4. Capillary electrophoresis assays of thymidine kinase

4.1. Introduction

Thymidine kinase (TK) is a key enzyme in the salvage pathway of nucleotide metabolism catalyzing the transfer of the terminal phosphate group of ATP to the 5'-hydroxyl group of thymidine (dT) and thus yielding thymidine monophosphate (dTMP). The product dTMP is subsequently phosphorylatad by nucleoside kinases to dTTP, which is used for the synthesis of DNA[102-104]. In mammalian cells the total TK activity is expressed by two genetically distinguishable isoenzymes: TK-1 and TK-2. TK-1 is cytosolic and is expressed only in S-phase cells and TK-2 is considered to be localized in mitochondria[105,106]. Of the two isoenzymes TK-1 is the most studied and the one generally referred to when talking about TK activity. As cytosolic activity (TK-1), which increases with cellular DNA synthesis, whereas the mitochondrial activity (TK-2) remains relatively constant during various phases of the cell cycle[107], it is assumed that TK-1 plays a role in the replication of DNA. In a variety of cancer and virus-infected cells the expression of TK-1 differs from the expression in normal cells. Generally, the level of TK activity is increased. Cancer cells with a changed ratio of TK-1 and TK-2, e.g. expression of TK-l in quiescent malignant cells and/or expression of TK enzymes with biochemical properties different from the normal counterparts have been observed[108]. In contrast to the cellular enzyme, the viral thymidine kinase from Herpes simplex virus (HSV) type 1 differs condiderably from the human host cell isoenzyme[109] and exhibits a broad range of acceptance for nucleosides[110,111]. HSV1 TK is a homodimer with 376 residues per subunit. The constituent subunits display the general α/β folding pattern. A central five-stranded parallel β-sheet is flanked on either side by helices. The central five-β-strand domain is referred to as the CORE domain. The LID domain of HSV1 TK consists of only eight residues, reminiscent of other small variants of nucleoside monophosphate (NMP) kinases[112] (Figure 4.1). Nucleoside analogs are modified nucleosides that, when phosphorylated by nucleoside kinases inhibit further DNA synthesis either by incorporation into DNA or by inhibition of DNA polymerases[113,114]. A very efficient application of nucleoside analogs is seen in the treatment of Herpes Simplex Virus type 1 infections. Herpes Simplex virus encodes for its own TK, which can phosphorylate the nucleoside analog acyclovir, an acyclic guanosine analog, while the human host TK cannot. Only cells infected with Herpes simplex virus will be affected – and killed - from the treatment with acyclovir and analogs.

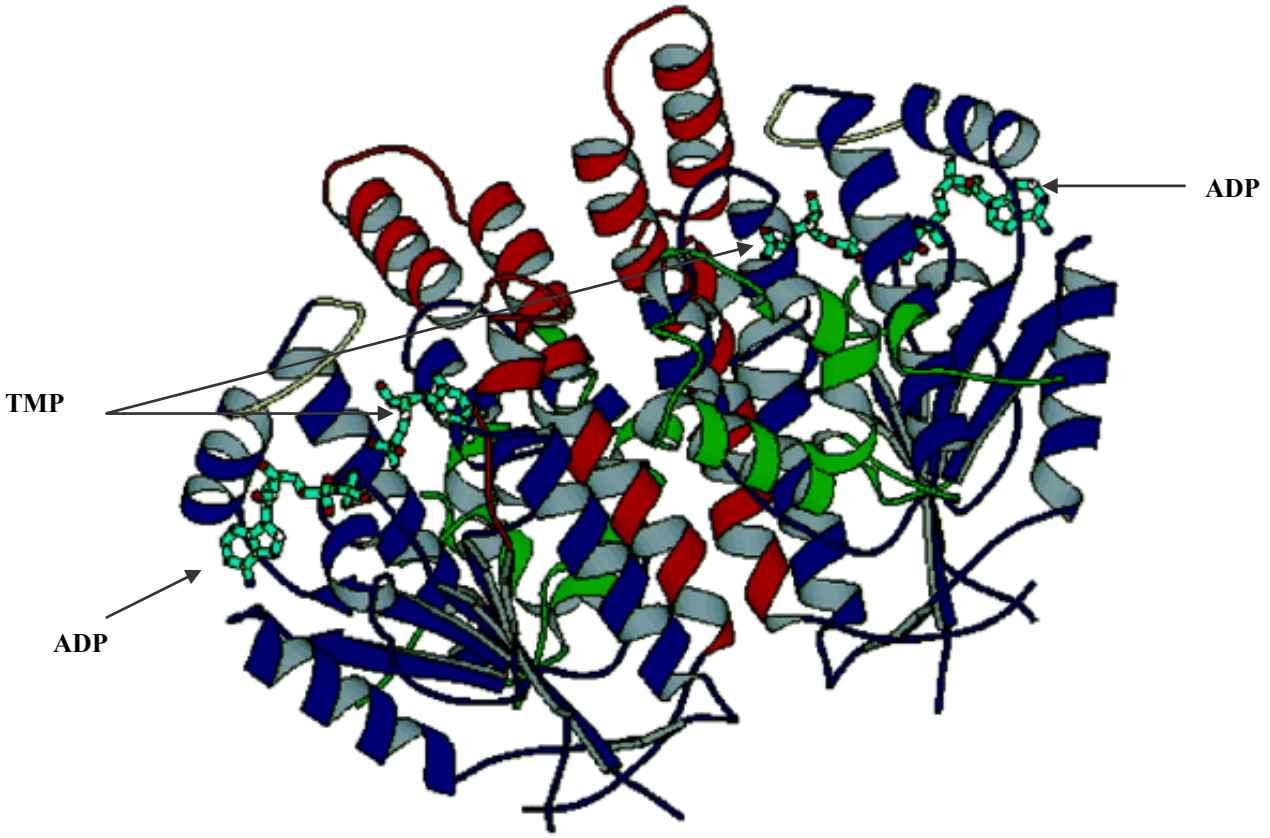


Figure 4.1. *Ribbon representation of the symmetric HSV1 TK dimer with bound ADP and dTMP. The CORE domain is depicted in blue, the TMP-binding domain in red, and the LID domain in yellow. The additional residues (250-322) are shown in green. Substrate and cofactor are depicted as cyan ball and stick models.*

4.1.1. Mechanism of action of antiviral drugs

More than 8 known viruses exist within the herpes virus family, the most important pathogenic being HSV-1 and -2[115]. Antiviral drug design could, in principle, be targeted at either viral proteins or cellular proteins. The viral approach is likely to yield more specific, less toxic compounds, with a narrow spectrum of antiviral activity and a higher likelihood of virus drug-resistance development, whereas the second approach might afford antiviral compounds with a broader activity spectrum and less chance of resistance development, but higher likelihood of toxicity. Both routes are worth exploring, the preferred route being dictated by both the nature of the virus and the targets that the virus or its host cell has to offer[116]. Despite the impressive safety record and efficacy of nucleoside analogs in general, and acyclovir in particular, for the treatment

of diseases caused by herpes viruses, a number of problems are associated with their use. As nucleoside analogs are incorporated into DNA and are chain terminators, they are potentially mutagenic. However, acyclovir is not mutagenic[115]. The treatment of choice for diseases caused by herpes viruses is acyclovir (ACV), which is a nucleoside analog of deoxyguanosine that has to be phosphorylated three times. The first phosphorylation is completed by the virally encoded thymidine kinase (TK) protein which allows ACV to become active only in virus-infected cells. The second and third phosphorylations are achieved by cellular thymidylate kinases.

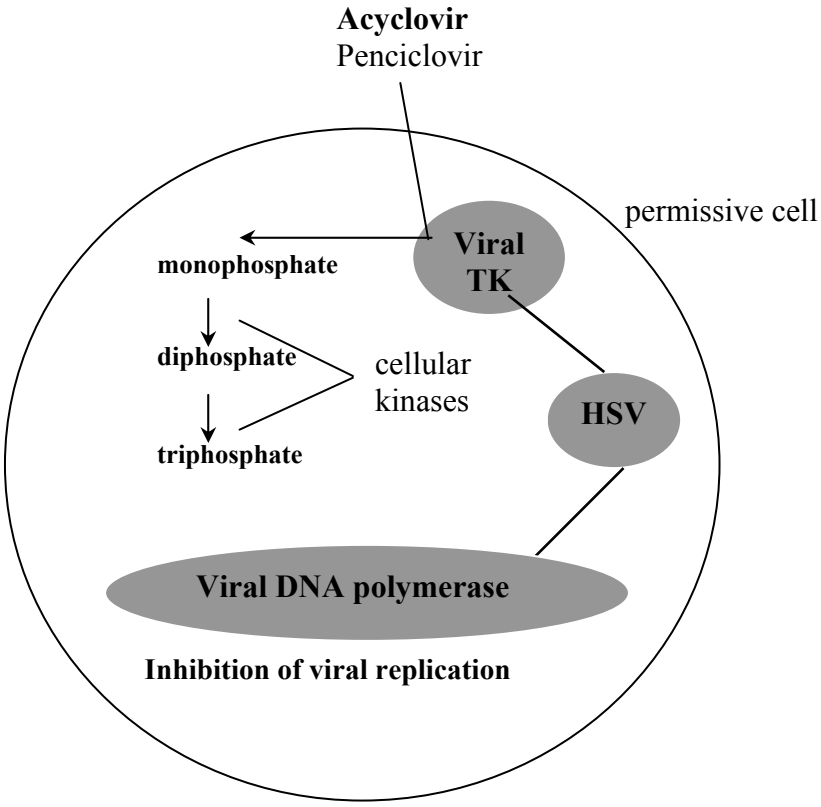


Figure 4.2. *Mechanism of action of antiviral drugs active against HSV*

4.1.2. Nucleoside analogs as substrates of thymidine kinase

Thymidine kinase catalyzes the phosphate transfer from ATP to thymidine (dT) in the presence of Mg^{2+} thus yielding thymidine monophosphate (dTMP) and ADP. Herpes simplex virus type 1 encodes for its own multifunctional TK. Unlike the very specific human cytosolic TK (TK1), it is able to phosphorylate pyrimidine as well as purine analogs[117]. At present, all the antiviral agents that are available for the treatment of herpes virus infections are nucleoside analogs: either

acyclic guanosine analogs (aciclovir (ACV), penciclovir (PCV), ganciclovir (GCV),) or their oral prodrug forms (valaciclovir (VACV), famciclovir (FCV) and valganciclovir) or thymidine analogs such as brivudin (*E*-5-(2-bromovinyl)-2'-deoxyuridine (BVDU), all being substrates of herpes virus TK[116,118]. Aciclovir is the drug of choice against HSV infections. All of these compounds target the viral DNA polymerase, and before they can interact with viral DNA synthesis, however, they need to be phosphorylated intracellularly to the triphosphate form[114].

4.1.3. Non-nucleoside analog inhibitors of herpes simplex virus 1 thymidine kinase

Nucleoside analogs can successfully cure the peripheral manifestations of viral infections, but are unable to prevent the initial viral infection or eradicate latent viral foci in neuronal cells. Aciclovir has a good potency as an antiherpetic drug, although it has rather poor efficacy, being a weak substrate for TK, its triphosphate exhibits a strong chain-terminating effect. As part of the search for novel TK inhibitors, which could be useful chemotherapeutic agents to prevent recurrent herpes infections, a group of compounds based on *N*-phenyl derivatives of guanine have been developed, including (N2-[m-(trifluoromethyl)phenyl]guanine (mCF3-PG))[119-121]. Some of these compounds display good competitive inhibition. The most potent inhibitor of HSV1 TK among the 9-substituted derivatives, 9-(4-hydroxybutyl)-N2-phenylguanine (HBPG), was a competitive inhibitor with respect to the substrate thymidine but was not a substrate for the enzyme[121]. The nucleoside analogs 5-substituted-6-aza-2'-deoxyuridines inhibit the phosphorylation of thymidine by HSV-1 TK but have no effect on the corresponding human enzyme[122]. The 5'-O-Trityl derivative of thymidine has been found to be a non-substrate inhibitor of HSV-1 TK[123]. Acyclovir (ACV) targets viral DNA herpes virus (HSV) polymerases. Before it can interact with viral DNA synthesis, it needs to be phosphorylated intracellularly, in three steps, into the triphosphate form. The first phosphorylation step is ensured by the HSV-encoded thymidine kinase (TK), and is therefore confined to virus-infected cells.

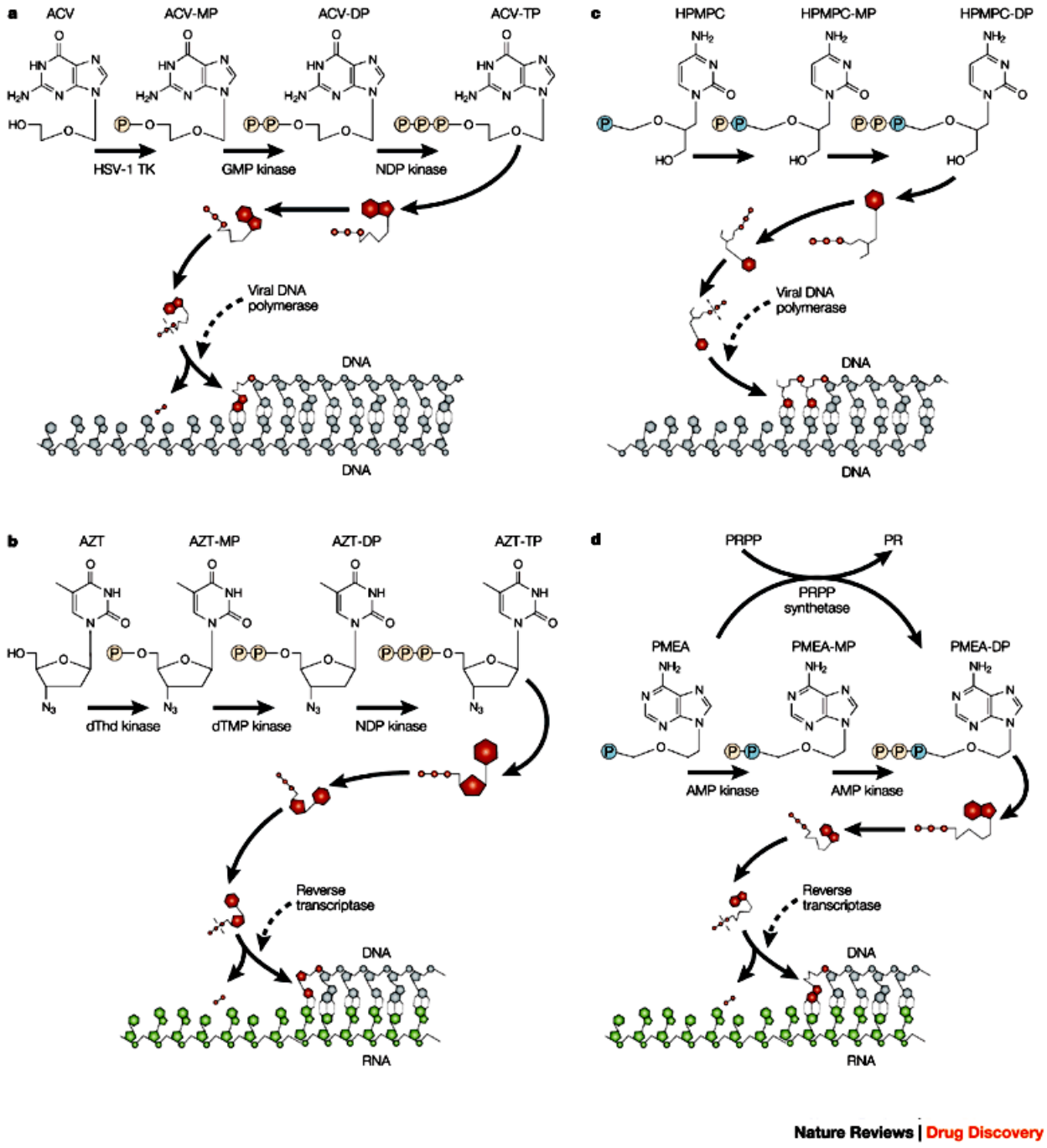


Figure 4.2. *Examples of antiviral nucleoside analogs acting by a chain termination mechanism*[116].

Cidofovir, (*S*-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; HPMPC); Adefovir (9-(2-phosphonylmethoxyethyl)adenine; PMEA); AZT, azidothymidine; DP, diphosphate; dThd,

thymidine; MP, monophosphate; NDP, nucleoside 5'-diphosphate; PR, 5-phosphoribose; PRPP, 5-phosphoribosyl-1-pyrophosphate; TP, triphosphate.

4.2. Development and validation of a capillary electrophoresis method for the characterization of herpes simplex virus type 1 thymidine kinase, its antiviral substrates and inhibitors

4.2.1. Introduction

Thymidine kinase (EC 2.7.121) is the key enzyme in the pyrimidine salvage pathway catalyzing the transfer of the γ-phosphate from ATP to thymidine to produce thymidine 5´-monophosphate in the presence of Mg^{2+} (see Figure 4.3)[107].

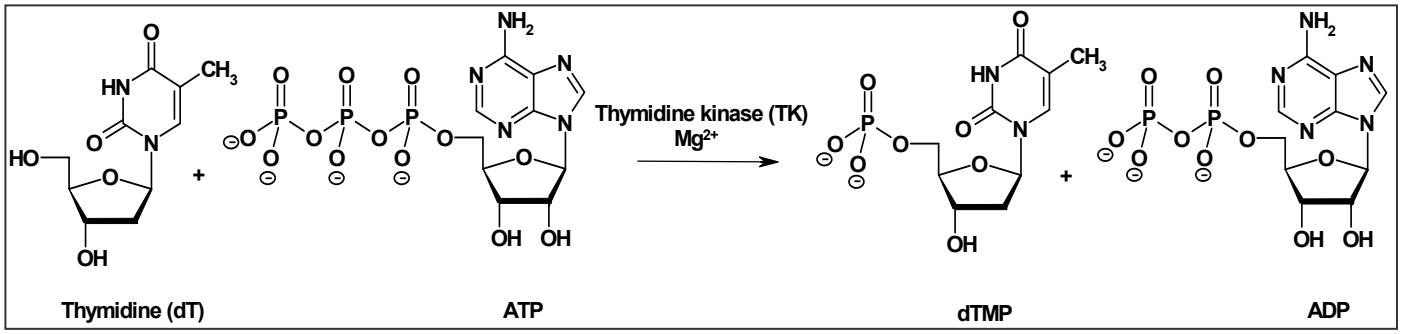


Figure 4.3. *Enzymatic reaction of thymidine kinase*

Herpes simplex virus type 1 encodes for an own thymidine kinase (HSV-1 TK), which has a very broad substrate specificity; it phosphorylates a wide range of substrates, including acycloguanosines (e.g. acyclovir, ganciclovir, penciclovir) and uracil derivatives (e.g. 2'-fluoro-2'-deoxy-1-arabinofuranosyl-5-iodouracil (FIAU))[102,124,125]. Nucleoside analogs are phosphorylated in three steps in HSV-1 infected cells. The first phosphorylation is completed by the virally encoded thymidine kinase, while the second and third phosphorylations to the corresponding di- and tri-phosphates are achieved by cellular kinases. Antiviral nucleoside triphosphate analogs act by competitive inhibition of viral DNA polymerase and they may act as DNA chain terminators[126]. In animal tumour models HSV-1 TK has been used extensively in gene therapy. Improvements in sensitivity of the HSV1-tk reporter gene imaging assay can be achieved either (*i*) by identifying substrates that exhibit higher V_{max}/K_m for HSV1-TK or (*ii*) by engineering TK enzyme(s) with improved V_{max}/K_m for a particular reporter substrate. Decreased V_{max}/K_m of HSV1-TK for thymidine (an endogenous competitor) also should improve HSV1-tk reporter gene assay sensitivity. It has been reported that mutant HSV1-TK enzymes that exhibit greater

cytotoxicity, due to increased substrate V_{max}/K_m, could also be better reporter genes for *in vivo* imaging of gene expression, in combination with positron-labeled acycloguanosines[127].

The compounds among the antiherpetic drugs that have demonstrated efficacy in the treatment of herpes virus infections in animals and humans are 9-[(2-hydroxyethoxy)methyl]guanine (acyclovir, ACV), 9-[(1,3-dihydroxy-2-propoxy-methylguanine (DHPG, ganciclovir), (E)-5-(2-bromovinyl)-2'-deoxuridine (BVDU), and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU)[112,118,124]. These nucleoside analogs have been identified as substrates of the herpes simplex virus type 1 encoded thymidine kinase (HSV-1 TK), but are not recognized as substrates by cellular kinases[128]. Endogenous thymidine within the cell competes with the nucleoside analogs for the active site. The rate of phosphorylation by thymidine kinase at a specific concentration of substrate can be determined by two ways, K_m (binding affinity) and V_{max} (maximum velocity). At low concentrations (equal to K_m) of substrate, the binding affinity plays a key role, but at higher concentrations, V_{max} is the key factor in determinig the phosphorylation[129].

For HSV-1 TK determination of specificity (K_{cat}/K_m) of prodrug toward natural substrate thymidine had been performed by several methods, including radioisotopic[130-132], HPLC[103,104,133] and spectrophotometric[134] assays. Unfortunately, these methods are not suitable for the determination of thymidine kinase in complex biological samples. Radiometric assays require tedious procedures and the use of radiolabeled substrates[125]. As the reaction proceeds, aliquots are removed and measured by liquid scintillation counting[103,135] and also there are risks of contamination and radiation exposure. In addition to the requirement for radioisotopes, the above method requires sampling and analysis of multiple single points per assay, therefore, these are complicated and hence difficult to use for kinetic studies of the substrates of the enzyme. High performance liquid chromatography among the separation techniques has been used for the characterization of new antiviral lead compounds but this technique suffers from relatively high prices of its consumables and also biological matrices which have a tendency to shorten the column life time. Spectrophotometric methods[134] are also employed for the determination of K_{cat} values of HSV-1 TK substrates. These are not suitable because of the use of two enzymes which require their isolation and purification. It is a time-consuming method and not enough sensitive. It also requires high amounts of materials and is prone to interference from other biological matrices.

Capillary electrophoresis (CE) is an excellent and easy analytical technique offering the advantage of high resolution, shorter separation times and consumes extremely small amounts (nanoliters) of sample; in addition, there is no need of radiolabeled substrate. CE offers a high capability of automatization and easily to adopt in high-throughput screening (HTS) assays. CE assays are highly advantageous when the antiviral lead compounds are not availabe in radiolabeled form. It has previously been successfully used for the determination of K_{cat}, K_m and V_{max} of UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase[136].

In the present study, we have developed a capillary electrophoresis method that allows to measure substrates and potential inhibitors for thymidine kinase within a few minutes with high accuracy and reproducibility. The new method is suitable for the high-throughput screening of test compounds.

4.2.2. Materials and methods

4.2.2.1. Materials

Adenosine-5'-triphosphate (ATP), adenosine-5'-diphosphate (ADP), adenosine-5'-monophosphate (AMP), thymidine (dT) and uridine-5'-monophosphate (UMP), (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) and thymidine-5'-monophosphate (TMP) from Sigma (Steinheim, Germany). Magnesium chloride and tris(hydroxymethyl)aminomethane (Trizma Base) were from Sigma (Taufkirchen, Germany). The dipotassium hydrogen phosphate for the CE buffer was obtained from Fluka (Neu-Ulm, Germany). Ganciclovir (GCV) was purchased from Roche, Mannheim, Germany.

4.2.2.2. Expression and purification of TK_{HSV1}

TK_{HSV1} was expressed as gluthathione *S*-transferase fusion protein in competent *Escherichia coli* BL21 using the vector pGEX-6P-2-TK. The protein was purified by gluthathione-affinity chromatography followed by on-column PreScission protease cleavage. Purification was monitored by SDS-polyacrylamide gel electrophoresis and led to a >90% pure $TK_{HSV1.}$ Total protein concentration was measured using the Bio-Rad protein assay.

Recombinant HSV-1 viral thymidine kinase was obtained from Prof. Dr. G. Folkers and Dr. L. Scapozza, Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology Zürich, Switzerland.

4.2.2.3. Capillary electrophoresis apparatus and conditions

A P/ACE capillary electrophoresis (CE) system MDQ glycoprotein (Beckman Coulter Instruments, Fullerton, CA, USA) equipped with a UV detection system coupled with diode-array detector (DAD) was used in this study. The CE-instrument was fully controlled through a PC, which operated with the analysis software 32 KARAT obtained from Beckman Coulter. The evaluation of the electropherograms was done using the same software. The capillary temperature was kept constant at 25 °C. The temperature of the sample storing unit was adjusted to 25 °C.

4.2.2.4. Fused silica capillary with pressure injection: Method A

The electrophoretic separations were carried out using an eCAP fused-silica capillary [40 cm (30 cm effective length) x 75 µm internal diameter (I.D) x 375 µm outside diameter (O.D) obtained from Beckman Coulter]. On-line UV detection was performed in the range of 190-350 nm. The runs were performed under the following conditions: T = 25 °C, λ_{max} = 260 nm, current = 95 µA, running buffer 20 mM sodium phosphate buffer, pH 7.5. The capillary was washed with 0.1 N NaOH for 2 min, deionized water for 1 min, and running buffer for 1 min before each injection. Injections were made by applying 0.1 psi of pressure to the sample solution for 25 s. Sodium phosphate buffer (20 mM), pH 7.5, was used for the separations.

4.2.2.5. Neutral capillary with electrokinetic injection: Method B

The electrophoretic separations were carried out using an eCAP coated neutral capillary [(30 cm (20 cm effective length) x 50 µm internal diameter (I.D.) x 360 µm outside diameter (O.D.) and (30 cm (20 cm effective length) x 75 µm internal diameter (I.D.) x 360 µm outside diameter (O.D.), obtained from CS-Chromatographie (Langerwehe, Germany)].
An electrokinetic injection for 30 seconds was applied for introducing the sample into the capillary. The separation was performed using an applied current of -60 µA and a data acquisition rate of 8 Hz. Analytes were detected using direct UV absorbance at 210 nm. The capillary was conditioned by rinsing with water for 1 min and subsequently with buffer (phosphate 50 mM, pH 6.5) for 1 min. Sample injections were made at the cathodic side of the capillary.

