A new entry to adenosine analogues via purine nitration - Combinatorial synthesis of antiprotozoal agents and adenosine receptor ligands
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Introduction
1.1 SOLID PHASE SYNTHESIS AND COMBINATORIAL CHEMISTRY

The progress that molecular biology has made in the past two decades allowed for the automated biological screening of drug candidates, a procedure which is known as high-throughput screening (HTS). In order to speed up the costly drug development process, medicinal chemists were now challenged to provide numerous drug candidates for many novel therapeutic targets in a short time and combinatorial chemistry was the device. In initial approaches, gigantic numbers of oligomeric molecules were prepared as mixtures of all possible combinations of a given set of building blocks. The philosophy was to create such a diverse set of compounds that screening was bound to produce a hit. But the often undefined quality of these ‘real’ combinatorial libraries soon emerged as a serious drawback and focus shifted to the automated parallel synthesis of single compounds of defined structure, which started a period of turbulent evolution of solid phase synthesis.

The founding father of solid phase synthesis is undoubtedly R. Bruce Merrifield, who revolutionised peptide synthesis in 1963 by reporting the preparation of a tetrapeptide on insoluble cross-linked polystyrene beads. Twenty-one years later, he was rewarded the Nobel prize for his ‘development of methodology for chemical synthesis on a solid matrix’. Shortly after Merrifield’s principal solid phase peptide synthesis Letsinger and Khorana each reported a modified strategy, which formed the groundwork for automated solid phase oligonucleotide synthesis, a technique that has proved invaluable in modern genetic engineering. The exemplary method of Khorana, who’s contribution to polynucleotide synthesis was rewarded with the Nobel prize in 1968, is outlined in Scheme 1.1. It involved the initial attachment of a nucleoside, thymidine 2, to previously synthesised 4-methoxytrityl-chloride polystyrene polymer 1. The resulting polymer 3 was subjected to repeated nucleotide coupling steps and final cleavage of the desired oligonucleotide from the polymer was achieved under mild acidic conditions furnishing TpTpT 6 in 84% yield from polymer bound thymidine 3. This example clearly demonstrates the benefits of synthesis on a solid support: easy work-up by simple washing and filtration steps and the use of reagents in excess to drive the reactions to completion. These procedures soon turned out to be quite applicable to automation.

The high-throughput synthesis and screening innovation in medicinal chemistry required translation of ‘classical’ solution phase organic chemistry to solid supported chemistry in order to produce the desired drug-like molecules. This demanded a serious understanding of polymer properties. Although a growing number of new solid supports become available for application in solid phase chemistry, the classic Merrifield-type polystyrene cross-linked with 1 or 2% divinylbenzene is still the most widely used support for solid phase operations. Functionalisation of the phenyl groups in the polystyrene matrix permits the attachment of
substrates. Since more than 99% of the reactive sites are inside these resin beads, they must be swollen in the reaction solvent in order to be accessible to reagents.\(^6\) The choice of reaction solvent is therefore crucial in polymer supported reactions and the optimum solvent may not be the same as used in analogous reactions in solution. In fact, the swollen cross-linked polymers are said to be the solvents in which the reactions are performed.\(^5\) The gel-phase polymer-solvent system does not allow the use of heterogeneous reagents and accordingly precipitation of side-products from the gel-phase is a typical nuisance in solid phase chemistry.

The attachment of the substrate to the polymer, sequential polymer supported synthesis steps and final detachment, poses divergent demands on the linkage between polymer and substrate. On the one hand, the linkage has to remain stable during the diversification steps preventing premature cleavage; on the other hand, facile release from the support must be guaranteed, without affecting the desired end-product. Obviously, much of the research in solid phase organic synthesis is devoted to the development of practical linker systems.\(^7\)
Given the long development time associated with transferring chemistry to solid phase and the difficulties with monitoring solid supported reactions, the practice of synthesising drug candidates entirely on a solid support has seen a decline in use over the past few years. Other solid phase applications are becoming increasingly important in organic synthesis, such as the use of polymer supported reagents, that allow the easy work-up by filtration, and polymer supported catalysts, that can be re-used, and scavenger resins to trap odorous or toxic species or reagents used in excess.

Judging from the commodious collection of solid phase reactions that has been reported in the past decade and the large and ever expanding amount of products that is commercially available for solid phase and combinatorial chemistry, including new solid supports, solid supported reagents, pre-loaded resins and building blocks, solid phase techniques have become an established arsenal for the synthetic chemist.

The philosophy behind combinatorial library design has changed radically since the early days of vast, diversity driven libraries. A reconsideration was imperative because the large numbers of compounds synthesised did not result in the increase in drug candidates that was originally envisaged. Whereas the amount of new chemical entities has remained relatively constant between 1990 and 2000, averaging approximately 37 per annum, the number of compounds synthesised and screened has increased by several orders of magnitude. The idea of maximising diversity is now mostly abandoned and current design strategies involve interactions between various disciplines with inputs from biotechnology, biomedical engineering, informatics, proteomics and genomics. In this collective approach the library is fashioned to bind to specific biological targets such as enzymes or receptors. Because these 'thematical' - as opposed to 'diverse' - libraries focus on processes with a common biochemical mechanism, they can potentially cross over to multiple therapeutic areas. Therefore, a drug-discovery strategy based on these thematic libraries may offer an increased probability for therapeutic success.

1.2 ADENOSINE RECEPTORS

The endogenous nucleoside, adenosine, is ubiquitous in mammalian cell types. Adenosine is related both structurally and metabolically to the bioactive nucleotides adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and cyclic adenosine monophosphate (cAMP); to the biochemical methylating agent S-adenosylmethionine (SAM); and it is present as a structural element in RNA and the coenzymes NAD, FAD and
coenzyme A. Together adenosine and these related compounds are important in the regulation of many aspects of cellular metabolism.

Adenosine itself is produced in many cell types with a basal concentration in the micromolar range. The concentration of endogenous adenosine in interstitial fluid has been estimated between 30 and 300 nM. The biosynthesis of adenosine proceeds primarily by two pathways. The first is a cascading hydrolysis pathway, from ATP to ADP to AMP to adenosine catalysed by 5'-nucleotidase enzymes and this process can occur both intracellularly and extracellularly. The second is the intracellular enzymatic hydrolysis of S-adenosyl-homocysteine to adenosine. Transport of intracellularly produced adenosine out of the cell proceeds primarily by facilitated diffusion through equilibrative or concentrative nucleoside transporter proteins. The lifetime of adenosine in circulation is in the order of several seconds and this rapid degradation means that adenosine acts locally, close to the site where it first enters circulation. The elimination of extracellular adenosine generally takes place by uptake back into the cell through the nucleoside transporters or by deamination to inosine catalysed by adenosine deaminase. Intracellular adenosine is either phosphorylated to AMP by adenosine kinase or metabolised enzymatically by adenosine deaminase to inosine.

### Adenosine and G-Proteins

Adenosine mediates many of its physiological effects via cell surface receptors named adenosine receptors. To date four adenosine receptor subtypes have been identified: three with affinity for the endogenous ligand adenosine in the nanomolar range, (A₁, A₂A and A₃) and one with affinity in the micromolar range (A₂B). The