4.2.2.6. Preparation of standard solutions for method validation

10 mM stock solutions of nucleotides (TMP, UMP and GMP) were prepared by dissolving the compounds in deionized water. Then they were further diluted to get 1 mM stock solutions of

nucleotides in deionized water. The 1 mM stock solutions were further diluted for the standard calibration curves in reaction buffer containing 20 mM Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and 1 mM ATP. A series of standards of nucleotides was obtained from the stock solutions by dilution with the same buffer over a concentration range from 0.01 to 50 µM. UMP (20 µM) was used as an internal standard for method validation of TMP, while cAMP was used as an internal standard for the method validation of GMP. Injections of standards were performed in triplicate. The calibration curve was obtained by plotting the corrected peak area of the TMP peak against its concentration.

4.2.2.7. Investigation of the influence of reaction time on enzyme velocity

A set of experiments was performed in order to investigate whether the reaction proceeds linearly with time for a fixed concentration of substrate, using method A. The reaction mixture for the determination of the enzymatic velocity over different intervals of time contained: 2 mM thymidine, 50 mM Tris-HCl buffer, pH 7.2, 5 mM ATP and 5 mM $MgCl_2$. The reaction was started by adding 10 µl of thymidine kinase (4 µg). The reaction was carried out at 37 °C and after various time intervals; the reaction mixture was heated for 5 min at 99 °C to stop the reaction. Then 45 µl of the solution were transferred to a small CE vial containing 5 µl of an aqueous UMP solution (200 µM) as an internal standard. At each time interval three injections were made.

4.2.2.8. Effects of Mg^{2+} on the activity of thymidine kinase

Effects of different concentrations of Mg^{2+}, 0.5, 1.0, 2.5, 5 and 10 mM of Mg^{2+} on the enzyme activity were monitored, using method B. The reaction mixture contained 1 mM ATP, 50 µM thymidine, 20 mM Tris-HCl buffer, pH 7.1, and different concentrations of Mg^{2+}. The reaction was started by adding 10 µl of suitably diluted thymidine kinase (4 µg) and the mixture was incubated at 37 °C for 35 min, and then stopped by heating at 99 °C for 5 minutes. The TMP formed was measured in duplicate by capillary electrophoresis.

4.2.2.9. Capillary electrophoresis method for determination of kinetic parameters of HSV-1 thymidine kinase substrates

Kinetic parameters of HSV-1 TK substrates were determined by a modification of the previously published protocol[134], using method B. In the enzyme assay tube containing 100 µl of total

volume, the enzyme was preincubated at 37°C for 2 min in a reaction mixture containing 20 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM ATP and different concentrations of the substrates ranging from 15.625 to 1000 µM. The reactions were initiated by adding 10 µl of the HSV-1 thymidine kinase to give a final amount of 2-4 µg and then allowed to proceed at 37°C for 15 min. After incubation of the reaction mixture at 37°C for 15 min, it was immediately heated to 99 °C for 5 min using an Eppendorf Thermomixer Comfort in order to inactivate the enzyme, 45 µl of the reaction mixture was transferred to the mini CE vials containing 5 µl of internal standard (UMP for dT, BVDU and cAMP for ACV, GCV). The final concentration of the internal standard in the assay was 20 µM. The absorbance at 210 nm was monitored continuously and the nucleotide concentrations were determined from the area under each absorbance peak. The experiments were repeated 3 times. Control experiments were performed in the absence of substrate and in the absence of enzyme in order to take into account the spontaneous hydrolysis of ATP under the experimental conditions.

4.2.2.10. Capillary electrophoresis method for screening of potential inhibitors of herpes simplex virus -1 thymidine kinase

HSV-1 TK inhibition assays were performed as described by a modification of the published protocol[102], using method B. Thymidine phosphorylation was measured in the absence and presence of inhibitor (aciclovir) at three different concentrations (0.0625, 0.5 and 5 mM). In addition to the substrate and inhibitor, the reaction mixture contained 20 mM Tris buffer, pH 7.4, 5 mM $MgCl_2$ and 1 mM ATP in a final volume of 100 µl. Reactions were incubated at 37°C for 2 min and then 4 µg of HSV-1 thymidine kinase was added in order to initiate the reaction, similarly as for the substrate assay (see above). The reaction was stopped after 15 min by heating to 99°C for 5 min.

4.3. Results and discussion

4.3.1. Optimization of nucleotide analysis by capillary electrophoresis

Our initial studies were done using a fused silica capillary, however, with subsequent experiments we observed that in enzyme assays it is important to use a coated capillary to avoid adsorption of protein and analytes to the capillary wall. In case of an untreated fused silica capillary, a strong EOF in the direction from the anode to the cathode exists, which significantly increases the migration time of nucleotides and in some cases reverses the direction[24]. In

untreated fused silica capillary columns reproducibility was achieved by subsequent rinsing of the capillary with 0.1 N NaOH; such strong alkaline solution is required to remove the cations from the capillary wall to provide the same silica surface for each run[34]. Thymidine kinase is dependent on Mg^{2+} for its activity. In the fused silica capillary it was not possible to use 5 mM or even a higher concentration of Mg^{2+}, whereas 5 mM Mg^{2+} was optimal for the enzyme assay. Higher concentrations of Mg^{2+} gave broad peak shapes and poor resolution. Therefore, we used a polyacrylamide-coated capillary in further investigations. Capillary electrophoretic separations can be optimized with respect to efficiency, selectivity, and running time. CE is still in development, therefore there is a lack of systematic approaches for optimizing experimental conditions. The separation and selectivity in CE depends on the buffer type, buffer ionic strength, buffer pH, column diameter, capillary length and operating voltage. In method B, for high resolution, sodium phosphate buffer pH 6.5 was used as a separation buffer because the nucleotides were relatively stable at higher pH, but readily hydrolyzed at low pH values[27]. Sodium phosphate buffer is the preferred buffer for the separation of nucleotides[13], because it gives high efficiency and resolution in short analysis times. The resolution in CE is affected by the electroosmotic flow (EOF), which is due to the high buffer flow caused by the electrical double layer on the inner surface of the capillary. In the present study polyacrylamide-coated fused silica capillary columns were used to get high reproducibility and resolution. Ucon-coated and polyacrylamide-coated fused silica capillary columns have been used for the separation of nucleotides[13,27,37]. It was found that the electroosmotic flow of these columns was significantly reduced and coating was inert to biological samples[13,30]. Reproducibility of migration time of coated capillary columns is significantly higher than that of untreated fused silica capillary columns as shown in table 4.1. In coated capillaries rinse buffer has to be applied to prepare the capillary for the next run. A shorter capillary length (30 cm (20 cm effective length)) was used in order to reduce the analysis time (Figure 4.4) and for improving the rinsing cycles because in shorter capillary more rinsing solution could be passed through within the same period of time. The eluting sequence was triphosphates, diphosphates, and followed by monophosphates. Due to the relatively high detection limits of CE because of a small internal diameter of 50-75 μm of the capillary this method lacked the sensitivity to perform assays for determining a full Michaelis-Menten analysis[137] of substrates having low K_m values like dT. In such cases sample preconcentration could be used to increase the sensitivity. Special detectors, such as laser-induced fluorescence and electrochemical detectors, are one solution for the problem of low

sensitivity of CE. However, another simple alternative is sample stacking. It is very convenient to utilize the electrical current not just for separation but also for sample concentration directly in the capillary[138,139]. The electrokinetic injection method was used to increase the sensitivity by on-line preconcentration as compared to the hydrodynamic injection. Stacking by hydrodynamic injection requires a long time for sample transfer, which can exceed the separation time. Another approach is to increase the capillary internal diameter in order to increase the sensitivity. Therefore, a 75 µm internal diameter capillary was used. However, the precision of migration time was reduced with the larger internal diameter of the capillary (Table 4.1). In previous studies it was observed that by increasing the time for the electromigration to 60 s (at 5 kV) gives a concentration of about 120 times greater than that obtained by hydrodynamic injection under non-stacking conditions[138]. The limits of detection and quantitation by both electrokinetic injection and hydrodynamic injection methods are given in table 4.1. In the previous enzymatic studies[13] a similar method was developed using a longer capillary length (60 cm (50 cm effective length)).

Table 4.1. *Method validation parameters: Limits of detection and quantitation, precision of migration time, peak area and linearity of the standard calibration curve.*

Compound	**dTMP 50 µm NC**[a]	**dTMP 75 µm NC**	**dTMP 75 µm FC**[b]	**UMP 75 µm FC**	**GMP 50 µm NC**
Limit of detection (µM) ± SD[c]	0.36 ± 0.20	1.03 ± 1.4	2.60 ± 0.60	2.16 ± 0.38	0.86 ± 0.13
Limit of quantification (µM) ± SD	1.08 ± 0.20	3.11 ± 1.4	6.80 ± 0.60	3.77 ± 0.38	3.31 ± 0.13
Coefficient of correlation; R^2	1.000	0.990	0.970	0.998	0.999
Mean value of migration time (min) ± SD (n= 12)	6.11 ± 0.01	7.96 ± 0.042	5.85 ± 0.15	6.08 ± 0.16	6.34 ± 0.02
RSD[d] of migration time (%)	0.16	0.53	2.56	2.63	0.32

[a]NC = neutral capillary

[b]FC = fused silica capillary

[c]SD = standard deviation

[d]RSD = relative SD

In the present study we used a shorter capillary length (30 cm (20 cm effective length)), and therefore, there was no need to dilute the sample prior to injection. The method was also optimized with regard to injection time and the applied voltage. Longer injection times give low reproducibility while very short injection times decrease the sensitivity. Different voltages and injection times like -10 kV for 5 sec, -6 kV for 15 sec and -6 kV for 30 sec were used with this shorter capillary length. The latter method was selected for the enzyme assays due to high sensitivity and reproducibility. The assay buffer concentration was optimized for electrokinetic injection, 20 and 50 mM Tris-HCl buffer, pH 7.4 was used; no difference in thymidine kinase activity was found. 20 mM Tris-HCl buffer, pH 7.4 was selected for the assay, because low concentration of the sample buffer improves the sensitivity in electrokinetic injection[138-140]. Chloride ions and other electrolytes in the buffer have faster mobility and thus, concentrate ahead of the analytes at the tip of the capillary thus decreasing the field strength and consequently decreasing the amount of the analyte being injected.

4.3.2. The use of an internal standard

The use of an internal standard (IS) has been generally accepted to be crucial for reproducibility in CE to compensate for injection errors and minor fluctuations of the migration times. The major source of imprecision remaining when using commercial CE instrumentation is injection volume variability. Therefore, the use of an IS will improve precision of quantification[141]. An ideal IS is water-soluble, and UV-detectable at 210 nm. Moreover, the migration time of the IS should differ from the migration times of the reaction compounds and not prolong the total analysis time. UMP, being UV-detectable at 210 nm, was chosen as an internal standard for the determination of kinetic parameters of thymidine and BVDU with a migration time of less than 8 min, while cAMP was used as an internal standard for ACV and GCV. As illustrated in table 4.1, with the use of the IS, the RSD of migration time after 12 runs was calculated to be 0.16, 0.53 and 2.56% for the coated capillary 50 µm ID, the coated capillary 75 µm ID and the untreated capillary 75 µm ID for dTMP, respectively, and 2.63 % with the untreated capillary of 75 µm ID for UMP.

4.3.3. Washing and re-equilibration

Using a coated capillary the overall rinsing time is reduced. The capillary was conditioned every day by rinsing it with water for 20 minutes before starting measurements and then with separation buffer for 5 minutes. The standard rinsing periods consisted of rinsing with water for one minute

followed by rinsing with buffer for one minute. The capillary was stored in vials containing separation buffer inside the CE-instrument overnight after rinsing with water. For rinsing procedures we applied 40 p.s.i. of pressure for buffer and water. Under this standard rinsing method, the capillary could be used for several hundred runs.

4.3.4. Validation of the capillary electrophoresis method

4.3.4.1. Limits of detection, quantitation and linearity

Three different capillary columns with different internal surfaces and diameters were used for the determination of limits of detection, quantitation and linearity of dTMP, while validation parameters for GMP were obtained with a neutral capillary of 50 µm ID. The concentrations of dTMP in this study were 0, 0.1, 0.5, 1, 5, 10 and 50 µM with the neutral capillary and 0, 2, 5, 10, 20, 30 and 40 µM for the fused silica capillary. CE employing a neutral capillary with electrokinetic injection gave low detection limits as compared with a fused silica capillary with pressure injection, therefore, low concentrations were used with the neutral capillary. UMP (20 µM) was used as an internal standard. The concentrations of GMP used in this study were 0, 1, 5, 10, 20 and 50 µM. Each sample was measured four times. Each peak area for different concentrations was entered into a diagram as a function of concentration. The limit of detection and quantitation for dTMP with polyacrylamide-coated capillaries of 50 and 75 µm ID were 0.36 and 1.03 µM, respectively which are low as compared to the fused silica capillary (2.60 µM). The 75 µm (ID) polyacrylamide-coated capillary showed an unexpectedly high detection limit along with a higher standard deviation of migration time. It could be reasoned that with high ID electrokinetic injection could inject more buffer ions and other species into the capillary and thus resulted in a more unfavorable detection and quantitation limit. May be due to this reason many CE instrument companies (like Beckmann Coulter) do not produce 75 µm ID diameter coated capillaries. The regressive linearity coefficients with a 50 µm ID polyacrylamide-coated capillary, 75 µm ID polyacrylamide coated capillary and 75 µm ID untreated fused silica capillary for dTMP were 1.000, 0.990 and 0.970, respectively. Measurement with a 50 µm ID polyacrylamide-coated capillary proved to exhibit a good linearity of calibration curve in the range from its lowest determined concentration to the highest concentration in the enzyme assays. The linearity of calibration curve equation with a 50 µm ID polyacrylamide-coated capillary for the measurement of dTMP was determined as $y = 40980 x - 8254$ ($R^2 = 1.000$) and the relative standard error of the slope was 2.02 %, whereas relatively poor linearity was observed in the 75

μm ID coated capillary. The limit of detection and quantitation for GMP with polyacrylamide-coated capillaries, 50 μm ID, was 0.86 and 3.31 μM. The mean values and standard deviations were calculated as shown in table 4.1.

4.3.4.2. Repeatability of migration time

The repeatability of migration time was calculated by injecting dTMP and GMP solutions made in separation buffer for 12 times. A high repeatability was found with coated capillaries, while it was lower for untreated capillaries (Table 4.1). The reason for the good precision and reproducibility in migration times for neutral capillaries is due to the absence of an EOF. In addition, the coated capillary is very inert to biological samples, which increased the reproducibility of the analysis.

4.3.5. Biological assays

4.3.5.1. Michaelis-Menten analysis

A typical single substrate based enzyme-catalyzed reaction can be expressed as:

$$\mathbf{E + S} \underset{}{\overset{K_1/K_{-1}}{\leftrightarrow}} \mathbf{ES} \overset{K_{cat}}{\leftrightarrow} \mathbf{E + P} \quad (1)$$

where E is the enzyme, ES is the intermediate enzyme-substrate complex, S is the substrate and P is the product. When all of the enzyme is in the ES state the initial reaction velocity v reaches its maximum, V_{max}. The relation between initial reaction velocity v and substrate concentration [S] can be described by the Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

where v is the initial velocity of the reaction, V_{max} is the maximum velocity, [S] is the substrate concentration and K_m is the Michaelis constant, the substrate concentration at half of the maximum of the velocity. The Lineweaver-Burk plot is obtained by inversion of this equation and is far more accurate and convenient to use experimentally, because of its linear relation between $1/v$ and $1/[S]$:

$$1/v = \frac{K_m}{V_{max} + [S]} + \frac{1}{V_{max}} \quad (3)$$

4.3.5.2. Determination of K_m and K_{cat} values

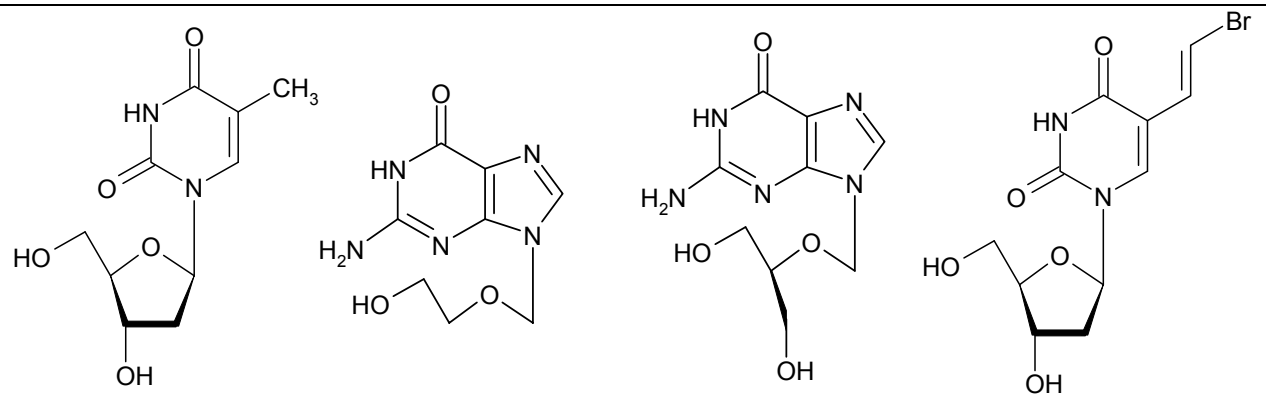
Binding affinity (K_m) and catalytic constant (K_{cat}) of the substrate are both important parameters. The catalytic constant (K_{cat}) is the measure of the turnover rate of the enzyme. An increased (K_{cat} / K_m) for the substrate toward the natural substrate dT correlates well with enhanced production of the active, phosphorylated drug within the cell[111]. Different concentrations of the substrates in the range of 15.625 –1000 μM were used in order to determine their kinetic parameters.

The K_{cat} values were calculated directly from V_{max} using the equation (4):

$$K_{cat} = V_{max} / [E] \quad (4)$$

where [E] is the final active enzyme concentration.

Table 4.2. *Kinetic analysis of substrates of HSV-1 thymidine kinase*



Thymidine (dT) **ACV** **GCV** **BVDU**

Substrate	Capillary electrophoresis[a]		Literature values	
	K_m (µM)	K_{cat} (S^{-1})	K_m (µM)	K_{cat} (S^{-1})
dT	n.d[d]	0.016 ± 0.003	0.2[109b], 0.38[142c], 0.9 ± 0.08[143c]	0.44[111b], 0.35 ± 0.004[133b]
ACV	238 ± 94	0.011 ± 0.002	200[109b 103], 417[142]	0.02[132c], 0.13[111b]
GCV	27.2 ± 18.6	0.12 ± 0.05	69±4[143c], 47.6[142c], 48[111b],	0.1 ± 0.017[133b], 0.10[111b]
BVDU	25.3 ± 13.4	n.d[e]	50[109b]	

[a] n = 3

[b] determined by standard spectrophotometric assays.

[c] determined by standard radiometric assays.

[d] n.d = could not be determined due to low K_m value and the limited sensitivity of the assay.

[e] n.d = could not be determined due to unavailability of isolated reaction product BVDUMP for measuring a calibration curve.

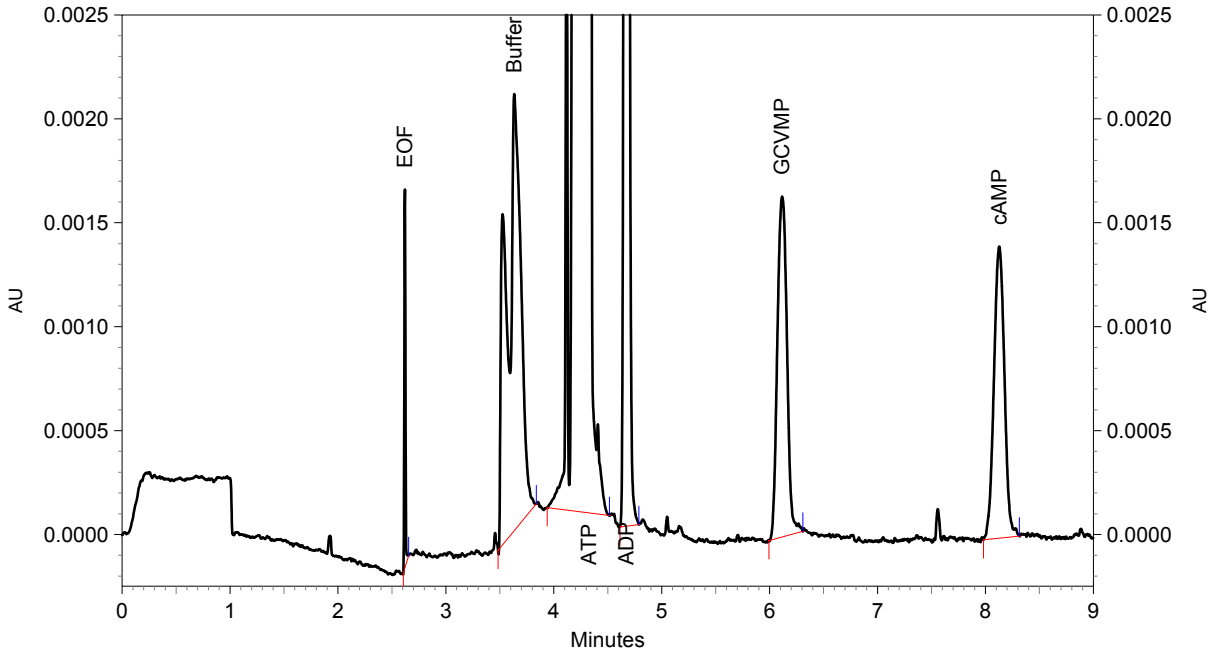


Figure 4.4. *Typical electropherogram for the determination of K_{cat} of GCV. The concentration of thymidine kinase was 4 µg/µl of protein; GCV: 500 µM, ATP: 1 mM, cAMP (internal standard): 20 µM. CE conditions: running buffer: 50 mM potassium phosphate, pH 6.5; constant current of -60 µA; detection at 210 nm, capillary cartridge temperature: 25 °C.*

The substrates were phosphorylated to the corresponding monophosphates by HSV-1 TK, as monitored by capillary electrophoresis.

The K_m and K_{cat} values were calculated by fitting the initial reaction rate for the formation of product (phosphorylated substrates) as a function of substrate concentration into the Michaelis-Menten equation (**3**). An average value of 238 µM (n = 3, SD = 94) from three independent measurements was determined to be the K_m of ACV for HSV-1 thymidine kinase (see Table 4.2). Figure 4.5 shows the Michaelis Menten plot of ACV at a HSV-1 TK concentration of 4 µg in the assay. The other K_m values thus obtained were K_m = 27.2 µM for GCV (n = 3, SD = 18.6) and K_m = 25.3 µM for BVDU (n = 3, SD = 13.4) (Table 4.2). The Michaelis constant (K_m) for HSV-1 TK substrates cannot be compared exactly with the K_m values from other studies, as the latter ones employed different analysis methods and K_m values can vary depending on the procedure for the purification of HSV-1 TK[132]. However, our results were in the same range as the reported values obtained by spectrophotometric and radiometric assays for ACV[103,109,144], GCV[80,132,144] and BVDU[80,132,133].

The K_{cat} values were calculated using the equation $V_{max} = K_{cat}/[E]$, where [E] is the total enzyme concentration and is based on one active site per monomer. Calculating the V_{max} values based on the product requires a calibration curve to convert the product peak areas of the reaction to the product concentration. Due to the unavailability of isolated reaction products, we could not obtain a product calibration curve directly. However, the V_{max} values were calculated using the calibration curve of thymidine monophosphate for dT, while for ACV and GCV, GMP was used which has no significant difference in UV absorption as compared to the phosphorylated substrates of ACV and GCV. The K_{cat} values of dT, ACV and GCV were determined as 0.0160 (n = 3, each concentration, SD = 0.003), 0.011 (n = 3, each concentration, SD = 0.002), and 0.12 (n = 3, each concentration, SD = 0.05), respectively (Table 4.2). Due to the limited sensitivity of the assay, the K_m value of dT could not be determined. The K_{cat} value of BVDU was not determined due to the unavailability of isolated reaction product for determining a calibration curve. However, K_m could be determined from the added concentration of BVDU.

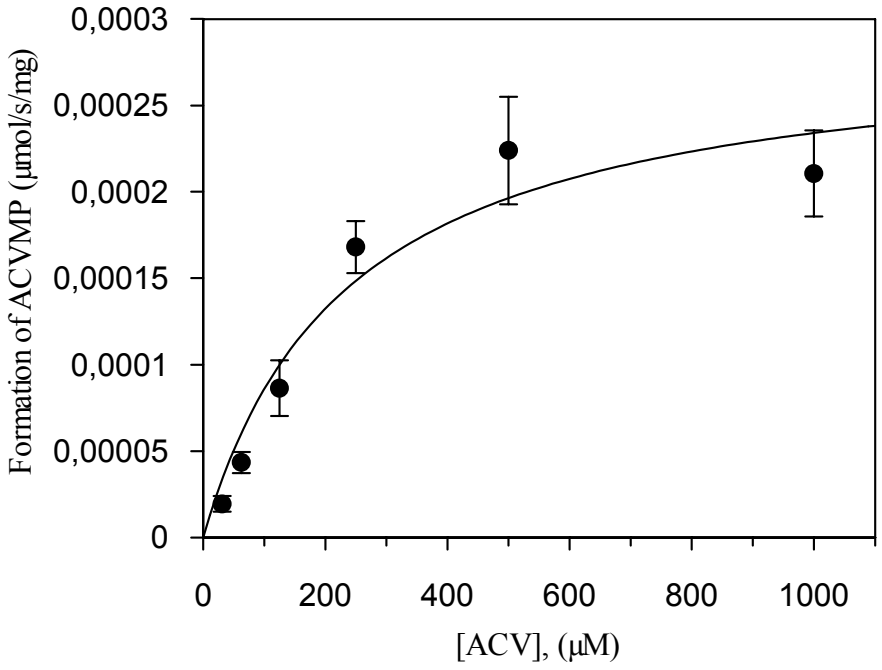


Figure 4.5. *Michaelis-Menten plot of initial ACV concentration with respect to the reaction velocity (µmol of ACVMP produced in one second per mg of enzyme) for the determination of K_m and K_{cat} values by the capillary electrophoresis method. Assay was conducted at pH 7.4 using 1 mM ATP, 5 mM $MgCl_2$ and various concentrations of ACV. Data points represent means ± SD from three separate experiments each run in duplicate.*

The activity of thymidine kinase as a function of various concentrations of $MgCl_2$ had been examined[45,95]. Mg^{2+} was found as a co-substrate of this enzyme. Thymidine kinase showed maximal activity at a concentration of 5 mM of $MgCl_2$. After increasing the concentration of $MgCl_2$ above this limit, there was no further increase in the activity of thymidine kinase, whereas lower concentrations of $MgCl_2$ resulted in reduced enzymatic activity (Figure 4.6). Thymidine kinase did not show any activity in the absence of magnesium, it showed an absolute requirement of magnesium. The dependence of the thymidine kinase activity on Mg^{2+} and ATP suggests that the ATP-Mg complex is the true substrate for the enzyme.

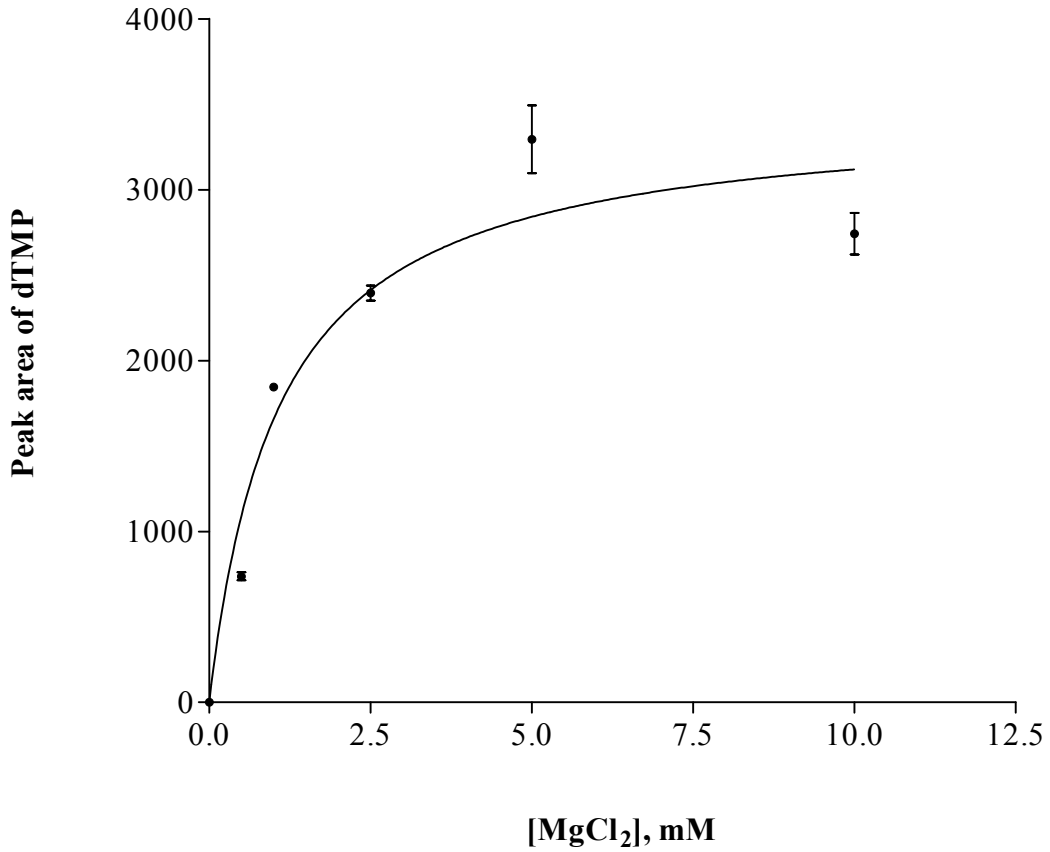


Figure 4.6. *Effects of different $MgCl_2$ concentrations on the activity of thymidine kinase at a fixed concentration of ATP, determined by capillary electrophoresis. K_m was determined at pH 7.1 using 1 mM ATP, 50 µM thymidine and various concentrations of $MgCl_2$ (0.5 to 10 mM). The separation conditions were 50 mM phosphate buffer, pH 6.5, a neutral capillary, 30 cm length (20 cm to the detector), 50 µm I.D.; 60 µA; 25 °C; detection at 210 nm; electrokinetic injection*

for 30 s at –6 kV. Data points represent means ± SD from three separate experiments each run in duplicate.

4.3.6. Enzyme inhibition assay

The assay results of HSV-1 TK with the competitive inhibitor ACV are presented in table 4.3 as percent inhibition of phosphorylated thymidine (dTMP). The reactions were performed with varying ACV concentrations while the substrate, thymidine, was held constant at 100 µM, and the preparation of the HSV-1 TK enzyme was 4 µg in a total assay volume of 100 µl. As the concentration of ACV increased, the percent formation of dTMP decreased, as shown in table 4.3.

Table 4.3. *Percent phosphorylation inhibition of thymidine kinase by ACV determined by capillary electrophoresis (CE). All values are means ± S.E.M of three separate experiments.*

No.	Concentration of ACV (mM)	Phosphorylation of dT (%)[a] (n = 3) [b]
1	0 (control)	100 ± 0
2	0.0625	97 ± 5.4
3	0.5	71 ± 1.6
4	5.0	62 ± 0.8

[a]Percentage of phosphorylated product obtained without ACV was set at 100%

[b]All values are means ± S.E.M of three separate experiments

In the presence of 0.5 and 5 mM of ACV the dTMP formation was reduced by 29 and 38 %, respectively.

4.4. Summary

We investigated and developed a very simple and robust method for the determination of substrates of HSV-1 TK which are used as antiviral drugs, and their binding affinity (K_m) and catalytic constant (K_{cat}/K_m). This CE method is also very useful for the investigation of novel potential inhibitors HSV-1 TK. The phosphorylated products of the substrates for the reaction of

HSV-1 TK could be separated successfully. Furthermore, enzymatic kinetics and inhibition assays were easily performed. Determination of K_m and K_{cat} by the new capillary electrophoresis method has multiple advantages such as no need for expensive radiolabeled substrates and a minimal use of reagents. The assay is fully automated and can be carried out in less then 10 min. CE has particular advantage over other techniques in that both, loss of substrate as well as the increase in product(s), can be monitored simultaneously. With the development of combinatorial chemistry in drug discovery, a simple, non-radioactive and automated screening assay like this will be essential for the discovery process.

Chapter 5. Capillary electrophoresis assays of nucleoside triphosphate diphosphohydrolases

5.1. Introduction

Extracellular nucleotides such as ATP, ADP, UTP and UDP, and a variety of diadenosine polyphosphates (Ap_nA) act as extracellular signalling molecules in virtually all tissues and can be inactivated by hydrolysis via ectonucleotidases. In particular, extracellular ATP is involved in a large variety of physiological and pathological functions. These involve roles for ATP in the following areas: (1) as a neurotransmitter and neuromodulator in the central and peripheral nervous system; (2) as a key messenger in nociception; (3) as a tumour-inhibiting agent; (4) in the control of secretion from a variety of endocrine glands; (5) in the modulation of platelet aggregation by ADP; (6) in Cl^- transport in the airway epithelia; (7) in renal function; and (8) in bone and cartilage disease[145]. Nucleotides are hydrolyzed by an extracellular hydrolysis cascade of surface-located enzymes that results in the formation of the respective nucleoside and free phosphate[146]. Nucleoside 5-tri- and –diphosphates may be hydrolyzed by members of the E-NTPDase family (ectonucleoside triphosphate diphosphohydrolase family), the E-NPP family (ectonucleotide pyrophosphatase/phosphodiesterase family), and by alkaline phosphatases. Nucleoside 5-monophosphates are subject to hydrolysis by ecto-5-nucleotidase and also by all alkaline phosphatases. Members of the various ectonucleotidase families reveal overlapping substrate specificity and tissue distribution whose functional significance needs to be further elucidated[146].

5.1.1. The ecto-nucleoside triphosphate diphosphohydrolases family

Members of ecto- nucleoside triphosphate diphosphohydrolases (E-NTPDase) family can hydrolyze nucleoside 5'-triphosphates and nucleoside 5'-diphosphates albeit with varying preference for the individual type of nucleotide. Therefore they represent ecto-nucleoside 5'-triphosphate diphosphohydrolases[147,148]. All members of the family share five highly conserved sequence domains (pyrase conserved regions) that presumably are of major relevance for their catalytic activity[147]. All nucleotidases belonging to the E-NTPDase family contain the actin-hsp 70-hexokinase β - and γ -phosphate binding motif[148]. Members of the E-NTPDase family may be separated into two groups according to their presumptive membrane topography (Figure 5.1).

Members of the first group include E-NTPDase1 to 4 and are predicted to have a transmembrane domain at the N- and at the C-terminus. Of the second group, including NTPDase5 and putative NTPDase6, NTPDase5 lacks the C-terminal hydrophobic domain. Its N-terminal hydrophobic leader sequence is cleaved, resulting in a soluble and secreted form of the enzyme. The enzymes hydrolyze not only ATP or ADP but have in common broad substrate specificity towards purine and pyrimidine nucleotides. They differ, however, regarding their preference for nucleotide 5'-tri- and nucleotide 5'-diphosphates[149,150].

Table 5.1. *Nomenclature of vertebrate members of the E-NTPDase (ecto-nucleoside triphosphate diphosphohydrolase) family*[151,152].

Name	Previously used names	Human gene symbol	Species (accession number)
NTPDase1	CD39, ecto-ATP diphosphohydrolase, ecto-apyrase	ENTPD1	human (S73813, U87967, AJ133133); rat (U81295); mouse (AF037366); bovine (AF005940); porcine (AJ133746)
NTPDase2	CD39L1, ecto-ATPase	ENTPD2	human (U91510, AF144748); rat (Y11835; AF276940); mouse (AF042811); chicken (U74467)
NTPDase3	CD39L3, HB6	ENTPD3	human (AF034840, AF039917); chicken (AF041355)
NTPDase4	UDPase (hLALP70v), hLALP70	ENTPD4	human (AF016032, AJ131358)
NTPDase5	CD39L4, ER-UDPase	ENTPD5	human (AF039918); mouse (AJ238636, AF006482)
NTPDase6	CD39L2	ENTPD6	human (AF039916); rat (AJ277748)
NTPDase7		ENTPD7	human (AAF90135)
NTPDase8		ENTPD8	mouse (AY364442); rat (AY536920)

NTPDases 1, 2, 3, and 8 are oligomeric cell surface membrane proteins. NTPDases 4, 5, 6 and 7 are intracellular membrane proteins.

Table 5.1. provides the new nomenclature for vertebrate NTPDases together with previously used names as well as the new human gene symbols and the accession numbers (including variants). Note that the family name is E-NTPDase and the name of individual enzymes NTPDase1 to NTPDase6. The accession numbers are given for the human enzymes as well as for the vertebrate species ortholog. The functional expression of NTPDase1 to NTPDase6 has been verified for at least one of the sequences listed.

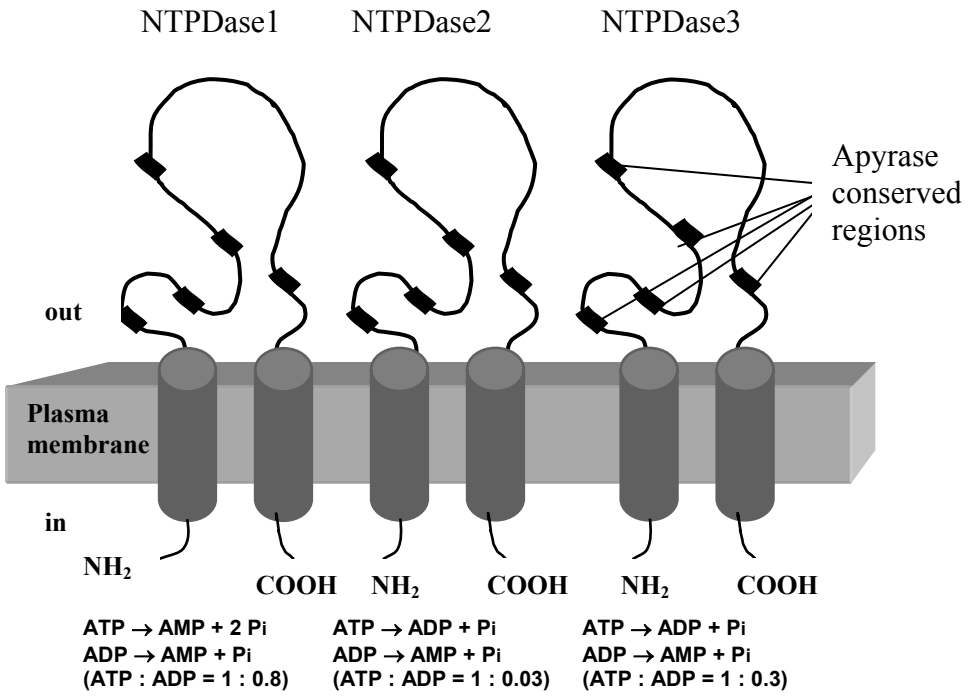


Figure 5.1. *Membrane topography and catalytic properties of NTPDase1 to 3 of the E-NTPDase family. The apyrase conserved regions are situated in the N-terminal half of all sequences. The catalytic properties are not restricted to the examples given but include other purine and pyrimidine nucleoside 5'-tri- or -diphosphates. The ratios indicate the relative preference of the ecto-nucleotidases for ATP and ADP.*

5.1.2. General properties of nucleoside triphosphate diphosphohydrolases1-3

Members of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family are dominant ectonucleotidases. These three closely related enzymes namely NTPDase1, NTPDase2 and NTPDase3 are located at the surface of the plasma membrane and hydrolyze nucleotides in

the range of concentration that activate P2 receptors[151]. NTPDase1 (CD39, ecto-apyrase, ecto-ATP diphosphohydrolase) hydrolyzes ATP and ADP at a molecular ratio of about 1:0.5 to 1:0.9[149]. In contrast, NTPDase2 (CD39L1, ecto-ATPase) has a strong preference for ATP with molecular ratios of ATP : ADP of 1 : 0.03 or less. NTPDase3 (HB6) is a functional intermediate and reveals a molecular ratio of ATP : ADP of approximately 1:0.3. The activity of all three types of ecto-nucleotidases depends on millimolar concentrations of divalent cations such as Ca^{2+} or Mg^{2+} [148-150]. Four out of the eight members of this family, namely NTPDases1, 2, 3 and 8 have two plasma membrane spanning domains with an active site facing the extracellular milieu[151].

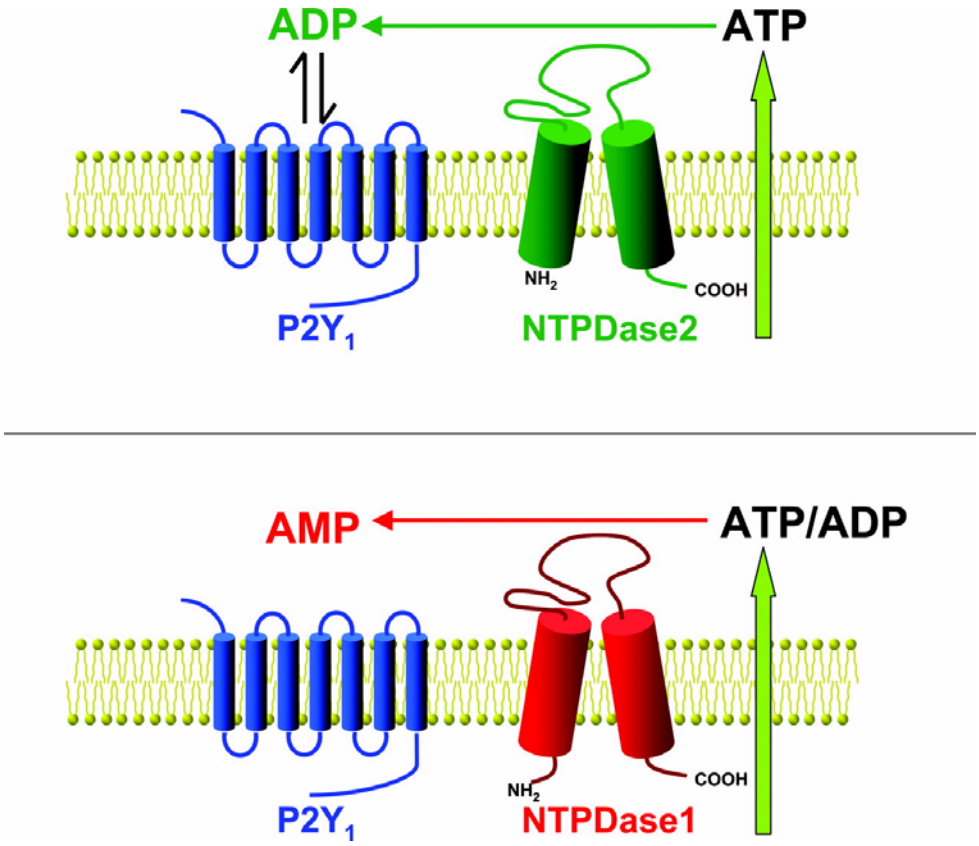


Figure 5.2. *Formation of the cognate agonist of the $P2Y_1$ receptor by NTPDase2 and inactivation of $P2Y_1$ receptor-activating nucleotides by NTPDase1. The scheme illustrates the release of cellular ATP and its conversion by NTPDase2 into the cognate agonist ADP for the $P2Y_1$ receptor (top) and the release of cellular ATP and the conversion of ATP (and ADP) to AMP (bottom), which is not an agonist of the $P2Y_1$ receptor.*

Even though NTPDases 5 and 6 may be present at the surface of the plasma membrane and secreted as soluble enzymes following a proteolytic cleavage, their high K_m values and low specific activities[153] make it unlikely that these enzymes regulate P2 receptor signalling. E-NTPDase activity is coupled with ecto-5'-nucleotidase activity to produce adenosine that exerts its effect through P1 receptors[154]. It can be concluded that NTPDase1 would hydrolyze nucleoside tri- and diphosphates equally well, resulting in the formation of nucleoside monophosphates and the inactivation of all P2 receptors agonists. NTPDase2 would selectively inactivate nucleoside triphosphates and act as an extracellular producer of nucleoside diphosphates. NTPDase2 preferentially hydrolyzes nucleoside triphosphates, and the presence of NTPDase2 under either coexpression or coculture conditions did not change the activation of the $P2Y_1$ receptor (Figure 5.2)[155]. NTPDase3, with its intermediate properties, is expected to effectively hydrolyze nucleoside triphosphates, but the hydrolysis of nucleoside diphosphates may be delayed and therefore the nucleoside diphosphate may be more long-lasting[151].

5.1.3. Nucleoside triphosphate diphosphohydrolase inhibitors

The analysis of nucleotide release or of the potency of externally applied ATP or its analogs requires the availability of inhibitors of ectonucleotidases that should preferably have no or only a small effect on P2 receptor activation[151]. It has been demonstrated that stable analogs of ATP can elicit tissue contractions up to a hundred times more effectively than ATP. This suggests that the effects of exogenously applied ATP on P2-receptors are limited by its enzymatic degradation. Inhibitors of ecto-nucleotidases could thus serve as drugs that increase the lifetime of extracellular ATP in situ. Regarding the diversity of enzyme families involved in extracellular nucleotide metabolism and the multiplicity of enzyme members, the task of developing specific inhibitors is demanding[146]. New molecules have been found to inhibit ecto-nucleotidases and particularly the E-NTPDases. These compounds can be grouped in to two categories: 1) nucleotides analogs, some of which are also P2 receptor agonists or antagonists (Figure 5.3) and 2) other molecules, most of which are related to the suramin-Evans blue families of dyes (Figure 5.4), also known as P2 receptor antagonists.

5.1.3.1. Nucleotide analogs

A large number of compounds have previously been investigated, representing nonhydrolyzable nucleotide analogs, as inhibitors of NTPDases. Until recently, the only compound that has been

shown to effectively inhibit hydrolysis of ATP in a variety of tissues (albeit with moderate potency) without significantly acting on purinoceptors was the structural analogue of ATP, ARL 67156 (FPL 67156) (N^6-diethyl-β,γ-dibromomethylene-ATP)[23,156]. ARL 67156 potentiates purinergic synaptic transmission, supporting the notion that endogenous ectonucleotidases reduce the effective concentrations of released ATP. Recently, 8-thioether-ATP derivatives, found inactive at P2Y- and P2X-receptors, and stable to NTPDase hydrolysis, were developed as specific and potent NTPDase inhibitors, and therefore hold promise as compounds that can inhibit ectonucleotidases without affecting P2 receptors. 8-Thiobutyladenosine-5'-triphosphate (compound **5.3**), a nonhydrolyzable analogue of ATP, acts as a competitive inhibitor of ATP with an estimated K_i value of 10 μM at nucleoside triphosphate diphosphohydrolase (NTPDase)[157,158].

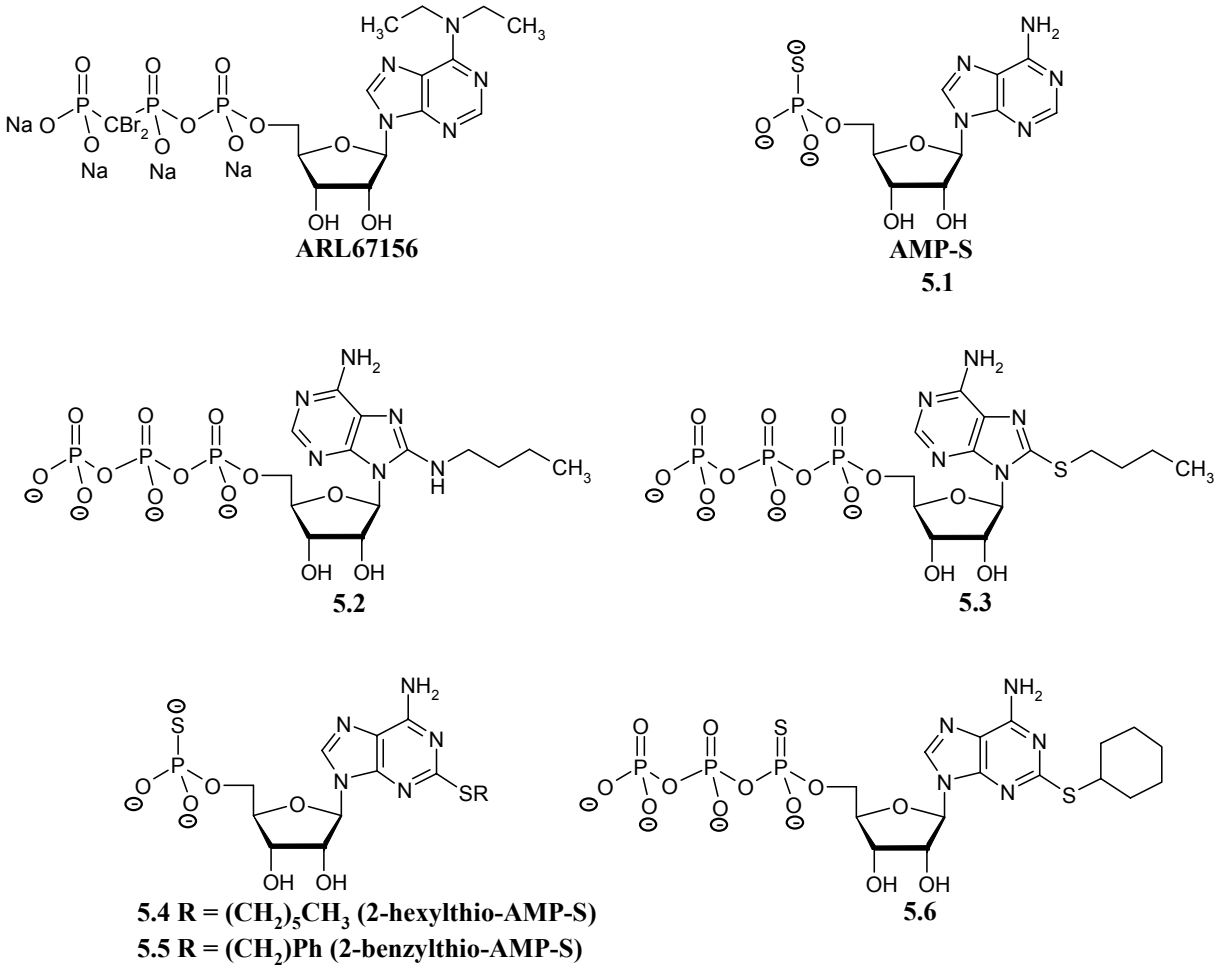


Figure 5.3. *Structures of nucleotide analogs as NTPDase inhibitors.*

Some 2-thioether-5'-mono-phosphoro-thioate adenosine derivatives (2-thioether-AMP-S) are inhibitors of E-NTPDase from pig pancreas. The monophosphate derivatives, 2-hexylthio-AMP-

S, and 2-benzylthio-AMP-S, were not hydrolyzed by pig pancreas E-NTPDase. The rate of hydrolysis of 2-hexylthio-5'-O-(1-thiotriphosphate)adenosine (2-hexylthio-ATP-α-S) by E-NTPDase was 28% of that of ATP. Hence, 2-hexylthio-AMP-S, 2-benzylthio-AMP-S and AMP-S were competitive inhibitors of E-NTPDase[159]. The ideal NTPDase inhibitor should not be a P2 receptor agonist and should not be dephosphorylated by the enzyme.

5.1.3.2. Non-nucleotide analogs

Inhibitors of P2-receptors antagonists such as suramin and related compounds[21,160], Evans blue and Trypan blue[161], Reactive red[162,163], Reactive blue[162,164], PPDAS (pyridoxal phosphate-6-azophenly-2',4'-disulphonic acid)[165,166] or a variety of textile and protein dyes also attenuate hydrolysis of ATP. These compounds inhibit members of different enzyme families albeit with different potency. The development of effective ecto-nucleotidase inhibitors with weak or lacking P2 receptor antagonism would considerably facilitate the functional analysis of nucleotides in intact organ preparations. There is a possibility to develop inhibitors with differential potency on ecto-ATPase and P2 receptors. Evans Blue is a very effective inhibitor of ecto-apyrase, and suramin preferentially inhibited ecto-ATPase[149].

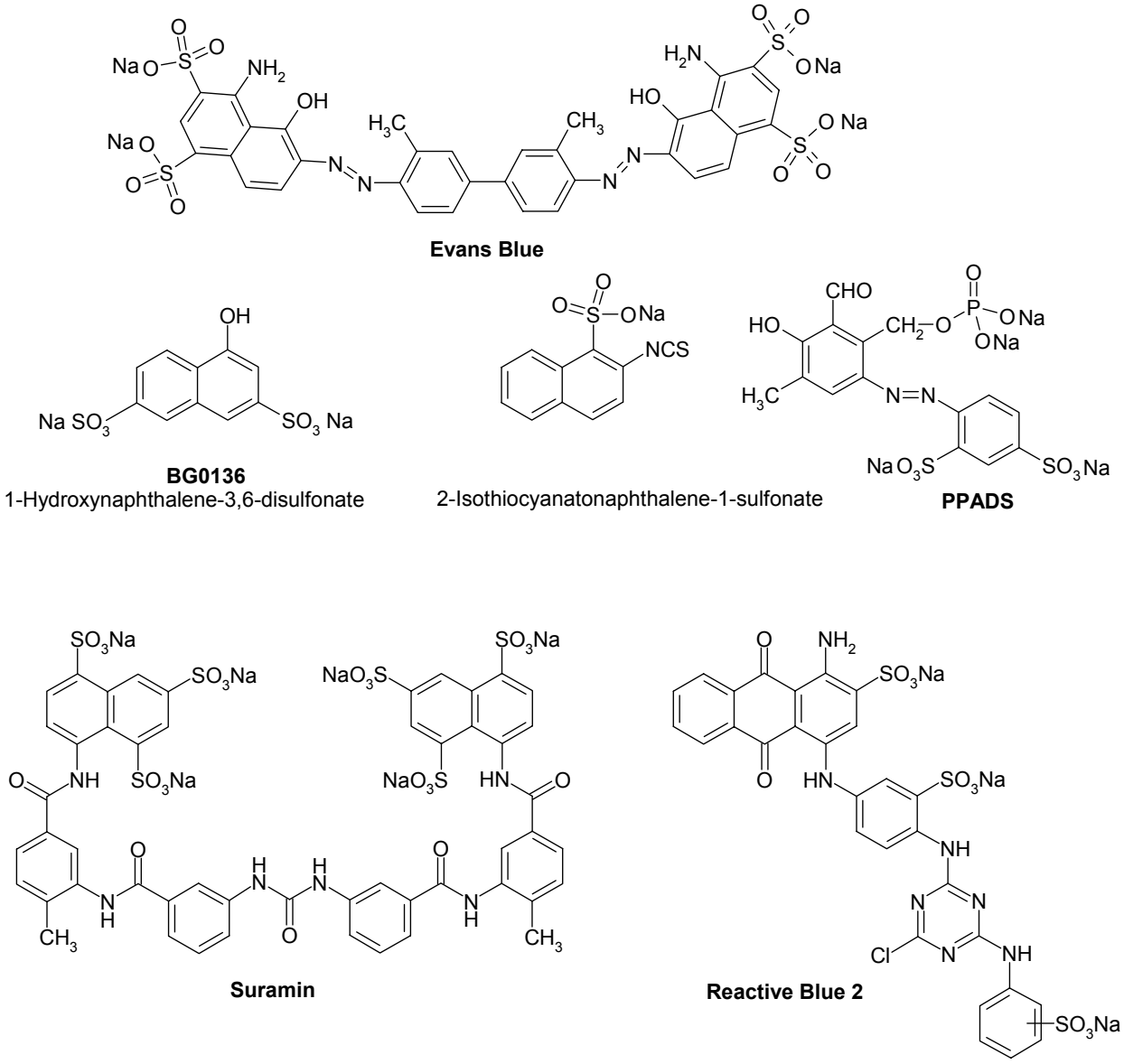


Figure 5.4. *Structures of sulfonate derivatives of the Suramin and Evans blue family that act as NTPDase inhibitors.*

These molecules share some common structural characteristics, including an aromatic core (e.g. naphthalene) substituted with sulfonate residues. From structure- activity relationship data obtained with P2 nucleotide receptors and ecto-nucleotidases, three structure-activity requirements have been found for these inhibitors: 1) the position of the sulfonate groups on the

naphthalene (or anthraquinone) core is important; 2) the amino-hydroxy-naphthalene disulfonate structures of these antagonists are involved in the binding to ectonucleotidases; 3) the inhibitory potencies and affinities of these antagonists at P2 purinoceptors increase with the size of the compounds[161]. BG0136 (1-naphthol-3,6-disulfonic acid disodium salt) exhibited inhibitory properties on E-NTPDase as well as antagonistic activity towards the $P2Y_2$ receptor. From related derivatives of BG0136 it is clear that a hydroxyl group is essential for inhibition of E-NTPDases. Substitution of the hydroxyl group by an amine caused a loss of the inhibitory property. Sulfonate groups ($-SO_3H$) seem to play a role but do not appear to be essential for inhibition. Molecules with a sulfonate group in the meta position with respect to the hydroxyl group are more efficient. BG0136 exhibited a mixed type of inhibition on E-NTPDases with a K_i value of 380 μM[167]. Small aromatic isothiocyanatonaphthalene-sulfonates were found to be selective inhibitors of P2-purinoceptors, the isothiocyanato residue as well as the aromatic core being essential for P2-purinoceptor blockade. 2-Isothiocyanatonaphthalene-1-sulfonate was better because of relatively high P2X-selectivity versus both the P2Y-purinoceptor and ecto-nucleotidases[168].

5.2. A capillary electrophoresis method for the characterization of ecto-nucleoside triphosphate diphosphohydrolases and the analysis of inhibitors by in-capillary enzymatic microreaction

5.2.1. Introduction

Extracellular nucleotides such as ATP, ADP, UTP, and UDP can act on a variety of nucleotide receptors (P2 receptors)[18]. The activation of P2 receptors is controlled by ecto-nucleotidases capable of hydrolyzing nucleoside tri- and diphosphates[146]. Inhibition of ecto-nucleotidases can result in a potentiation of purinergic signalling, supporting the notion that endogenous ecto-nucleotidases reduce the effective concentration of the released nucleotide[21,169,170]. Similarly, metabolically stable analogs of ATP are considerably more effective in causing a biological response than ATP itself. Inhibitors of ecto-nucleotidases could thus represent valuable tools for amplifying the biological effects induced by extracellularly released nucleotides. In addition, inhibition of ecto-nucleotidases is mandatory for both, studies of nucleotide release and the analysis of the potency on P2 receptors of nucleotides or their hydrolyzable analogs.

Inhibitors of ecto-nucleotidases should have no effect on P2 receptors and should not be dephosphorylated by ecto-nucleotidase. Ideally they would also reveal selectivity for individual E-NTPDase isoforms. Many inhibitors of P2 receptors also act as inhibitors of ecto-nucleotidases. These include suramin, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and reactive blue 2 (Figure 5.4). To date only the ATP analog ARL67156 (FPL67156, N^6-diethyl-β,γ-dibromomethylene-ATP, Figure 5.4)[169,170] and 8-thiobutyladenosine 5′-triphosphate (8-Bu-S-ATP)[157] reveal enzyme inhibitory potential without significantly affecting nucleotide receptors. However, these compounds have not been tested on defined NTPDase isoforms. The development of novel inhibitors of ecto-nucleotidases requires fast and precise methods for analyzing catalytic activity.

Capillary electrophoresis (CE) has recently emerged as a versatile technique for enzyme assays [79,171]. CE systems have been successfully applied for assaying enzyme activity[5,82,141], including the determination of Michaelis-Menten constants (K_m values)[79], and inhibition constants (K_i values for enzyme inhibitors)[80], exhibiting a number of advantages over conventional methods.

These include rapid separation of substrate and product, ultra-low sample volume requirements, and high throughput by automation. CE is particularly useful for investigating enzymatic reactions involving charged substrates or products, e.g. for the monitoring phosphorylation or dephosphorylation reactions[30,84]. Electrophoretically mediated microanalysis (EMMA), first described by Bao and Regnier[85] has been successfully used for in-line enzyme assays[86]. In this technique, the capillary is used as a microbioreactor as well as for the separation of substrates and products. There are two major types of EMMA methods. In the first continuous format [85], the capillary is filled with an appropriate substrate solution and upon injection of a zone of enzyme, the product will continuously form during the electrophoretic mixing of enzyme and substrate. One drawback of this method is that the separation buffer has to allow the enzymatic reaction to proceed.

In the second plug-plug technique[87], substrate and enzyme are introduced into the capillary as distinct plugs. Upon application of an electric potential, these zones mix with each other due to differences in their electrophoretic mobilities. The reaction proceeds during the mixing process. The resultant product is transported to the detector separately from enzyme and educts under the influence of an applied voltage, where they are individually detected. Even double enzyme-catalyzed reactions were studied by the latter method by injecting plugs of substrate and two different enzymes separately in reaction buffer. This technique was used to study the enzymatic reactions of hexokinase and apyrase as well as lactate dehydrogenase and glucose-6-phosphate dehydrogenase[31,31,32].

In the present study, a method for analyzing the enzymatic reactions of NTPDase1, 2 and 3, three surface-located members of the ecto-nucleoside triphosphate diphosphohydrolase family (EC 3.6.1.5) [146] has been developed, performing the enzymatic reaction inside the capillary at the capillary inlet followed by electrophoretic separation of the reaction products. In the at-capillary inlet reaction technique, the plugs of enzyme, substrate and inhibitor are introduced into the capillary, where they are allowed to react by simple diffusion and not by voltage as in EMMA according to a previously described procedure for angiotensin-converting enzyme assays [172,173].

5.2.2. Materials and methods

5.2.2.1. Reagents and chemicals

Reactive blue 2, suramin, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), dipyridamole and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were obtained from Sigma, Steinheim, Germany. ARL-67156 was from Tocris Cookson, Bristol, UK. ATP, ADP, AMP, UMP, $MgCl_2 \cdot 6H_2O$, and tris(hydroxymethyl)aminomethane (Trizma Base), were from Sigma (Taufkirchen, Germany). Culture medium was obtained from Invitrogen (Karlsruhe, Germany). Penicillin and streptomycin were purchased from Sigma-Aldrich (Deisenhofen, Germany). Leupeptin, pepstatin A, chymostatin, and antipain were from Calbiochem (Schwalbach, Germany).

5.2.2.2. Cell transfection and preparation of membrane fractions containing nucleoside triphosphate diphosphohydrolases

Chinese hamster ovary (CHO) cells were cultured in HAM's F-12 medium containing 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. They were transfected by electroporation with plasmid-DNA containing rat NTPDase1 (GenBank Accession number U81295)[174], NTPDase2 (Y11835)[174], and NTPDase3 (AJ437217), all cloned into the pcDNA3 plasmid. Transfection with the empty plasmid pcDNA3 served as a control. Transiently transfected CHO cells were used for preparation of membrane fractions 48 h after electroporation.

After removal of culture medium, cells were washed twice with buffer A (in mM: 140 NaCl, 5 KCl, 0.5 EDTA, 20 Hepes, pH 7.4) and scraped off with 5 ml of ice-cold buffer B (in mM: 140 NaCl, 5 KCl, 20 Hepes, pH 7.4) containing protease inhibitors (in µg/ml: 2, chymostatin; 1, pepstatin A; 150 benzamidine; 2, antipain; 2, leupeptin) and iodoacetamide (2 mM). Cells were centrifuged at 300 g for 10 min at 4 °C. The cell pellet was resuspended in buffer B, homogenized using a Potter-Elvehjem homogenizer and sonicated. The homogenate was centrifuged for 10 min at 300 g at 4 °C and the resulting supernatant fraction was centrifuged at 100,000 g for 1 h at 4 °C. The pellet fraction was resuspended in buffer C containing 50% (v/v) glycerol, 2 mM iodoacetamide, 20 mM Hepes (pH 7.4), and stored at –20 °C. ATPase activity of individual membrane fractions was determined by analysis of free phosphate formed according to

Lanzetta et al. [175]. Protein was determined according to the method of Spector [176]. The membrane preparations contained 4-6 µg of protein/µl.

Cell transfections and preparations of membranes were performed by Prof. Dr. H. Zimmermann and coworkers, Institute of Zoology Department of Neurochemistry university of Frankfurt, Germany.

5.2.2.3. Capillary electrophoresis instrumentation

All experiments were carried out using a P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a UV detection system coupled with a diode-array detector (DAD). Data collection and peak area analysis were performed by the P/ACE MDQ software 32 KARAT obtained from Beckman Coulter. The capillary temperature was kept constant at 37 °C. The temperature of the sample storing unit was adjusted to 10 °C. The electrophoretic separations were carried out using an eCAP polyacrylamide-coated fused-silica capillary [(30 cm (20 cm effective length) x 50 µm internal diameter (I.D.) x 360 µm outside diameter (O.D.), obtained from CS-Chromatographie (Langerwehe, Germany)]. The separation was performed using an applied current of -60 µA and a data acquisition rate of 8 Hz. Analytes were detected using direct UV absorbance at 210 nm. The capillary was conditioned by rinsing with water for 2 min and subsequently with buffer (phosphate 50 mM, pH 6.5) for 1 min. Sample injections were made at the cathodic side of the capillary.

5.2.2.4. Nucleoside triphosphate diphosphohydrolase inhibition assays outside the capillary

The reaction mixture for the NTPDases inhibition assays contained 400 µM ATP in a phosphate-free physiological saline solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, pH 7.4) and 10 µl of different concentrations of NTPDases inhibitors or test compounds were added over a range of 7 to 10 concentrations to determine the K_i values. Test compounds were first screened at 1 and 0.3 mM concentrations. The reaction was started by adding 10 µl of membrane preparation containing NTPDase enzyme. The reaction was carried out at 37 °C in a final volume of 100 µl for 10 min, then stopped by heating at 99 °C for 3 minutes. Then 50 µl were transferred to eppendorf tubes containing 50 µl of an aqueous UMP solution with a final concentration of 10 µM as an internal standard and 400 µl of deionized water. After mixing 50 µl of the reaction mixture was transferred to a small CE vial for measurement. The AMP or ADP formed was repeated three times in two separate experiments. The absorbance at 210 nm was

monitored continuously and the nucleotide concentrations were determined from the area under each absorbance peak.

5.2.2.5. At-inlet reaction procedure and automation of analytical process

The CE running buffer consisted of dipotassium hydrogen phosphate 50 mM, pH 6.5. The reaction buffer contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes, pH 7.4. Before use, a new capillary was washed with deionized water for 10 minutes. The automation cycle consisted of (i) washing with water for 2 minutes (40 p.s.i; 1 p.s.i = 6894.76 Pa) (ii) equilibration with the CE running buffer for 1 minute (40 p.s.i), (iii) injection of a plug of reaction solution containing 320 µM ATP (substrate) in reaction buffer and various concentrations of inhibitor stock solutions in reaction buffer, (iv) injection of a plug of suitably diluted enzyme, (v) injection of another plug of reaction solution as in (iii), (vi) and finally injection of a plug of water. The plugs were then allowed to react, while the capillary ends were dipped into water, for a predetermined waiting period of 5 min. Then, a current of -60 µA was applied and the reaction products moved towards the detector end of the capillary. After each analysis the capillary was rinsed with CE running buffer for 2 min followed by deionized water for 1 min.

Each electropherogram was recorded over 7 min. The diluted membrane fractions containing enzyme were placed in the sample storage unit whose temperature was kept constant at 10 °C. At this temperature, enzyme activity remained unaffected during the approximately 24 h of instrument run. Substrate and buffers used in the enzyme reaction were also kept at 10 °C in the autosampler reservoir.

5.2.2.6. Quantitative determination of AMP and ADP and method validation

AMP and ADP were dissolved in enzyme assay buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, pH 7.4) to obtain 1 mM stock solutions. Standard calibration curves were obtained with final concentrations of 2, 5, 10, 20, 30, and 50 µM. For validating the method, 20 µM of UMP was used as an internal standard. A polyacrylamide-coated capillary was used for the separation and quantitation of AMP and ADP. The procedure was as described above using enzyme preparations inactivated by heating to 99 °C for 3 min using an Eppendorf Thermomixer Comfort. Determinations were performed in triplicate. The calibration curves were obtained by plotting the corrected peak area of AMP or ADP, respectively, against their concentrations.

5.2.2.7. Investigation of standard nucleoside triphosphate diphosphohydrolase inhibitors by in-capillary reaction

For the determination of the IC_{50} and K_i values of NTPDase inhibitors, 6-8 different concentrations of inhibitor spanning about three orders of magnitude were used (see Figure 5.8-5.10), while a fixed substrate concentration of 320 μM of ATP was employed for all three NTPDases. Under the applied conditions less than 10 % of substrate was converted by the enzymes. Membrane preparations derived from transfected cells and containing NTPDase1, NTPDase2, or NTPDase3, were appropriately diluted with reaction buffer for the inhibition assays. Control experiments were performed using membrane preparations of cells transfected with the empty plasmid (pcDNA3). Substrate and inhibitors were dissolved in the reaction buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, pH 7.4. The Cheng-Prusoff equation was used to calculate the K_i values from the IC_{50} values, determined by the non-linear curve fitting program PRISM 3.0 (GraphPad, San Diego, California, USA).

$$K_i = \frac{IC_{50}}{1 + \frac{[ATP]}{K_m\,(ATP)}}$$

5.2.2.8. Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) determination

For the determination of the Michaelis-Menten constants (K_m) and the maximum velocity (V_{max}) eight different substrate concentrations of ATP were used, 10, 20, 30, 50, 100, 200, 250 and 1000 μM of ATP for NTPDase1 dissolved in reaction buffer, while the following ATP concentrations were used for NTPDase2 and 3: 25, 50, 100, 150, 200, 250, 300, 500 and 1000 μM. The capillary inlet reaction method was used as described above.

5.2.2.9. Investigation of Reactive blue 2 derivatives (anthraquinones), polyoxometalates and uridine nucleotide mimetics as potential inhibitors of nucleoside triphosphate diphosphohydrolases by capillary electrophoresis

For the determination of the IC_{50} and K_i values of Reactive blue 2 derivatives and polyoxometalates (Table 5.5.) as NTPDases inhibitors, 6-8 different concentrations of inhibitor spanning about three orders of magnitude were used, while a fixed substrate concentration of 400

μM of ATP was employed for all three NTPDases. Under the applied conditions less than 10 % of substrate was converted by the enzymes. Membrane preparations derived from transfected cells containing NTPDase1, NTPDase2, or NTPDase3, were appropriately diluted with reaction buffer for the inhibition assays. Control experiments were performed using membrane preparations of cells transfected with the empty plasmid (pcDNA3). Substrate and inhibitors were dissolved in the reaction buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, pH 7.4.

The anthraquinone derivatives related to RB-2 were synthesized by Dr. Stefanie Weyler Pharmaceutical Institute, University of Bonn. The polyoxometallates (Table 5.5.) were obtained from Dr. Holger Stephan, Forschungszentrum Rossendorf, Institut für Bioanorganische und Radiopharmazeutische Chemie, Dresden, Germany. The uridine nucleotide mimetics were synthesized by Andreas Brunschweiger, Pharmaceutical Institute, University of Bonn.

5.3. Results

5.3.1. Development of the on-capillary reaction technique

The on-capillary reaction technique has previously been successfully applied for inside capillary enzymatic reactions using electrophoretically mediated microanalysis (EMMA)[85,86,86]. A related inside capillary enzymatic reaction methodology in which the enzymatic reaction was performed at the capillary inlet without electrophoretic mixing of enzyme and substrate had been applied for the determination of the Michaelis-Menten constant[173] and for inhibition studies of the angiotensin-converting enzyme[172]. In the present study we developed a CE method for the monitoring of reactions of NTPDase1, 2 and 3 by a modification of the described at-capillary inlet enzymatic reaction[172,173]. Suitable conditions for the separation and quantitative determination of nucleotides and the monitoring of enzymatic nucleotide metabolism using CE had previously been developed in our group[30]. However in those studies, the enzymatic reaction was performed in a vial outside the capillary and the samples were injected into the capillary and subjected to CE analysis only after the enzymatic reaction had been stopped[30]. A modification of the developed separation protocols was now used after performing the enzymatic reaction directly in the capillary close to the capillary inlet. Thus, a small aliquot of substrate-bearing reaction buffer was hydrodynamically injected into the capillary followed by enzyme and then again substrate, effectively sandwiching an aliquot of enzyme on either side by substrate. After

the final plug of substrate a small plug of water was injected resulting in a stacking effect which improved the resolution of the peaks[30].

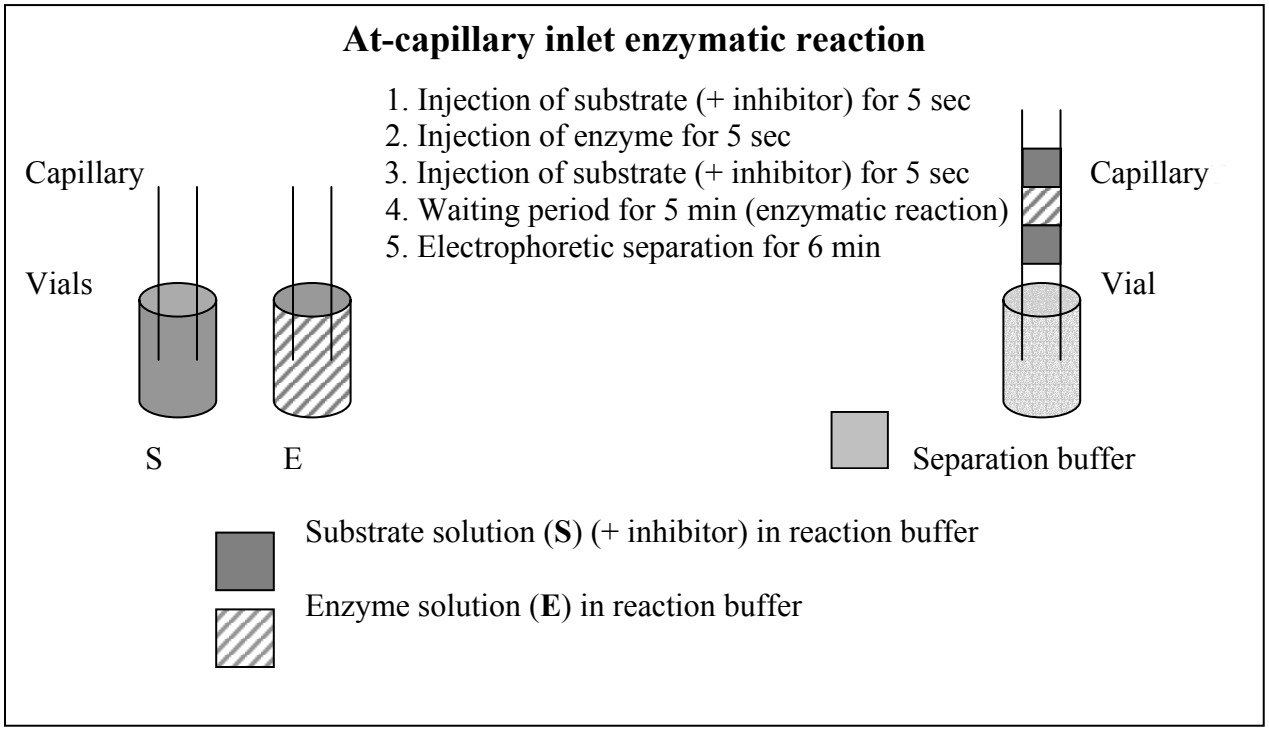


Figure 5.5. *Schematic illustration of microscale reaction of NTPDases at capillary inlet. 1. Injection of a sample of 4 nl of 320 µM of ATP (substrate) in reaction buffer containing UMP (20 µM) as an internal standard in the absence or presence of test compound (potential inhibitors) (0.3 p.s.i., 5 s); 2. Injection of enzyme (0.3 p.s.i., 5 s); 3. Injection of 320 µM of ATP (substrate) in reaction buffer containing UMP (20 µM) as an internal standard in the absence or presence of test compound (0.3 p.s.i., 5 s); 4. Overlayed plugs are then allowed to stand during a predetermined period of 5 min; 5. Subsequently a –60 µA current is applied and the reaction products migrate to the detector. Electrophoresis conditions were as described in the experimental section.*

The sandwich mode was required because otherwise not enough product was formed. An inverse sandwich mode of two plugs of enzyme on either side of substrate as described by van Dyck et al.[172] for the reaction of angiotensin converting enzyme, proved to be unfavorable in our case

since the rate of conversion of substrate to product was more than 10%. Figure 5.5 provides a schematic overview of the different steps of the capillary inlet reaction procedure. A reaction buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM Hepes, pH 7.4, was found suitable for the enzymatic reaction, which was allowed to take place for 5 min. The separation of substrate and product(s) was then initiated by applying a constant current of -60 µA using a 50 mM phosphate buffer (pH 6.5) resulting in a voltage of 7 kV. The separation was completed within less than 6 min (see Figure 5.6).

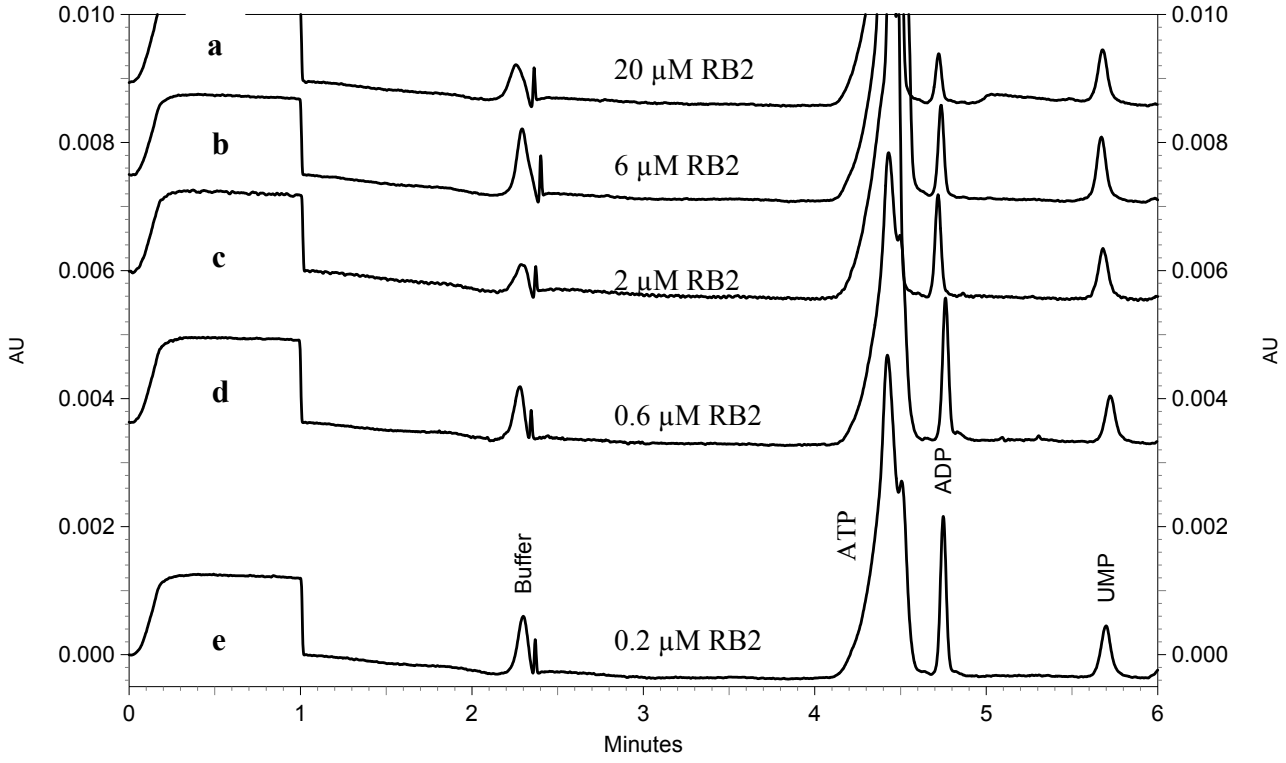


Figure 5.6. *Overlay of five electropherograms after NTPDase3 on-line reaction at the capillary inlet with different concentrations of reactive blue 2 (inhibitor) added to the substrate plug. The concentration of NTPDase3 was 0.05 µg/µl of protein, ATP: 320 µM, UMP (internal standard): 40 µM. Waiting period (duration of enzymatic reaction): 5.0 min. CE conditions: running buffer: 50 mM potassium phosphate, pH 6.5; constant current of -60 µA; detection at 210 nm, capillary cartridge temperature: 37 °C.*

Reactive blue 2 (RB2) concentrations: a) 20, b) 6, c) 2, d) 0.6 and e) 0.2 µM. The lowest concentration (0.2 µM), gave virtually the same electropherogram as the control without inhibitor.

A wavelength of 210 nm was chosen for the detection of the nucleotides due to the higher sensitivity that can be reached at this wavelength in comparison with higher wavelengths. The washing time and pressure had to be optimized for the enzyme assay, because inside the capillary there was a high concentration of salts, buffer ions, lipids and proteins. A period of 2 min of washing with buffer followed by 1 min with water at 40 p.s.i pressure was found to be sufficient for cleaning the capillary. In the method described for monitoring angiotensin converting enzyme [172,173], van Dyck et al. had encountered some drawbacks, such as unpredictable migration time shifts and current breakdown. We believe that these problems were due to the adsorption of enzyme to the capillary wall because a fused silica capillary was used. In our study we used a polyacrylamide-coated capillary, which is not expected to show protein adsorption, thus we did observe neither current breakdown nor unpredicted migration time shifts. However, when we used a fused-silica capillary the method was not successful due to the high concentration of salts required in the reaction buffer and probably because of enzyme and lipid adsorption to the capillary wall resulting in peak broadening. Using a short, coated capillary, high precision of migration time and very good peak resolution was observed up to several hundreds of runs.

5.3.2. Quantitative analysis of AMP and ADP

The NTPDase activity and inhibition was determined by measuring the corrected peak area of AMP for NTPDase1, and ADP for NTPDase2 and 3, respectively. Validation of CE measurements of AMP and ADP were performed exactly the same way as for enzyme activity assays, i.e (1) injection of plugs of different concentrations of AMP or ADP in reaction buffer containing UMP (20 μM) as an internal standard, (2) a suspension of a membrane preparation containing inactived enzyme, followed by (3) another injection of AMP or ADP in reaction buffer containing UMP (20 μM) as an internal standard. An overview of the quantitative parameters of the method validation is provided in Table 5.1 A strictly linear correlation between AMP and ADP concentrations and the corrected peak area ratio was found: a correlation coefficient (R^2) of 0.998 for AMP and 0.999 for ADP (n = 3) was calculated for a concentration range from 2.0 to 50.0 μM. The limit of quantification (LOQ) was found to be 2.95 μg/ml for AMP and 1.41 μg/ml for ADP. The limit of detection (LOD) of AMP was determined to be 0.80 μg/ml for AMP and 0.36 μg/ml for ADP. Standard deviations of migration times were generally low (Table 5.1).

Table 5.1. *Limits of detection, limits of quantification, migration times and linearity of calibration curve for AMP and ADP determination.*

Compound	**AMP**	**ADP**
Limit of detection ± SD[a] (µg/ml)	0.80 ± 0.20	0.36 ± 0.10
Limit of quantification ± SD (µg/ml)	2.95 ± 0.21	1.41 ± 0.13
Coefficient of correlation; R^2	0.998	0.999
Mean value of migration time ± SD (min) (n= 12)	6.00 ± 0.03	4.77 ± 0.01
RSD[b] of migration time (%)	0.50	0.21
Regression equation	y = 724.5x + 1.53, $S_{y,x}$= 775	y = 432.2x + 0.70, $S_{y,x}$= 241

[a]SD = standard deviation

[b]RSD = relative standard deviation

5.3.3. Determination of Michaelis-Menten constant (K_m) and maximum velocity (V_{max})

The newly developed method was subsequently used to characterize the catalytic properties of defined members of the E-NTPDase family. Using the optimized conditions, Michaelis-Menten constants (K_m) and maximal velocity (V_{max}) for NTPDases were determined. The enzyme velocity was determined by measuring the peak areas of the products of the enzymatic reaction. K_m values were obtained by using different concentrations of the substrate ATP. Each substrate concentration was analyzed in triplicate. The Michaelis-Menten plots are depicted in Figure 5.7. Estimated K_m values of 76, 203, and 311 µM were obtained for NTPDase1, 2 and 3, respectively. The initial reaction velocities were calculated from the amounts of product formed, AMP in the case of NTPDase1 and ADP in the case of NTPDase2 and 3. V_{max} values were 0.023, 0.021 and 0.010 µmol/min/mg of protein (membrane preparation) for NTPDase1, 2, and 3, respectively (Table 5.2).

Table 5.2. *Kinetic parameters of NTPDases. The results are means ± SEM of three separate experiments each run in duplicate.*

	NTPDase1	NTPDase2	NTPDase3
K_m ± SEM [μM]	76 ± 12	203 ± 8	311 ± 4
V_{max} ± SEM [μmol/min/mg protein]	0.023 ± 0.002	0.021 ± 0.002	0.010 ± 0.001

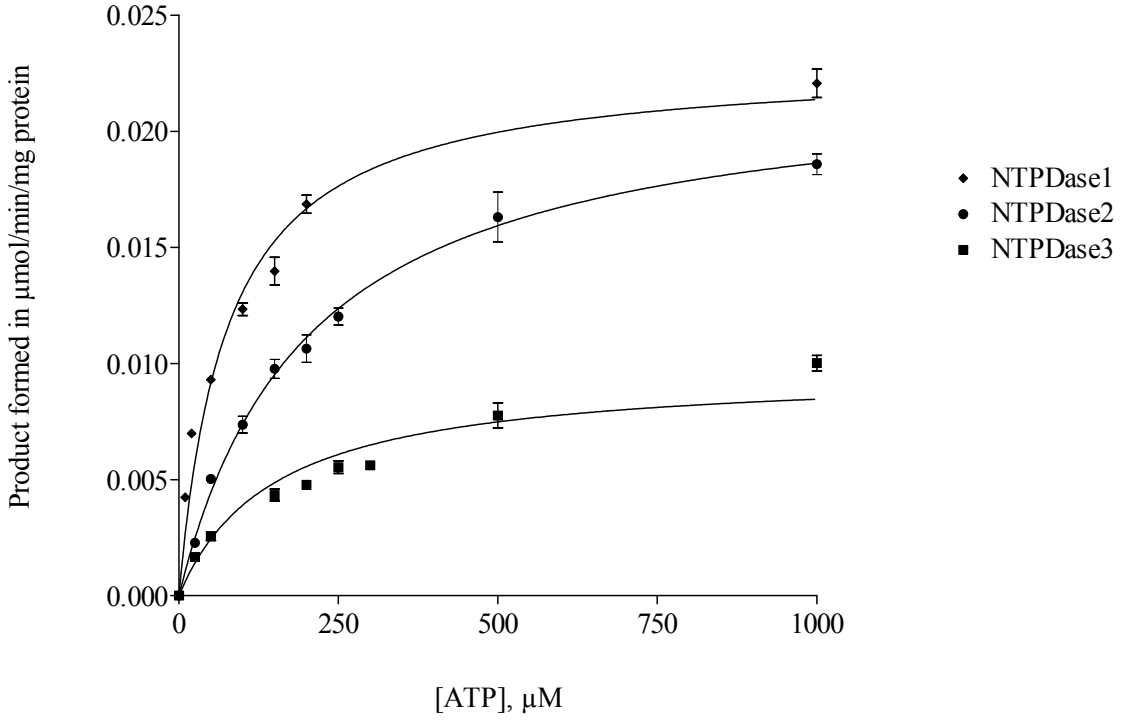


Figure 5.7. *Michaelis-Menten plots for the enzymatic reaction of NTPDase1 (◆), NTPDase2 (●) and NTPDase3 (■) of initial ATP concentrations with respect to the reaction velocity for the determination of K_m and V_{max} values by using in-capillary reaction at capillary inlet. For enzyme activity assay see section 2.4 and for CE conditions see Figure 5.6. Data points represent means*

± SD from three separate experiments each run in duplicate. For determined K_m and V_{max} values see table 5.2.

5.3.4. Development of an enzyme inhibition assay

Four selected NTPDase inhibitors were investigated: The nucleotide analog ARL67156 (Figure 5.3), the anthraquinone dye Reactive blue 2, the pyridoxal phosphate derivative PPADS, and the symmetrical naphthalenesulfonic acid derivative suramin (Figure 5.4). While ARL67156 has been reported to be a weak but selective ectonucleotidase inhibitor without significant effects at P2 receptors, the other three compounds are also antagonists at certain P2 receptors [166,169].

Inhibition of NTPDases 1, 2 and 3 was determined by a range of concentrations of inhibitors spanning 3 orders of magnitude. The compounds entered the capillary inlet together with the substrate by hydrodynamic injection and the injected plugs of substrate/inhibitor and enzyme were then allowed to react for 5 min. The compounds reacted inside the capillary without applying any voltage, unlike in EMMA, where compounds are mixed electrophoretically by applying voltage before the reaction takes place. After the reaction, a constant current of –60 μA with reverse polarity was applied to separate the reaction products. As an example, an overlay of six electropherograms of the NTPDase3 enzymatic reaction is shown in Figure 5.6, in which the inhibitor concentration (reactive blue 2) was varied from 0.2 to 20 μM and the concentration of ATP as a substrate was fixed at 320 μM. By increasing the concentration of the inhibitor reactive blue 2, the peak height for ADP was decreased. Using NTPDase1, NTPDase2 and NTPDase3 and the newly developed CE method, a concentration-dependent inhibition by the standard inhibitors was observed for each of the enzymes (Figure 5.8-5.10). This allows a direct comparison of the effects of these inhibitors on a variety of identified NTPDases. The K_i values derived are summarized in Table 5.3.

The K_i values clearly show that the various NTPDases are differentially susceptible to the individual inhibitors. The values for reactive blue 2 were similar for NTPDase1 and 2, but lower by a factor of 20 for NTPDase3. Similarly, K_i values for PPADS were similar for NTPDase1 and NTPDase2 but 15-fold lower for NTPDse3. ARL67156 revealed the highest inhibitory potency for NTPDase1, was considerably less effective on NTPDase3 and essentially ineffective on NTPDase2. In contrast, suramin revealed the lowest K_i values for NTPDase3 and was less effective on NTPDase2 and NTPDase1. Dipyridamole was not an inhibitor of the E-NTPDases investigated.

Table 5.3. *K_i values for NTPDase1, 2 and 3 obtained for Reactive blue 2, PPADS, suramin, and ARL67156, using the in-capillary electrophoresis method. The results are means ± SEM of three separate experiments each run in duplicate.*

Inhibitor[a]	**K_i ± SEM [μM]**		
	NTPDase1	NTPDase2	NTPDase3
RB2	20.0 ± 0.003	24.2 ± 0.06	1.10 ± 0.03
PPADS	46.0 ± 0.01	44.2 ± 0.03	3.0 ± 0.001
Suramin	300 ± 0.1	65.4 ± 0.01	12.7 ± 0.03
ARL 67156	27.0 ± 0.004	≥ 1,000[b]	112.1 ± 0.05
Dipyridamole	> 1,000[c]	> 1,000[c]	> 1,000[c]

[a] For structures see Figure 5.4

[b] 50 % inhibition at 1 mM concentration

[c] no inhibitory activity up to a concentration of 1 mM

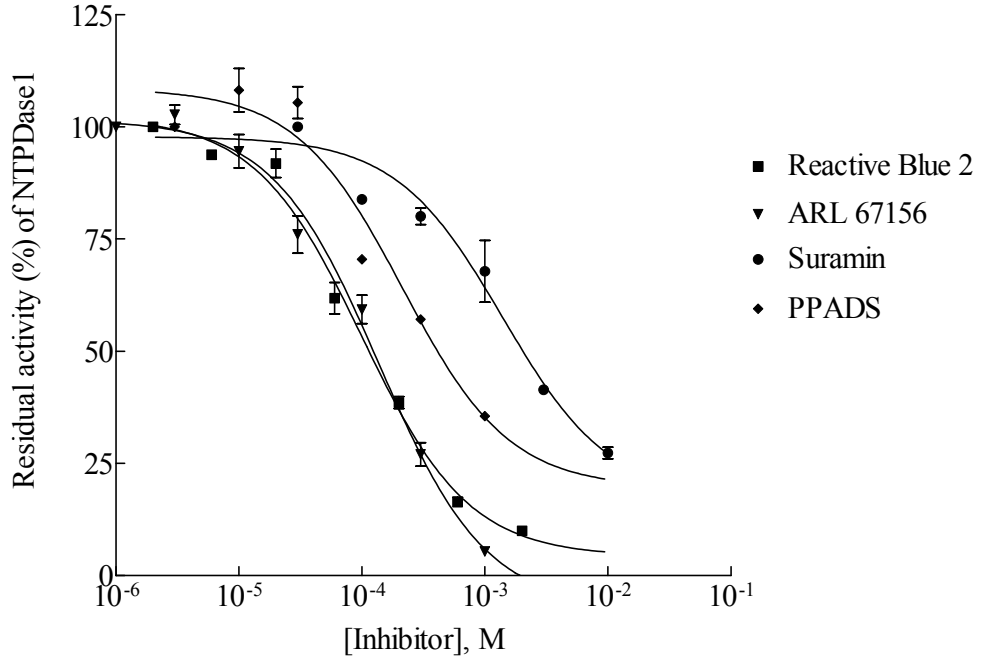


Figure 5.8. *Concentration-dependent inhibition of NTPDase1 by reactive blue 2 (■), ARL67156 (▼) suramin (●) and PPADS (◆) determined by capillary electrophoresis using in-capillary reaction at capillary inlet, using a substrate concentration of 320 µM ATP, a reaction buffer consisting of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes, pH 7.4, and various concentrations of inhibitor. The separation conditions were 50 mM phosphate buffer, pH 6.5, neutral capillary, 30 cm length (20 cm to the detector), 50 µm I.D.; -60 µA, 7 kV; capillary cartridge temperature 37 °C; detection at 210 nm, pressure injection. Data points represent means ± SD from three separate experiments, each run in duplicate.*

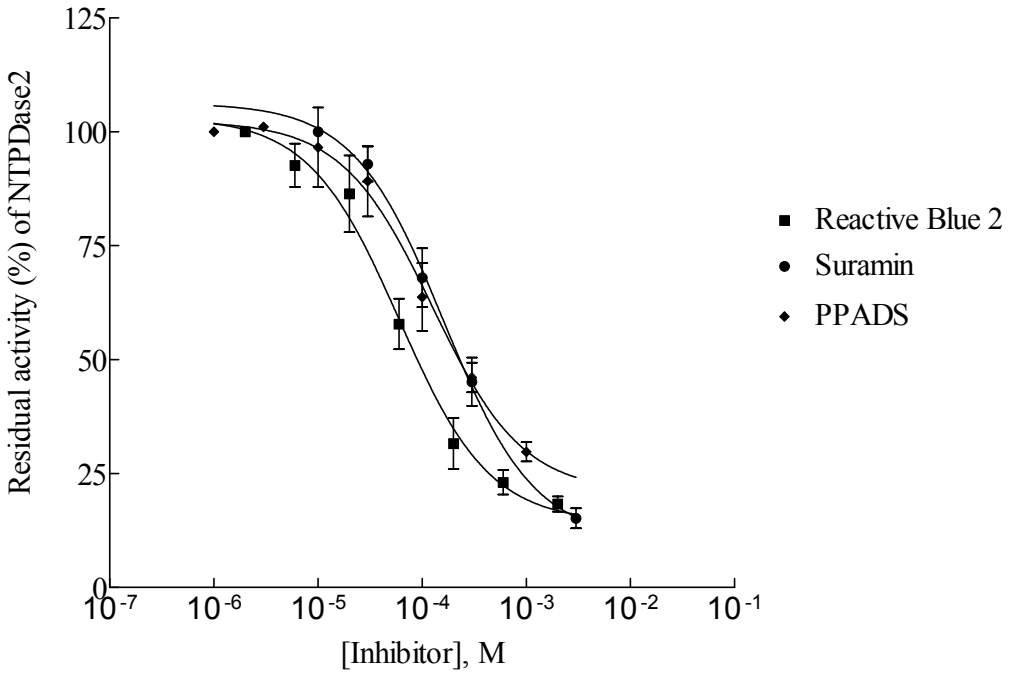


Figure 5.9. *Concentration-dependent inhibition of NTPDase2 by reactive blue 2 (■) suramin (●) and PPADS (◆) determined by capillary electrophoresis using in-capillary reaction at capillary inlet. For CE and assay conditions see Figure 5.6. Data points represent means ± SD from three separate experiments, each run in duplicates. ARL67156 (not shown) exhibited only 50 % inhibition at a concentration of 1 mM.*

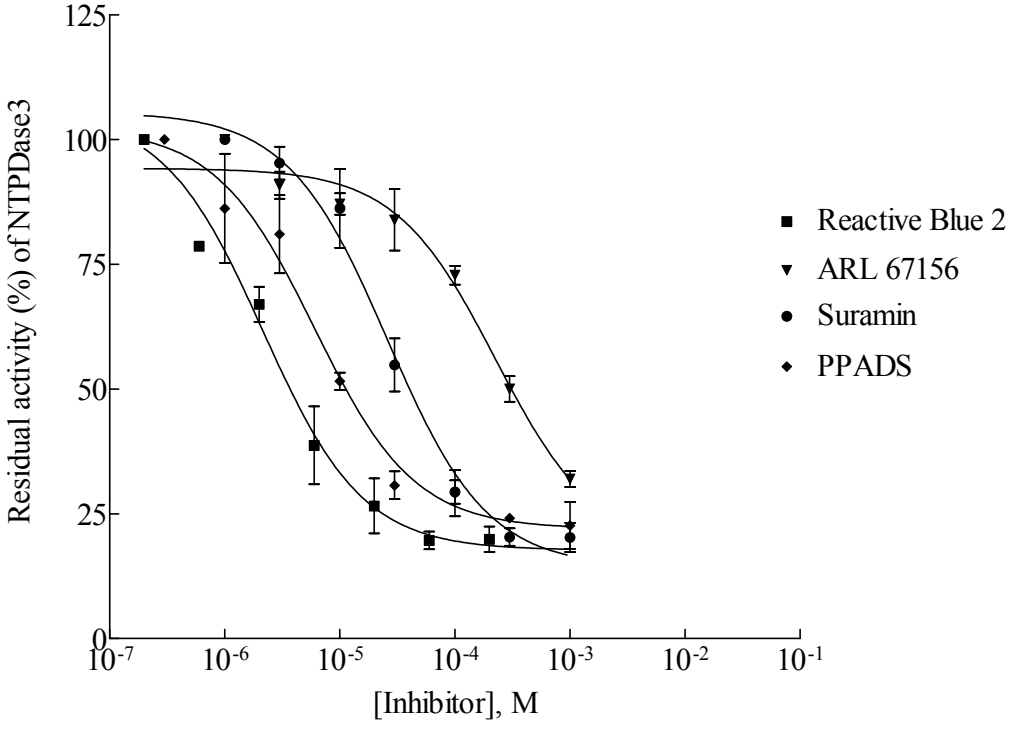
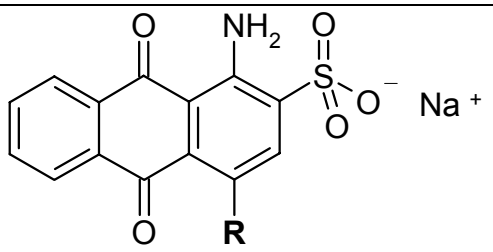


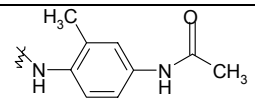
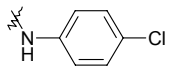
Figure 5.10. *Concentration-dependent inhibition of NTPDase3 by reactive blue 2 (■), ARL67156 (▼), suramin (●) and PPADS (◆) determined by capillary electrophoresis using in-capillary reaction at capillary inlet. For CE and assay conditions see Figure 5.6. Data points represent means ± SD from three separate experiments each run in duplicate.*

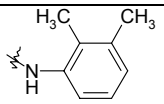
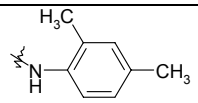
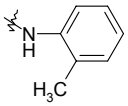
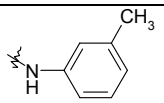
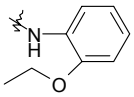
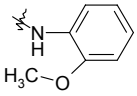
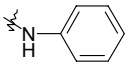
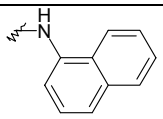
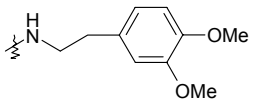
5.3.5. Investigation of Reactive blue 2 derivatives and polyoxometalates as nucleoside triphosphate diphosphohydrolase inhibitors by capillary electrophoresis

Reactive blue 2 (RB-2) had been characterized as a relatively potent NTPDase inhibitor with some selectivity for NTPDase3 (see table 5.3.). In search for the pharmacophore and to analyze structure-activity relationships we investigated a series of truncated derivatives and analogs of RB-2. Anthraquinone dervatives **5.2.1-5.2.11** (see table 5.4) inhibited the NTPDases in a concentration-dependent manner.

Table 5.4. *K_i values for NTPDase1, 2 and 3 of anthraquinone derivatives determined by capillary electrophoresis. The results are means ± SEM of three separate experiments each run in duplicate; $P2Y_2$ receptor inhibitory activity for assessing enzyme versus receptor selectivity is given.*



Inhibitor		K_i [μM] ± SEM			IC_{50} ± SEM [μM], $P2Y_2$-Receptor[d]
		NTPDase1	NTPDase2	NTPDase3	
RB-2 (lead structure)		20.0 ± 0.003	24.2 ± 0.06	1.10 ± 0.03	
No.	R				
5.2.1		> 1 mM (15 %[a])	486 ± 18	343 ± 63	12 ± 4
5.2.2		15.7 ± 3.4	18.0 ± 2.0	16.4 ± 1.6	21 ± 7

5.2.3		> 100 µM[c] 1 mM (67 %[a,c])	22.7 ± 3.4	38.5± 5.0	14 ± 9
5.2.4		18.0 ± 3.5	15.6 ± 2.5	41.8 ± 5.5	10 ± 1
5.2.5		> 1 mM (25 %[a])	25.7 ± 5.1	23.0 ± 2.5	11 ± 2
5.2.6		51.5 ± 0.4	12.8 ± 0.9	19.1 ± 6.0	22 ± 7
5.2.7		> 1 mM (17 %[a])	40.8 ± 11.1	58.0 ± 12.7	18 ± 4
5.2.8		≈ 1 mM (57 %[a])	53.8 ± 5.7	17.6 ± 6.6	10 ± 2
5.2.9		49.1 ± 5.1	35.8 ± 6.1	14.3 ± 1.5	11 ± 3
5.2.10		>>1 mM (0 %[a])	>>1 mM (0 %[a])	**1.5 ± 0.1**	11 ± 1
5.2.11		173 ± 6	54.1 ± 6.8	23.4 ± 0.4	n.d[b]

[a]inhibition at 1 mM.

[b]not determined.

[c]A curve could not be determined. Inhibition was not concentration-dependent; there was no inhibition at 100 µM.

[d]Dissertation, S. Weyler, University of Bonn, 2004.

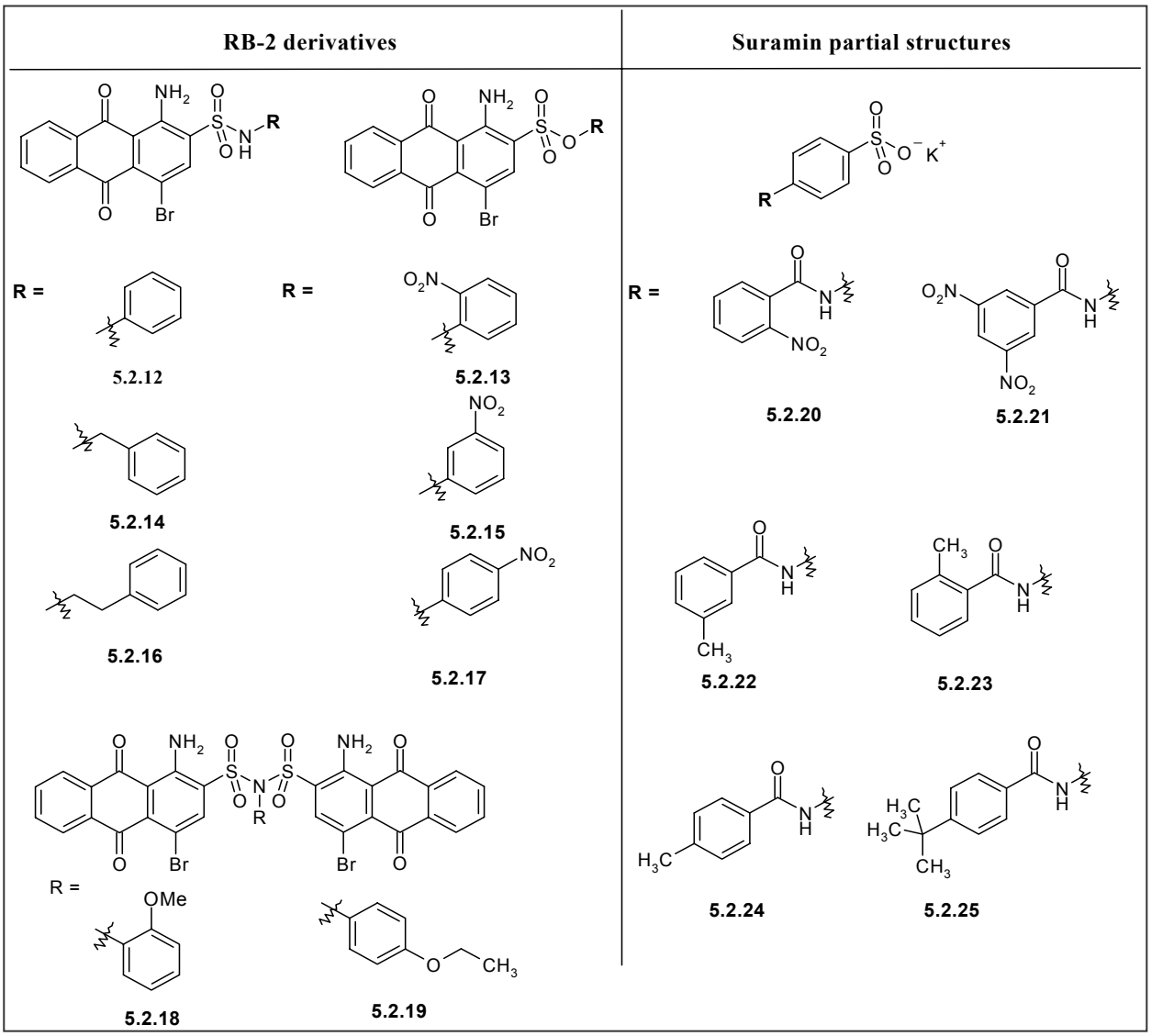


Figure 5.11. *Structures of some anthraquinone derivatives and partial structures of suramin inactive as NTPDase1, 2 and 3 inhibitors.*

Structure-activity relationships

Even though the investigated anthraquinone derivatives exhibited only a partial structure of RB-2 and were much smaller than the parent compound, many of them were similar or even more active as NTPDase inhibitors. For example, compound **5.2.2** bearing a p-chlorophenylamino residue was a potent, non-selective NTPDase inhibitor (K_i 16-18μM). While NTPDase 2 and 3 were quite tolerant with regard to the substituent **R** accepting a large variety of different substituted arylamino residues and even an arylethylamino residue, SARs of NTPDase 1 were more restricted: phenylamino (**5.2.9**), p-chlorophenylamino (**5.2.2**), m-methyl- (**5.2.6**) and o, p-

dimethylphenylamino (**5.2.4**) were best tolerated, while monosubstitution in the ortho-position of the phenylamino residue (**5.2.5**, **5.2.7**, **5.2.8**) or replacement of the phenyl ring by 1-naphthyl (**5.2.10**) or a phenylethyl residue (**5.2.11**) were detrimental for NTPDase 1 inhibitory activity. Compound **5.2.10** was the most potent and selective inhibitor of NTPDase3 and not inhibitory to NTPDase1 and 2 even at high, millimolar concentrations. Anthraquinone derivatives with substitutions at the sulfonate and sulfanilamide groups were not inhibitors of NTPDases (compounds **5.2.12-5.2.19** and **5.2.32-5.2.35**) (Figure 5.11). Thus, the sulfonate group appeared to be required for NTPDase inhibition. Other classes of non-anthraquinone derivatives related to suramin that were tested, compounds **5.2.20-5.2.35,** were also inactive at NTPDases1-3 (Figure 5.11 and 5.12).

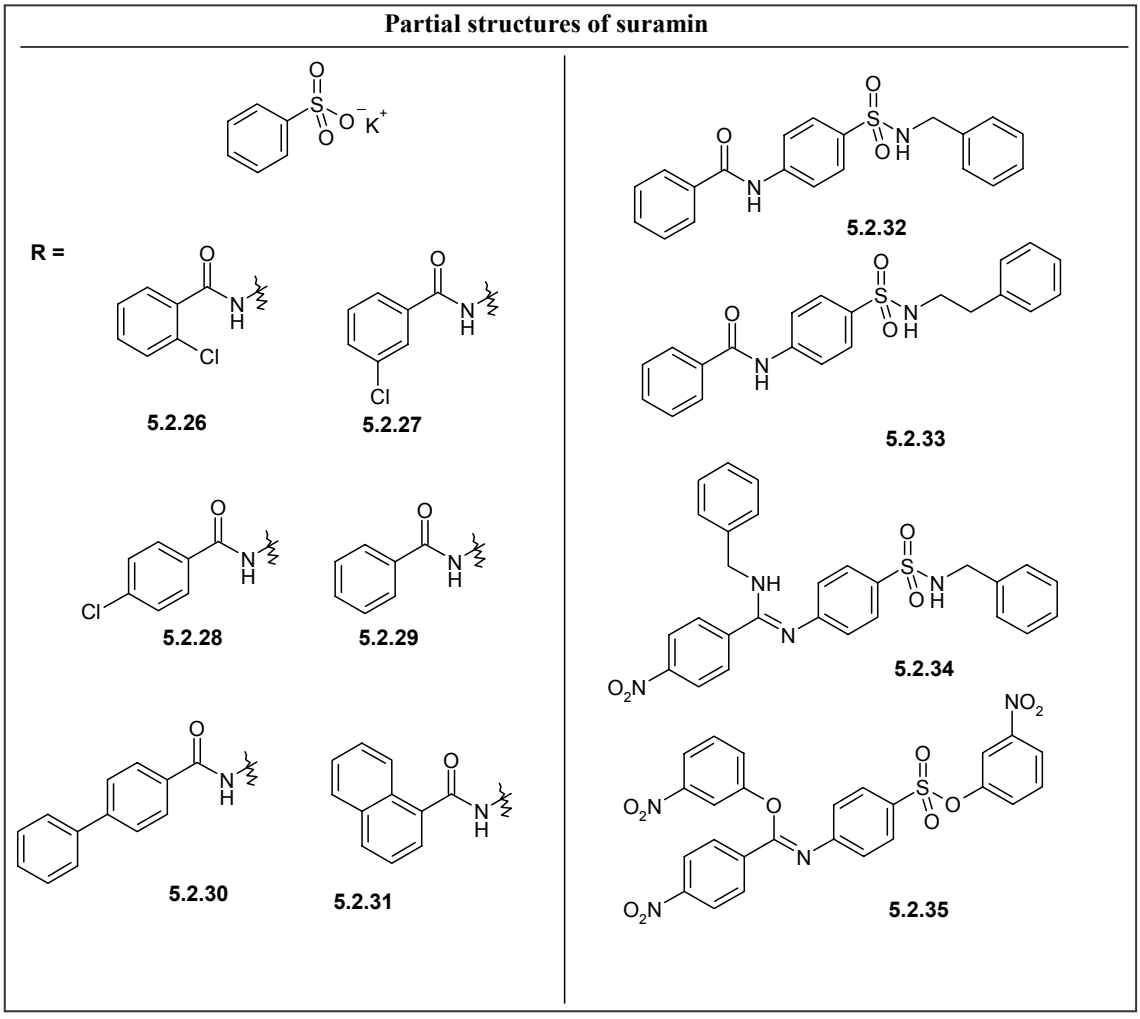


Figure 5.12. *Partial Structures of suramin*

Polyoxometalates are anionic complexes that have relatively high thermodynamic and/or kinetic stability in aqueous solutions at biological pH. In addition to well-developed applications in catalysis, separations, analysis and as electron-dense imaging agents, these complexes have been shown to exhibit biological activity ranging from enzyme inhibition to antitumour/antiviral properties[177]. All of the polyoxometalates tested, PV1 to PV6, were potent inhibitors of the NTPDases, with K_i values between 0.14 and 29 μM (Table 5.5). Compound PV1 and PV3 were selective inhibitors, they showed low K_i values for NTPDase1 and 2 but higher ones for NTPDase3, while PV2, PV4 and PV5 were equally potent inhibitors for all three NTPDases. PV6 was not an inhibitor of NTPDase1 but a potent inhibitor of NTPDase2 and 3. Previously, $[(O_3POPO_3)_4W_{12}O_{36}]^{16-}$ (polyoxometalate I) and $[(O_3PCH_2PO_3)_4W_{12}O_{36}]^{16-}$ (polyoxometalate II) which are structurally related, have been used successfully against HIV-1 infected cells. It was found that these compounds inhibit the DNA polymerases, with IC_{50} values ranging from 2 to 10 μM[177]. The cesium and tetramethylammonium (TMA) salts of polyoxotungstate anions with covalently attached organosilyl groups were found to be active against HIV-1 with EC_{50} values in the range of 3.3 to 50 μM[178]. The anti-HIV-1 activity of polyoxotungstates in human lymphocytes was tested and EC_{50} values were found to be around 1 μM[179]. But in our study the K_i values are even lower for NTPDase inhibition, with K_i values as low as 140 nM (PV4, NTPDase 1).

Table 5.5. *K_i values for polyoxometalate inhibitors of NTPDase1, 2 and 3 obtained by a capillary electrophoresis method. The results are means ± SEM of three separate experiments each run in duplicate. Formulae, molecular mass and stability of polyoxometalates tested as NTPDase inhibitors.*

Inhibitor	**General informations**			***K_i [μM] ± SEM***		
	Formula	**Stability at pH 7.4**	**M_r g/mole**	**NTPDase1**	**NTPDase2**	**NTPDase3**
PV1	$Na_6[W_{12}O_{39}]$ x H_2O	stable	2986.1	2.58 ± 0.30	28.8 ± 0.2	3.26 ± 0.18
PV2	$H_3[PW_{12}O_{40}]$ x H_2O	< 1 h	2880.2	3.49 ± 0.23	6.17 ± 0.15	8.72 ± 1.81
PV3	$K_7[Ti_2W_{10}PO_{40}]$ K_6H_2	stable	2878.9	2.00 ± 0.34	37.4 ± 1.3	4.00 ± 0.26
PV4	$[CoW_{11}TiO_{40}]$ x $12H_2O$	3 h	3221.9	0.140 ± 0.021	0.910 ± 0.041	0.563 ± 0.113
PV5	$K_{10}[Co_4(H_2O)_2(PW_9O_{34})_2]$ x $22H_2O$	24 h	5518.3	0.480 ± 0.010	1.53 ± 0.20	2.61 ± 0.97
PV6	$(NH_4)_{18}[NaSb_9W_{21}O_{86}]$	10 h	6680.2	> 1 mM (15 %)[a]	3.94 ± 0.78	3.77 ± 0.52

[a] Percent inhibition at 1 mM

5.3.6. Investigations of uridine-derived nucleotide mimetics as nucleoside triphosphate diphosphohydrolase1 inhibitors by capillary electrophoresis

Several different uridine-derived nucleotide mimetics (for structures see Figure 5.13) were tested at NTPDase1 using similar assay conditions as for P2 receptor antagonists but with 1.0 mM concentration of test compounds. But none of them was inhibitory at the tested 1.0 mM concentration.

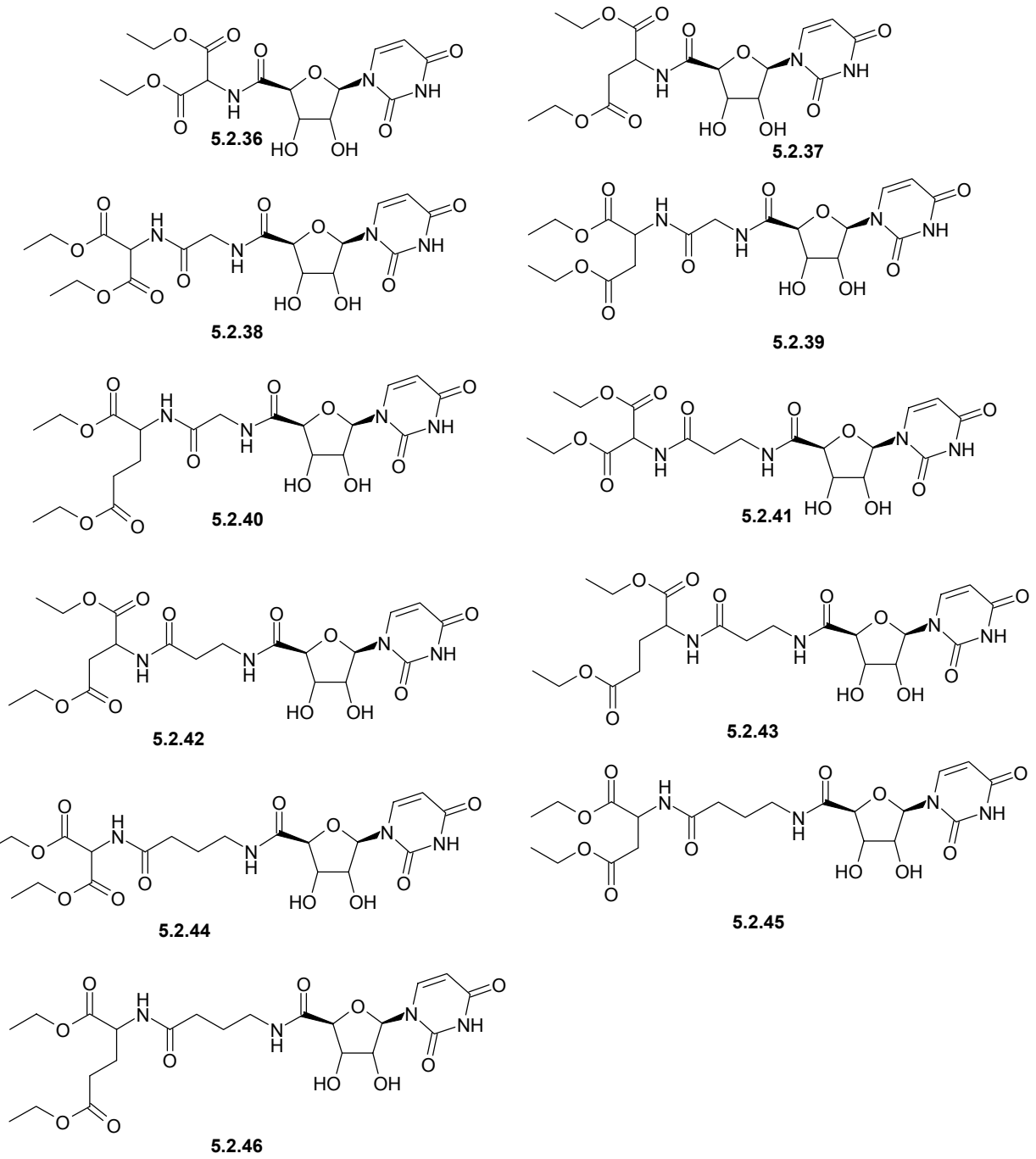


Figure 5.13. *Structures of uridine nucleotide mimetics*

5.4. Discussion

The ecto-nucleoside triphosphate diphosphohydrolases (EC 3.6.1.5) represent a major and ubiquitous family of ecto-nucleotidases. They catalyze the sequential hydrolysis of the γ- and β-phosphate residues of nucleoside tri- and diphosphates, producing the corresponding nucleoside monophosphate derivatives[146]. To date four different cell surface-located isoforms of the enzyme family have been cloned and functionally characterized (NTPDase1, 2 and 3, and very recently NTPDase8)[151,180,181]. The four enzymes differ in substrate specificity and in the pattern of product formation. Whereas NTPDase1 hydrolyzes ATP and ADP about equally well, NTPDase2 has a high preference for the hydrolysis of ATP over ADP. NTPDase3 and NTPDase8 are functional intermediates. NTPDase1 hydrolyzes ATP directly to AMP, ADP is the preferential product of ATP hydrolysis by NTPDase2, and NTPDase3 and NTPDase8 hydrolyze ADP formed from ATP efficiently to AMP.

In previous studies, a variety of compounds have been tested regarding their potency for inhibiting ecto-nucleotidases, often in intact tissues or on cells with undefined enzyme species. Only few studies have used recombinant enzymes to clearly identify the isoform investigated [149,165]. In addition, several methods have been used for the determination of Michaelis-Menten constants (K_m values), and inhibition constants (K_i values for enzyme inhibitors) of NTPDases, including radioisotopic[166], HPLC[148,182,182] and spectrophotometric assays[183]. All of these methods are time-consuming. Radiometric assays are very sensitive, but require tedious procedures and the use of radiolabeled substrates[166]. High performance liquid chromatography suffers from relatively high prices for columns, buffers and solvents; in addition, sample pretreatment to remove proteins and lipids is required. Spectrophotometric methods [157,183] require large amounts of material and are prone to interference from other biological matrices. In addition, the analysis of the total of free phosphate formed confuses the additive contribution of the ATPase and ADPase activity of the identical enzyme, if the nucleoside triphosphate is applied. Our newly developed in-capillary electrophoresis method represents an easy, fast and convenient method for analyzing ecto-nucleotidase activity, including substrate analysis, enzyme kinetics, and the screening for novel inhibitors.

The validity of the method is underpinned by a comparison with previously obtained K_m values. Reported K_m values vary between species and investigators. Since in many cases the identity of the enzyme species was not determined, the values cannot easily be related to our data. However,

the K_m values we have obtained for the rat orthologs of the enzymes are in very good agreement with values obtained for the recombinant enzymes from other species. Reported K_m-values (ATP, μM) are 17 for human NTPDase1[180] and 12 for mouse NTPDase1[180]; 70, 210 and 394, respectively for human NTPDase2[184,185] and 37 for mouse NTPDase2; 75 and 128, respectively for human NTPDase3[186] and 11 for mouse NTPDase3 [187]. Interestingly, the value obtained for rat NTPDase1 in our study (76 μM) is in excellent agreement with a previously reported value for recombinant rat NTPDase1 (75 μM[187]). The K_m values for NTPDase8, the most recently cloned member of the enzyme family are within the same range (13 μM for mouse and 46 μM for rat NTPDase8)[181].

Using identified enzyme species we can clearly show that NTPDase1, 2 and 3 reveal a differential susceptibility to inhibitors. An inhibitory effect of suramin[20,21,23,160,166,188-191], of PPADS[166], of Reactive blue 2[23,166,190], or of ARL67156[23,156,169] on the hydrolysis of ATP by mammalian ecto-nucleotidases has previously been reported for various cellular systems. However, these studies did not identify the NTPDase isoform(s) expressed nor did they exclude a contribution to ATP hydrolysis by other types of ecto-nucleotidases.

In our study, Reactive blue 2, PPADS and suramin inhibited NTPDase3 15- to 25-fold more effectively than NTPDase1. NTPDase2 was inhibited by Reactive blue 2 and PPADS to a similar extent as NTPDase1 but the K_i value for suramin was five fold higher for NTPDase1. An analysis of detergent-solubilized NTPDase1 and NTPDase2 purified from porcine brain, revealed K_i values (ATP) for suramin of 1.8 mM and 2.1 mM, respectively[82,147]. These values are much higher than those obtained for the membrane-bound rat enzymes reported in this study. In contrast to the other compounds tested, ARL67156 revealed the highest inhibitory potency for NTPDase1. It was considerably less effective on NTPDase3 and essentially ineffective on NTPDase2. Since ARL67156 is a widely used inhibitor of "ecto-ATPase" activity, this observation is a caveat for experiments in which this inhibitor is used without previous identification of the NTPDase involved. Dipyridamole was not an inhibitor of the NTPDases investigated but in a previous study it was found to be an effective inhibitor of ATP hydrolysis in rat superior cervical ganglionic cells at 10 μM concentration[192]. The compound has been found to be inactive in other systems [e.g.[13]].

The P2 receptors are currently under intense investigation in order to understand the pharmacology of these receptors and the physiological processes they mediate[162]. To determine the structural requirements for inhibition of NTPDases by Reactive blue 2 and suramin, we tested

a series of analogs. Several anthraquinone derivatives related to Reactive blue 2 were active (**5.2.1-5.2.11**). Compound (**5.2.1**) is more active at NTPDase1 than NTPDase2 and 3 but equipotent at P2Y receptors. It seems that amino-hydroxy-naphthalene-sulfonate structures are responsible for the binding with P2 receptors and NTPDases.

The position of sulfonate group is important for the activity in case of P2Y receptors and NTPDases. Within the anthraquinone derivatives the individual compounds are about equipotent, indicating that the position of the substituents has little effect on potency (**5.2.2-5.2.9**). Compound **5.2.10** was a selective inhibitor of NTPDase3 with K_i value of 1.5 μM, while being inactive at NTPDase1 and 2. Several- fold lower activity (IC_{50} = 11 μM) was observed at $P2Y_2$ receptors, compared to NTPDase3 inhibitory activity. It contains a naphthalene ring side chain. It is noteworthy that all of the inhibitors used in this study share two common structural feature- multiple negative charges (sulfonates) and hydrophobic fused rings- suggesting that these are key determinants for the binding specificity of both the purinoceptors and the NTPDases. Anthraquinone derivatives with substitution at the sulfonate group were not inhibitors of NTPDases (compounds **5.2.12-5.2.25**). The presence of an unsubstituted sulfonate group on the anthraquinone core is important for both $P2Y_2$ receptors and NTPDase inhibition of the analogs. Effects of Reactive blue 2 and related analogs had been studied on ecto-nucleotidases and P2 receptors but no structure-activity relationships were established[164], similar studies had been conducted with suramin and Reactive red analogs[193].

5.5. Summary

A capillary electrophoresis (CE) method for the characterization of recombinant NTPDases 1, 2, and 3, and for assaying NTPDase inhibitors has been developed performing the enzymatic reaction within the capillary. After hydrodynamic injection of plugs of substrate solution with or without inhibitor in reaction buffer, followed by a suspension of an enzyme-containing membrane preparation, and subsequent injection of another plug of substrate solution with or without inhibitor, the reaction took place close to the capillary inlet. After 5 min, the electrophoretic separation of the reaction products was initiated by applying a constant current of –60 μA. The method employing a polyacrylamide-coated capillary and reverse polarity mode provided baseline resolution of substrates and products within a short separation time of less than 7 min. A 50 mM phosphate buffer (pH 6.5) was used for the separations and the products were detected by their UV absorbance at 210 nm. In order to define the properties of individual

isoforms, we determined the K_m-values of heterologously expressed NTPDase1, NTPDase2 and NTPDase3 and the K_i values for selected inhibitors. The Michaelis-Menten constants (K_m) for the recombinant rat NTPDases 1, 2, and 3 obtained with this method were consistent with previously reported data. The inhibition studies revealed pronounced differences in the potency of reactive blue 2, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), suramin, and N^6-diethyl-β,γ-dibromomethylene-ATP (ARL67156) towards the NTPDase isoforms. Notably, ARL67156 does not inhibit all NTPDases, having only a minor inhibitory effect on NTPDase2. Dipyridamole is not an inhibitor of the NTPDase isoforms investigated. To determine the structural requirements for inhibition of NTPDases by suramin and Reactive blue 2, we also tested a series of analogs. Compound **5.2.10** was identified as a selective inhibitor of NTPDase3 with a K_i value of 1.5 μM, while being inactive at NTPDase1 and 2.

The scale of the enzymatic reaction could be dramatically reduced to the nanoliter scale as compared to off-line analysis of the reaction carried out in a microcentrifuge tube. Moreover, since the capillary is used as a reaction vessel, all the assay steps (mixing, reaction, separation, and quantitation) are combined in a fully automated assay. This process was carried out using a temperature-controlled autosampler in order to eliminate routine handling and to speed-up the process. The new method is fast and accurate, requires no sample pretreatment and can be fully automated; thus it is clearly superior to the current standard methods.

Chapter 6. Investigation of the extracellular metabolism of nucleotides in neuroblastoma x glioma NG108-15 cells determined by at-capillary inlet reaction

6.1. Introduction

Extracellular purine and pyrimidine nucleotides, such as ATP, ADP, UTP and UDP, are physiological signalling molecules which bind to membrane receptors termed P2 receptors and produce a wide range of physiological responses[18]. Nucleotides released into the extracellular space are rapidly degraded by ecto-nucleotidases[145]. Such metabolism is quite important in control of nucleotide-mediated cellular responses, not only terminating the activation of P2 receptors but also generating another signalling molecule, adenosine. Since P2 receptors usually co-exist with P1 adenosine receptors, extracellular adenine nucleotide metabolism converts the signal input derived from P2 receptors to P1 receptors. A major problem in pharmacological studies at P2 receptors using nucleotides is their fast enzymatic degradation. Some P2 receptor antagonists make the studies even more complicated by inhibiting the ecto-nucleotidases. Extracellular adenine nucleotide metabolism to adenosine proceeds by a cascade of several ecto-enzymes, such as ecto-ATPase, ecto-apyrase, ecto-nucleotide pyrophosphatase and ecto-5'-nucleotidase (CD73). These enzyme activities largely affect P2 receptor-mediated physiological responses. A group of enzymes was previously identified to metabolize nucleotides on NG108-15 cells based on degradation and inhibition studies: (1) ecto-alkaline phosphatase (E-ALP) responsible for AMP and UMP hydrolysis. In addition, the enzyme plays an important role in the breakdown of UDP, and to a lesser extent of ADP and UTP, while exhibiting only a minor effect on ATP degradation; (2) ecto-adenylate kinase, which leads to the phosphorylation of ADP to ATP; and (3) E-NTPDase which is mainly responsible for the beakdown of ATP and UTP[13].

Capillary electrophoresis (CE) appears to be well-suited to the study of whole, intact cells. It is possible to analyze samples with volumes ranging from nanoliters to picoliters with CE. It also provides fast and highly efficient separations of diverse analytes. It has recently been used for the screening of specific ligands of endothelin receptors by immobilizing whole cells on the capillary surface[194]. But this approach may encounter a problem that receptors or enzymes may lose their activity upon fixation to the capillary column. Also, coating of the capillary column with cells is very difficult and a non-reproducible method. Therefore, we developed an electrophoretically

mediated microanalysis (EMMA) method for studying the extracellular adenine nucleotide metabolism of intact live NG108-15 cells.

In this study, a simple and fast method for analyzing the extracellular enzymatic degradation of nucleotides has been developed, performing the reaction inside the capillary at the capillary inlet followed by electrophoretic separation of the reaction products. In the at-capillary inlet reaction technique, plugs of live NG108-15 cells and substrate are introduced into the capillary, where they are allowed to react by simple diffusion[173]. After that the products are separated by application of the voltage.

6.1.1. Experimental

6.1.1.1. Apparatus

The experiments were performed on a P/ACE capillary electrophoresis (CE) system MDQ glycoprotein (Beckman Coulter Instruments, Fullerton, CA, USA) equipped with a UV detection system. The electrophoretic separations were carried out using an eCAP polyacrylamide-coated fused-silica capillary [(30 cm (20 cm effective length) x 50 µm internal diameter (I.D.) x 360 µm outside diameter (O.D.), obtained from CS-Chromatographie (Langerwehe, Germany)]. An electrokinetic injection for 30 seconds was applied for introducing the sample. The separation was performed using an applied current of -60 µA and a data acquisition rate of 8 Hz. Analytes were detected using direct UV absorbance at 210 nm. The capillary was conditioned by rinsing with 10 mM HCl, 0.5 % SDS for 1 min, with water for 0.5 min and subsequently with buffer (phosphate 50 mM, pH 7.4) for 1 min. Sample injections were made at the cathodic side of the capillary. The CE-instrument was fully controlled through a PC, which operated with the analysis software 32 KARAT obtained from Beckman Coulter. The evaluation of the electropherograms was done using the same software. The capillary temperature was kept constant at 37 °C. The temperature of the sample storing partition was adjusted to 37 °C.

6.1.1.2. Chemicals

The nucleotides adenosine-5'-diphosphate, adenosine-5'-triphosphate, uridine-5'monophosphate, uridine-5'-diphosphate, uridine-5'-triphosphate were obtained from Sigma (Taufkirchen, Germany). Stock solutions of nucleotides were prepared in Hepes (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffer with nucleotide concentrations ranging from 1 to 10 mM. The dipotassium hydrogen phosphate for the CE buffer was purchased

from Fluka (Neu-Ulm, Germany). Sodium chloride, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, anhydrous D-glucose, Hepes (free acid), calcium chloride and magnesium sulfate (constituents of Krebs-Hepes buffer), Dulbecco's modified eagle's medium (DMEM), penicillin-streptomycin and fetal bovine serum for cell culture were obtained from Sigma. HAT (hypoxanthine, aminopterin, thymidine) supplement was from Gibco Life Technologies (Karlsruhe, Germany).

6.1.1.3. Biological experiments and sample preparation

Cells were cultured in DMEM containing 10% fetal bovine serum, HAT-supplement and penicillin-streptomycin until cells were 80% confluent. After rinsing off the cells from the culture flasks they were washed three times with Krebs-Hepes buffer at 37 °C. Then the pellet was suspended in buffer at a cell concentration of 10^5 cells per mL (final concentration in the assay). The Krebs-Hepes buffer consisted of sodium chloride (118.6 mM), potassium chloride (4.7 mM), potassium dihydrogen phosphate (1.2 mM), sodium bicarbonate (4.2 mM), anhydrous D-glucose (22 mM), Hepes (free acid, 10 mM), calcium chloride (1.3 mM) and magnesium sulfate (2.7 mM).

6.1.1.4. Sample preparation procedure

Nucleotides were first dissolved in Krebs-Hepes buffer to obtain 10.0 mM stock solutions. These were further diluted with the necessary amount of the same buffer to obtain a physiological concentration of nucleotides for the in-capillary NG108-15 cell metabolism studies. NG108-15 cell suspension (10^5 cells/mL) was placed in mini CE vials inside the instrument.

6.1.1.5. At-inlet reaction procedure and automation of analytical process

The CE running buffer consisted of dipotassium hydrogen phosphate 50 mM, pH 7.4. The reaction buffer (Krebs-Hepes buffer) consisted of sodium chloride (118.6 mM), potassium chloride (4.7 mM), potassium dihydrogen phosphate (1.2 mM), sodium bicarbonate (4.2 mM), anhydrous D-glucose (22 mM), Hepes (free acid, 10 mM), calcium chloride (1.3 mM) and magnesium sulfate (2.7 mM). Before use, a new capillary was washed with deionized water for 10 minutes. The automation cycle consisted of (i) washing with 10 mM HCl, 0.5 % SDS for 1 min (40 p.s.i; 1 p.s.i = 6894.76 Pa) (ii) with water for 0.5 minutes (ii) equilibration with the CE running buffer for 1 minute (40 p.s.i), (iii) injection of a plug of reaction buffer containing

NG108-15 cells, (iv) injection of a plug of reaction buffer containing ATP, 200 μM, (v) injection of another plug of reaction solution as in (iii), (vi) and finally injection of a plug of water. The plugs were then allowed to react, while the capillary ends were dipped into water, for a predetermined waiting period of 5 min. Then, a current of -60 μA was applied and the reaction products moved towards the detector end of the capillary. After each analysis the capillary was rinsed with 10 mM HCl, 0.5 % SDS for 2 min, CE running buffer for 1 min followed by deionized water for 0.5 min.

Each electropherogram was recorded over 7 min. The NG108-15 cells were placed in the sample storage unit whose temperature was kept constant at 37 °C. At this temperature, the cells' activity remained unaffected during the approximately 12 h of instrument run. Substrate and buffers used in the reaction were also kept at 37 °C in the autosampler reservoir.

6.2. Results and discussion

6.2.1. Optimization of washing procedure

In our previous studies[13,30] we performed enzymatic reactions outside the capillary and diluted the samples before injection into the capillary, which produced reproducible migration times and sharp peaks with simple washing procedures using water and separation buffer. Later on using an in-capillary enzymatic reaction procedure, there was a need to increase the rinsing time to get reproducible migration times, since with shorter rinsing times the capillary was blocked after several runs. However, in this study, investigating the nucleotide metabolism by NG108-15 cells by performing the reaction directly inside the capillary, we observed the adsorption of materials on the coated capillary. The accumulation of proteins at the capillary wall is probably caused by precipitation of proteins/lipids by the freely moving non-cross-linked polyacrylamide chains attached at a high ligand density to the capillary wall (free polymers are known to precipitate proteins)[195]. Therefore, previously established procedures of washing were not enough for reproducible results, which generated zone broadening after few runs. To prevent such distortions, the efficacy of different conditioning procedures was evaluated. Washing procedures applying 5 M and 2 M HCl for 2 min and 5 min, respectively, were tried followed by conditioning for 2 min with separation buffer, 50 mM phosphate, pH 7.4. However, this procedure was not efficient enough to remove the proteins accumulated at the capillary wall. Also, polyacrylamide coating did not withstand such strongly acidic conditions. It has previously

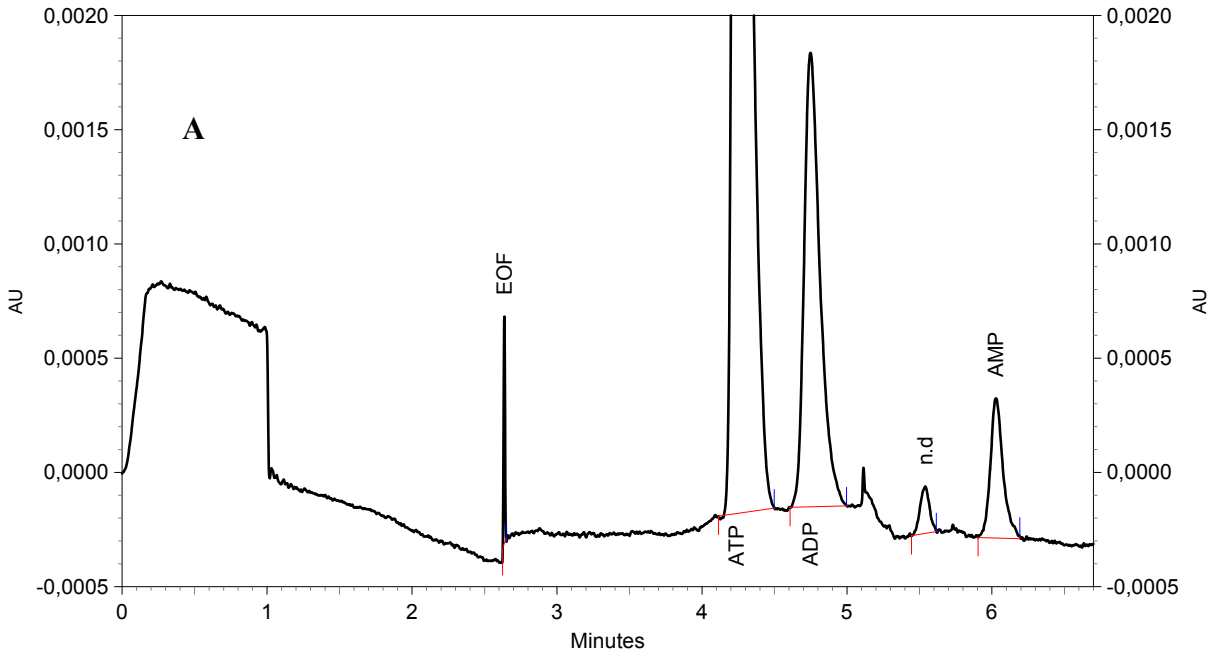
been reported that for polyacrylamide-coated capillaries washing with 2 M HCl between the runs and storage in water over night, is a prerequisite for their high performance. But in our case the capillary coating was found to be not stable under strongly acidic conditions[196]. Wätzig et al. investigated the use of washing solutions containing sodium dodecyl sulfate (SDS) in the analyses of both small molecules and proteins. It was observed that after the SDS wash, the electroosmotic flow was restored to values close to normal in a capillary which had previously been coated with plasma proteins, even when applying a reduced washing procedure (1 min)[197]. SDS binds to the proteins, denatures them and gives them a net negative charge which considerably reduces their tendency to adhere to the walls of the capillary. Plasma protein samples were analyzed by the direct injection method and subsequent washing with SDS gave reproducible results[198]. Capillary zone electrophoresis using polyacrylamide-coated capillaries had been used to study metallothionein isoforms (cysteine-rich metal-binding proteins), using sodium phosphate buffer for separation. 0.01 M HCl containing 0.5 % SDS was used as a washing procedure between each run[199]. We observed that the use of 0.01 M HCl containing 0.5 % SDS, pH 3.0, was a good separation buffer for obtaining reproducible migration times in a polyacrylamide-coated capillary.

6.2.2. Biological applications

6.2.2.1. Nucleotide metabolism by NG108-15 cells

Nucleotide metabolism is usually detected by HPLC or radioactive methods which are sensitive but costly and complicated. HPLC separations require large amounts of biological material and involve time-consuming procedures. In comparison to this, capillary electrophoresis offers a simple and cost-effective alternative. It allows short analysis times and requires only very samll amounts (nanoliters) of samples. Another major advantage of CE is the fact that it does not require sample pretreatment. The degradation of the nucleotides ATP and UTP by intact, live mammalian NG108-15 cells (mouse neuroblastoma x rat glioma hybrid cell line) was investigated using the developed CE method. As an example, electropherograms of ATP and UTP degradation by the cells are shown in Figure 6.1A and 6.1B. An electropherogram of NG108-15 cells without nucleotide added is shown in Figure 6.1 C. Plugs of assay buffer containing 10^5 cells/mL were injected and in between two plugs, a single plug of assay buffer containing 500 μM of nucleotide were injected by hydrodynamic injection into the capillary and then allowed to react for different time intervals. Subsequently, after electrophoretic separation,

the concentrations of nucleoside tri-, di-, and monophosphates were determined. The degradation of substrates (ATP and UTP) inside the capillary occurred without applying any voltage, unlike in EMMA, where compounds are mixed electrophoretically by applying voltage before the reaction takes place. After the reaction, a constant current of –60 µA with reverse polarity was applied to separate the reaction products. A time-dependent degradation of ATP and UTP was observed. On increasing the incubation time inside the capillary the formation of ADP, UDP and AMP and UMP was increased (Figure 6.2). Degradation of ATP to ADP was faster than that of ADP to AMP and UTP degradation was faster than that of UDP. Degradation of UTP was slower than that of ATP. This is the first time that the nucleotide metabolism by intact whole cells has been studied by capillary electrophoresis performing the enzymatic reaction directly inside the capillary.



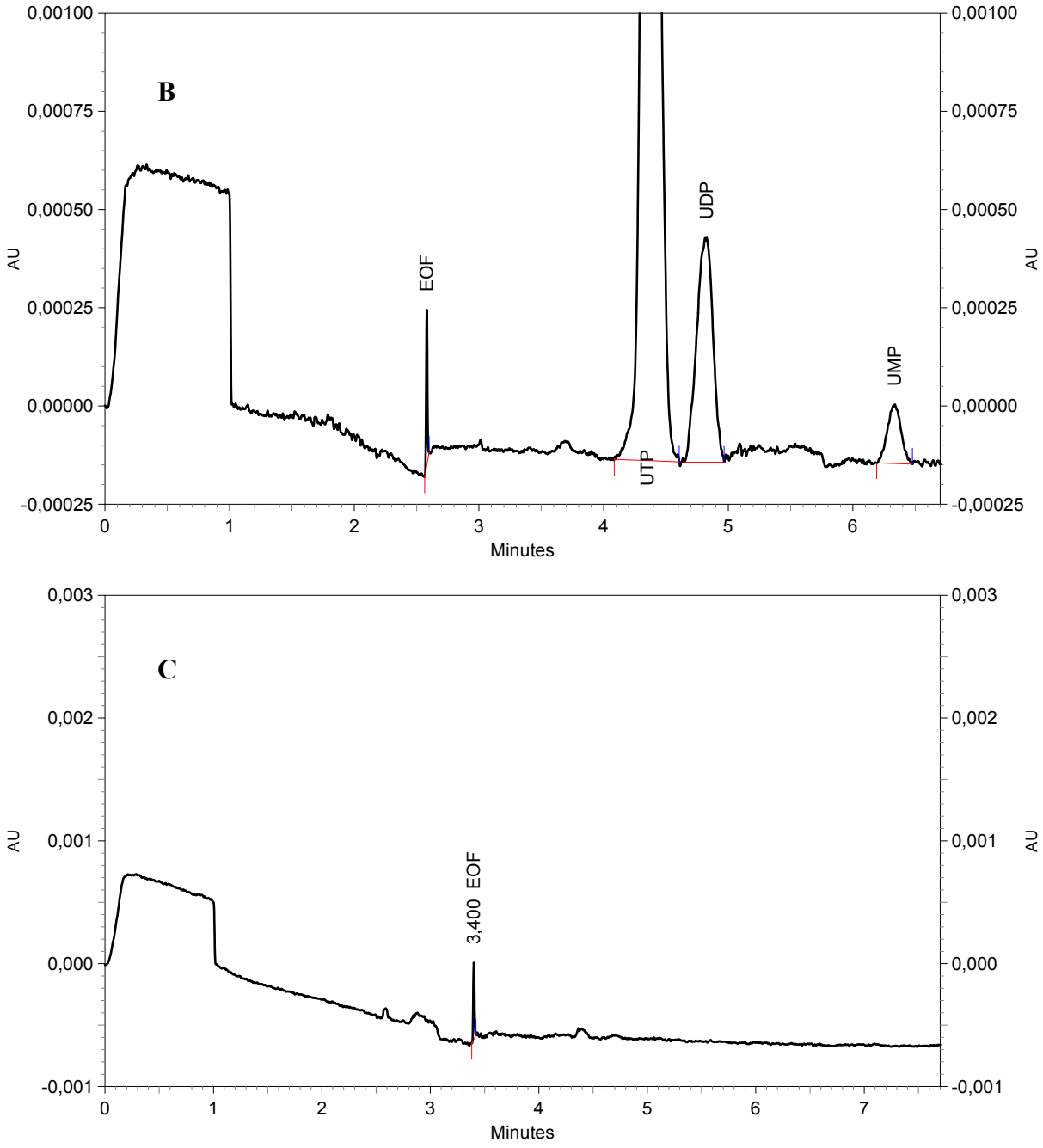


Figure 6.1. A*. Typical electropherogram of NG108-15 cell metabolism at capillary inlet using intact, live cells and performing the enzymatic reaction within the capillary. Concentration of NG108-15 cells was 10^5 cells/mL and 500 µM ATP was used as a substrate, in Krebs-Hepes buffer. The plugs of cells and substrates were allowed to stand during a predetermined period of 5 min and subsequently a –60 µA current was applied and the reaction products migrated to the detector. The separation conditions were 50 mM phosphate, pH 7.4, polyacrylamide coated*

capillary, 30 cm length (20 cm to the detector), 50 µm I.D.; -60 µA; 37 °C; detection at 210 nm; pressure injection; reverse polarity.

***B**. Typical electropherogram of NG108-15 cell metabolism at capillary inlet. Concentration of NG108-15 cell was 10^5 cells/mL and 500 µM UTP was used as a substrate, in Krebs-Hepes buffer.*

***C**. Typical electropherogram of NG108-15 cell metabolism at capillary inlet. Concentration of NG108-15 cell were 10^5 cells/mL without nucleotide added, in Krebs-Hepes buffer.*

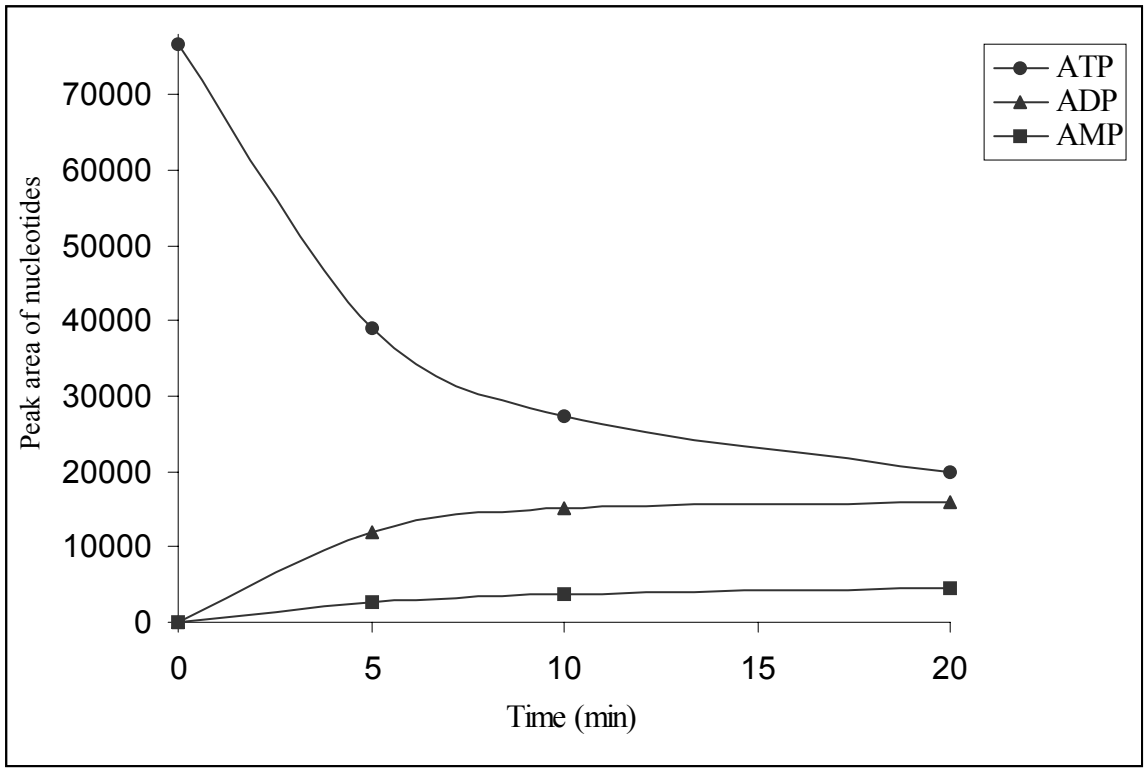


***Figure 6.2.** Time-dependent degradation of ATP by NG108-15 cells (10^5 cells/ml). Determined by in-line CE assay using live cells. The initial ATP concentration was 500 µM.*

6.3. Summary

Capillary electrophoresis can be a valuable tool for the on-line monitoring of bioprocesses. The metabolism of extracellular nucleotides on intact NG108-15 cells, a neuroblastoma x glioma hybrid cell line, was studied by means of capillary zone electrophoresis (CZE) using an in-capillary enzyme-catalyzed microreaction technique. The CZE method employing a neutral-bonded capillary and reversed polarity mode provided a good resolution and short analysis time of less than 7 min. Plugs of cells and nucleotides were injected hydrodynamically and allowed to react inside the capillary for a predetermined period of time, after that degraded products were detected by their UV absorbance at 210 nm. Constant current (–60 μA) was applied, and a phosphate buffer, pH 7.4, was used. Ecto-nucleotidases and ectophosphatases present on the extracellular surface of the cells hydrolyzed nucleotides, such as ATP, ADP, UTP, UDP, UMP and AMP. The cells were stable in the presence of the applied electric current. This could be confirmed by injecting the cells into the capillary and allowing them to stand for 10 min; after electrophoretic separation we did not see any nucleotide peak. Which inticates that interacellular nucleotides, present in high concentration, are not released. In a separate experiment, the cells were destroyed by heating at 99 °C for 10 min to detect the nucleotides released from inside the cells. Under such conditions a high quantity of nucleotides was released from the cells. By these experiments it was confirmed that cells had remained intact during incubation inside the capillary and also by applying a current to separate the nucleotides. The method is superior to the other methods in which the cells were incubated outside the capillary. No sample preparation at all was required.

Chapter 7. Development of a capillary electrophoresis method for the investigation of ester hydrolysis products

7.1. Introduction

Marine ascidians are known to be a rich source of chemically diverse secondary metabolites with often remarkable biological activities. In many cases these compounds are simple amino acid derivatives or more complex alkaloids. Especially, ascidians of the family Didemnidae have been investigated intensively because of their unusual, cytotoxic metabolites.

In the present study a CE-based assay was developed for the investigation of the chemical and enzymatic stability of a 5'-deoxy-5'-methylthioadenosine-2',3'-diester, a natural product isolated from a scidians[200] that had been collected at Heron Island's, Wistari Reef, the Great Barrier Reef, Australia. 5'-deoxy-5'-methylthioadenosine-2',3'-diester was investigated in radioligand binding assays in order to determine its affinity for AR subtypes. To make sure that the measured effects were actually due to intact 5'-deoxy-5'-methylthioadenosine-2',3'-diester, we examined a possible hydrolysis of compound 5'-deoxy-5'-methylthioadenosine-2',3'-diester by rat brain cortical membrane preparations as a potential source of esterase activity. To examine the enzymatic hydrolysis of compound 5'-deoxy-5'-methylthioadenosine-2',3'-diester, it was incubated with carboxylesterase.

7.2. Experimental

7.2.1. Materials and methods

7.2.1.1. Capillary electrophoresis instrumentation

CE separations were carried out using a P/PACE system MDQ glycoprotein (Beckman Coulter Instruments, Fullerton, CA, USA) equipped with a DAD detection system. The electrophoretic separations were carried out using an eCAP fused-silica capillary [40 cm (30 cm effective length) x 75 μm internal diameter (I.D) x 375 μm outside diameter (O.D) obtained from Beckman Coulter]. On-line UV detection was performed in the range of 190-350 nm. The runs were performed under the following conditions: T = 25 °C, λ_{max} = 260 nm, current = 95 μA, running buffer 25 mM sodium tetraborate buffer, 100 mM SDS, pH 8.5. The capillary was washed with 0.1 N NaOH for 2 min, deionised water for 1 min, and 25 mM sodium tetraborate buffer, 100 mM SDS, pH 8.5, for 1 min before each injection. Injections were made by applying 0.1 psi of

pressure to the sample solution for 25 s. The CE instrument was fully controlled through a personal computer, which operated with the analysis software 32 KARAT obtained from Beckman Coulter. The evaluation of the electropherograms was done using the same software. The temperature was kept constant at 25°C and the samples were also stored at the same temperature.

7.2.1.2. Chemicals

Tris(hydroxymethyl)aminomethane was obtained from Acros Organics, Geel, Belgium. Sodium tetraborate, boric acid and sodium dodecyl sulphate (SDS) were obtained from Sigma Aldrich, Germany. All other reagents were obtained from Sigma Aldrich, Germany.

7.2.2. Determination of ester hydrolysis by capillary electrophoresis

Ester hydrolysis of 5'-deoxy-5'-methylthioadenosine-2',3'-diester was performed with carboxylesterase from porcine liver (EC 3.1.1.1; 1840 I.U./mL) obtained from Sigma Aldrich, Germany. A 1 mM stock solution of 5'-deoxy-5'-methylthioadenosine-2',3'-diester in DMSO (12.5 µL) was diluted with Tris-HCl buffer, 5 or 50 mM, pH 8.0 (concentration of 5'-deoxy-5'-methylthioadenosine-2',3'-diester : 25 µM) and 2 µL of carboxylesterase were added. Incubation was carried out at 25°C for 60 min. A sample of 12.5 µL of a 1 mM stock solution of 5'-deoxy-5'-methylthioadenosine-2',3'-diester in DMSO was diluted with Tris-HCl buffer 50 mM, pH 7.4 (concentration of 5'-deoxy-5'-methylthioadenosine-2',3'-diester: 25 µM) and incubated with rat brain cortex (protein concentration ca. 70 µg/mL) at room temperature (23°C) for 90 min.

Incubation was terminated by heating the sample at 99°C for 10 min. The samples were centrifuged at 10,000 g for 5 min at room temperature (23°C) and the resulting supernatants were used for subsequent analysis by capillary electrophoresis (CE).

7.3. Results and discussion

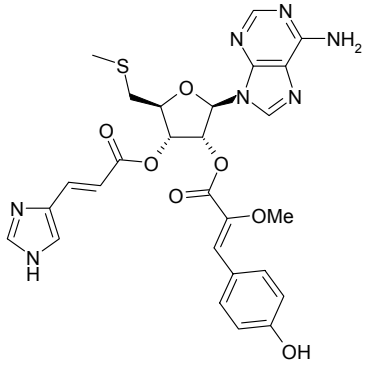
7.3.1. Investigation of ester hydrolysis products by capillary electrophoresis

Micellar electrokinetic capillary chromatography (MECC) was used for the separation of hydrolyzed products of a natural product, a 5'-deoxy-5'-methylthioadenosine-2',3'-diester (for structure see Table 7.1). 5'-deoxy-5'-methylthioadenosine-2',3'-diester consists of a nucleoside moiety (adenosine) with a methylthio group at the 5'-position of the ribose moiety instead of a hydroxyl group. The ribose is esterified with 3-(4-hydroxyphenyl)-2-methoxyacrylic acid at the

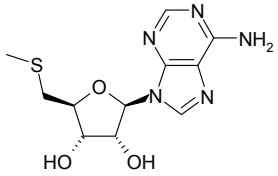
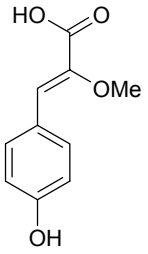
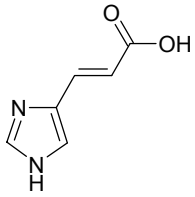
2'-position and with urocanic acid at the 3'-position. A standard solution of 5'-deoxy-5'-methylthioadenosine-2',3'-diester (25 μM) was injected into the capillary and the migration time was found to be 17.72 min with a wavelength of maximum absorption at 295 nm using borate buffer (25 mM) with 50 mM SDS. Then 5'-deoxy-5'-methylthioadenosine-2',3'-diester was incubated with esterase to examine the hydrolysis of 5'-deoxy-5'-methylthioadenosine-2',3'-diester (see Figure 7.1). Under these conditions, the 5'-deoxy-5'-methylthioadenosine-2',3'-diester was hydrolyzed to three products and peaks appeared at 4.77, 5.80, 6.28 and 17.72 min respectively, the last peak appeared with the same migration time as the standard, which could therefore be detected as the intact compound. Further detection of the hydrolyzed products was done by visualising their UV spectra. Each peak showed a different UV spectrum, wavelengths of maximum absorption with their characteristic UV band are collected in Table 7.1. By hydrolysis of 5'-deoxy-5'-methylthioadenosine-2',3'-diester the number of chromophores was changed, so that different UV spectra were obtained by photodiode array detection, which represented a key method to identify the hydrolyzed products.

To examine the hydrolysis of the 5'-deoxy-5'-methylthioadenosine-2',3'-diester by rat brain cortical membranes, as a possible source of esterase activity, the 5'-deoxy-5'-methylthioadenosine-2',3'-diester was incubated with rat brain cortex as described above. The resulting electropherogram showed only one peak that could be identified as the non-hydrolzsed intact 5'-deoxy-5'-methylthioadenosine-2',3'-diester (see Figure 7.2). In comparison to Figure 7.1 no additional peaks could be found within the migration time of the three peaks derived from hydrolysis products. Therefore it could be confirmed that no hydrolysis of 5'-deoxy-5'-methylthioadenosine-2',3'-diester occurred when incubating it with rat brain cortex. Further the stability of 5'-deoxy-5'-methylthioadenosine-2',3'-diester was examined by heating the 25 μM standard solution of the compound at 99°C for 10 min. When this solution was injected into CE no degradation products could be found, which confirmed that this compound was stable at high temperature. Therefore no degradation was caused in the termination process of the incubation by heating the samples at 99°C. In the sample preparation for ester hydrolysis two different buffer concentrations (5 and 50 mM) were used to dilute the 5'-deoxy-5'-methylthioadenosine-2',3'-diester. CE analyses showed that no differences in the peak area of the hydrolyzed products could be observed and that both buffer concentrations can be used for the ester hydrolysis experiments.

Table 7.1. *CE measurements of hydrolysis products of* 5'-deoxy-5'-methylthioadenosine-2',3'-diester *after incubation with esterase* [a]



5'-deoxy-5'-methylthioadenosine-2',3'-diester

Peak no.	Mean migration time ± SEM (min)	Structures of products	λ_{max} in UV-spectrum
A	17.72 ± 0.31	5'-deoxy-5'-methylthioadenosine-2',3'-diester [b]	295 nm
B	4.77 ± 0.06	 MTA	260 nm
C	5.80 ± 0.07		283 nm
D	6.28 ± 0.08		275 nm

[a] for incubation conditions see Figure 7.1 and Experimental Section

[b] 85 % of 5'-deoxy-5'-methylthioadenosine-2',3'-diester were unhydrolyzed

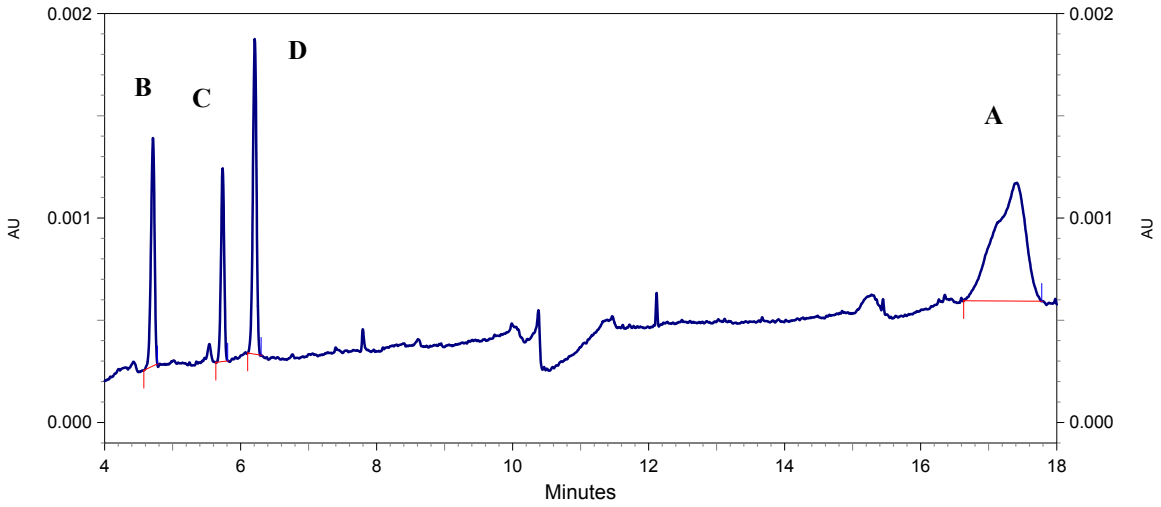


Figure 7.1. *Electropherogram of* 5'-deoxy-5'-methylthioadenosine-2',3'-diester *(concentration 25 µM) incubated with carboxylesterase from porcine liver (2 µL) at pH 8.0 and 25°C for 60 min. The separation conditions were 25 mM sodium borate buffer, 100 mM SDS, pH 8.5, fused silica capillary, 40 cm length (30 cm to the detector), 75 µm I.D.; 95 µA; 25°C; detection at 260 nm; 0.1 psi pressure injection (25 s). For structural assignment of peaks see Table 7.1.*

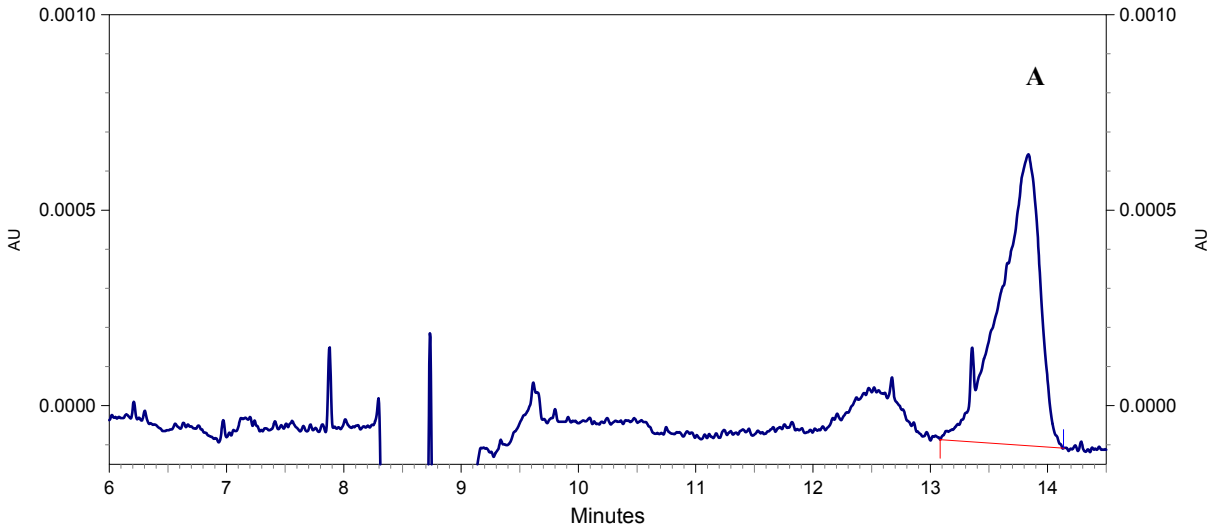


Figure 7.2. *Electropherogram of 5'-deoxy-5'-methylthioadenosine-2',3'-diester incubated with rat brain cortex for 90 min. The separation conditions were 25 mM sodium borate buffer, 100 mM SDS, pH 8.5, fused silica capillary, 40 cm length (30 cm to the detector), 75 µm I.D.; 95 µA; 25 °C; detection at 260 nm; 0.1 psi pressure injection (25 s).*

7.4. Summary

A capillary electrophoresis (CE) method was developed for the investigation of ester hydrolysis products of a marine natural product, 5'-deoxy-5'-methylthioadenosine-2',3'-diester. The method employing a fused-silica capillary and normal polarity mode provided good resolution of the products of the enzymatic reaction. After stopping the enzymatic reaction by heating, the samples were injected by pressure and detected by their UV absorbance at 260 nm. A constant current (95 μA) was applied, and a sodium tetraborate buffer containing 100 mM SDS, pH 8.5 was used for the separations. The stability of the 5'-deoxy-5'-methylthioadenosine-2',3'-diester was examined by heating a 25 μM standard solution of the compound at 99 °C for 10 min. It was shown that the compound is chemically stable at high temperature. Similarly, incubation of the ester with rat brain cortex for 90 min showed no hydrolysis. However, incubation with carboxylesterase resulted in slow hydrolysis of the compound.

Chapter 8. Development of a capillary electrophoresis method for the purity determination of [^{3}H]PSB-298, a new radioligand for adenosine A_{2B} receptors

8.1. Introduction

Adenosine receptors (ARs) are investigated as potential drug targets in many therapeutic areas. Adenosine has been suggested to play a role in asthma, possibly via activation of A_{2B} adenosine receptors on mast cells and other pulmonary cells. New A_{2B} receptor antagonists (1,8-disubstituted xanthine derivatives) were synthesized in our group to obtain highly potent and selective A_{2B} antagonists possessing good water solubility. For the development of a radioligand binding assay for A_{2B} ARs, a tritium-labeled adenosine receptor antagonist, [^{3}H]PSB-298 (see Fig.8.1) was prepared. In order to determine the purity of [^{3}H]PSB-298, we had to develop a capillary electrophoresis method.

8.2. Experimental

8.2.1. Materials and methods

8.2.1.1. Capillary electrophoresis instrumentation

Capillary electrophoresis was performed on a P/PACE System 5500 (Beckman Coulter Instruments, Fullerton, CA, USA) equipped with a photodiode array detection system. The instrument was controlled by P/ACE station software (Beckman instruments). The runs were performed under the following conditions: T = 25 °C, λ = 321 nm, V = 10 kV, in 150 mM Tris-HCl buffer containing 100 mM SDS, pH 9.1. The electrophoretic separations were carried out using an eCAP fused silica capillary (75.0 μm internal diameter (I.D.) x 375 μm outside diameter (O.D.), 37.0 cm length (30 cm to the detector). The capillary was washed with 0.1 N aqueous NaOH solution for 2 min, followed by deionized water for 1 min, and 150 mM Tris-HCl buffer containing 100 mM SDS, pH 9.1, for 1 min before each injection. Injections were made by applying a slight pressure (0.5 psi) for 5 s delivering approximately 5 nL of the sample solution.

8.2.1.2. Chemicals

(8-{4-[2-(2-Hydroxyethylamino)-2-oxoethoxy]phenyl}-1-propylxanthine (PSB-298) and 1-propargyl derivative (8-{4-[2-(2-hydroxyethylamino)-2-oxoethoxy]-phenyl}-1-propargylxanthine

(PSB-297) were synthesized in our laboratory[201] and PSB-297 was custom-labeled via catalytic hydrogenation using tritium gas by Nycomed Amersham, Buckinghamshire, UK through Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany, to obtain [^{3}H] PSB-298. The specific activity of [^{3}H] PSB-298 was 124 Ci (4.59 TBq)/mmol.

Tris(hydroxymethyl)aminomethane was obtained from Acros Organics (Leverkusen, Germany). Dimethylsulfoxide (DMSO) was from Fluka (Switzerland), sodium dodecyl sulfate (SDS) was from Sigma, Germany; HCl was from Merck, Germany.

8.2.1.3. Preparation of standard solutions of PSB-298 and PSB-297

Stock solutions of the unlabeled compounds 8-[4-(2-hydroxyethylamino)-2-oxoethoxy)phenyl-1-propylxanthine (PSB-298) and 8-[4-(2-hydroxyethylamino)-2-oxoethoxy)phenyl-1-propargylxanthine (PSB-297) were prepared in DMSO at a concentration of 1.0 mg/mL which were then diluted with a mixture of DMSO and deionized water (1 : 1) to obtain a range of different concentrations. The radioactive compound [^{3}H]PSB-298 was dissolved in DMSO at a concentration of 4.4 mg/mL and then diluted 1 : 1 with deionized water. For the determination of linearity and the limit of detection and quantitation, solutions of PSB-298 and PSB-297 were run in the concentrations 0.002, 0.005, 0.01, 0.02, 0.06, 0.08 and 0.1 mg/mL.

8.3. Results and discussion

The compound [^{3}H]PSB-298 was synthesized from PSB-297 (see Figure 8.1); therefore, a CE method had to be developed for the determination of potential contamination of [^{3}H]PSB-298 by its precursor PSB-297.

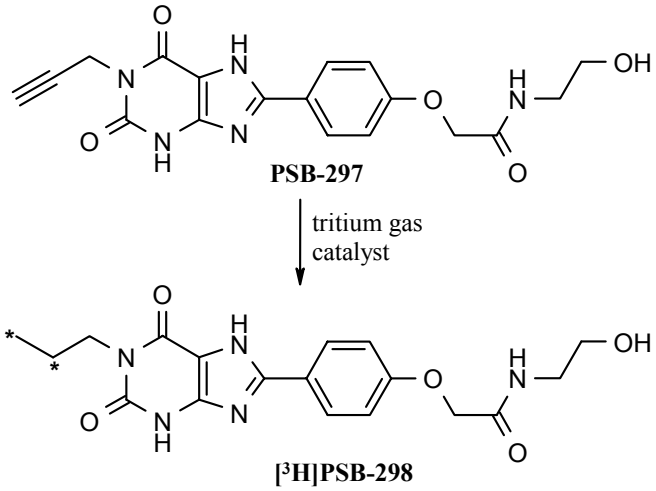


Figure 8.1. *Preparation of [^{3}H]PSB-298 from the propargyl precursor PSB-297 by catalytic hydrogenation (* denotes position of radiolabel).*

A Tris-HCl buffer with a high pH (pH 9.1) was chosen because at this pH the majority of the silanol groups were negatively charged. Hence, interactions between sample components, which have a negative charge in micelles, and the wall, should be reduced resulting in a shorter migration time. Additionally, the ionic strength of the selected buffer was rather high (150 mM). This is beneficial for the separation efficiency but at the same time limits the possibility of applying higher voltages. Thus, a moderate value of 10 kV was chosen. The use of SDS as a micelle-forming agent had two advantages; firstly, the compound-SDS aggregates are negatively charged and interaction with the negatively charged wall is reduced. Secondly, it separated the two compounds, which were very similar in molecular weight by additional partition chromatography because SDS micelles act as peudostationary phase.

8.3.1. Method validation

8.3.1.1. Linearity and limit of detection and quantitation

Figure 8.2 shows a plot of PSB 298 peak areas vs. concentrations, which was used to determine the concentration of [^{3}H]PSB-298. Linear relationships between peak areas and concentrations of compounds were found within the concentration range of the standards.

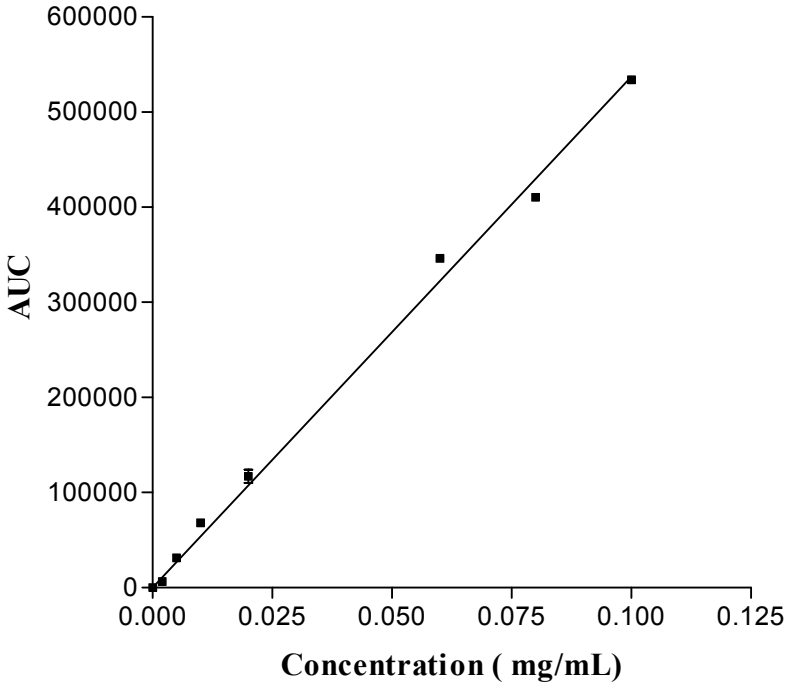


Figure 8.2. *Linearity of calibration curve was determined for the concentration range of 0.002-0.1 mg/mL of PSB-298). Regression equation;* $y = 5260000x + 8290$ *and* $R^2 = 0.996$

The determination of the compouds exhibited a high degree of linearity of calibration cuver in the range from their limit of quantitation (LOQ; calculated at a signal-to-noise ratio equal to 10, within the linear range) to the highest measured concentration at 0.1 mg/mL.

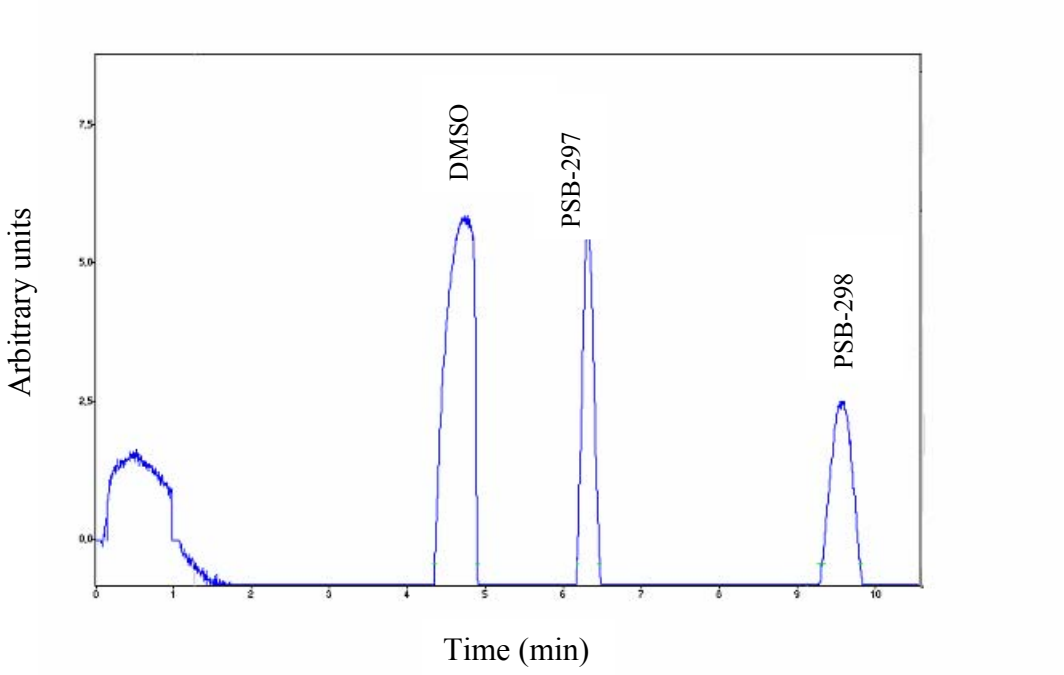


Figure 8.3. *Electropherogram of [*^{3}*H]PSB-298 in the presence of PSB-297 as an internal standard. The separation conditions were 150 mM Tris-HCl, 100 mM SDS, pH 9.1, fused silica capillary, 37 cm length (30 cm to the detector), 75 µm I.D.; 10 kV; 25* $^{\circ}$*C; detection at 321 nm; pressure injection (0.5 psi, 5 s).*

The coefficient of correlation for PSB-298 and PSB-297 were 0.996 and 0.993, respectively. The LOQs for PSB-298 and PSB-297 were 0.04 ± 0.001 (SEM) (n = 3) and 0.05 ± 0.002 (SEM) (n = 3) mg/mL, respectively. The limit of detection (LOD) was defined as a signal-to-noise ratio of 3. The LOD value for PSB-298 and PSB-297 was 0.002 mg/mL. Each sample was measured 3-4 times.

8.3.1.2. Purity determination of [^{3}H]PSB-298 by capillary electrophoresis

The concentration of the solution of [^{3}H]PSB-298 in DMSO was determined by using capillary electrophoresis. Peaks were identified by two means: (i) by comparing the migration times of the observed peak with that of the nonradioactive PSB-298 eluted under the same conditions, and (ii) by spiking the radioactive sample with non-radioactive PSB-298 standard. Under the same conditions the peak for the precursor compound PSB-297 appeared at 6.31 ± 0.07 min and that for PSB-298 (radioactive and non-radioactive) at 9.54 ± 0.13 min. Concentration in the original radioactive sample of PSB-298 was found to be 4.4 µg ± 0.0054 /50µl (SEM) (n = 3). The solution of the radioactive compound gave only a single peak, no peak of the starting compound was found indicating high purity (>95%). Therefore, quantitative determination could be performed in the presence of PSB-297 (0.1 mg/mL) as an internal standard. A typical electropherogram of [^{3}H]PSB-298 in the presence of its precursor PSB-297 used as internal standard is shown in Figure 8.3.

8.4. Summary

A micellar electrokinetic chromatographic (MECC) method has been developed for determining the purity of a newly synthesized antagonist radioligand for A_{2B} adenosine receptors [^{3}H]PSB-298 ((8-{4-[2-(2-hydroxyethylamino)-2-oxoethoxy]phenyl}-1-propylxanthine). MECC separation of [^{3}H]PSB-298 ((8-{4-[2-(2-hydroxyethylamino)-2-oxoethoxy]phenyl}-1-propylxanthine) and its precursor, the 1-propargyl analog (8-{4-[2-(2-hydroxyethylamino)-2-oxoethoxy]-phenyl}-1-propargylxanthine (PSB-297) was performed with the use of an untreated fused-silica capillary of 30 cm of effective length and 75 µm I.D., and a positive voltage of 10 kV in 150 mM Tris-HCl buffer containing 100 mM SDS, pH 9.1. The CE method was successfully validated: linearity of calibration curve, limit of quantitation, limit of detection and accuracy were evaluated.

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7. Publications

1. Kehraus, S.; Gorzalka, S.; Hallmen, C.; **Iqbal, J**.; Müller, C.E.; Wright, A.D.; Wiese, M.; König, G.M. Novel amino acid derived natural products from the ascidian Atriolum robustum: identification and pharmacological characterization of a unique adenosine derivative. *J. Med. Chem.* **2004**, *47*, 2243-2255.

2. **Iqbal, J**.; Vollmayer, P.; Braun, N.; Zimmermann, H.; Müller, C.E. A capillary electrophoresis method for the characterization of ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) and the analysis of inhibitors by in-capillary enzymatic microreaction. *Purinergic Signalling* **2005** (in press).

3. Diekmann, M.; Bertarelli, D.; Hayallah, A.M.; **Iqbal, J**.; Preiss, B.; Müller, C.E. Characterization of native rodent and recombinant human A_{2B} adenosine receptors with [^{3}H]PSB-298, a new antagonist radioligand. (Manuscript).

4. **Iqbal, J**.; Burbiel, J.C.; Müller, C.E. Development of off-line and in-line capillary electrophoresis methods for the screening and characterization of adenosine kinase inhibitors and substrates. (Manuscript).

5. **Iqbal, J**.; Scapozza, L.; Müller, C.E. Development of a capillary electrophoresis method for the quantitative determination of K_m and K_{cat} of Herpes Simplex Virus type 1 thymidine kinase substrates. (Manuscript).

Poster abstract

- Iqbal, J.; Müller, C. E. **Development of a novel, capillary electrophoresis based method for the screening of potential adenosine kinase inhibitors**. 14th Camerino-Noordwijkerhout Symposium "Ongoing Progress in the Receptor Chemistry" University of Camerino, Camerino, Italy, September 7-11, 2003

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3. 14th Camerino-Noordwijkerhout Symposium "Ongoing Progress in the Receptor Chemistry"
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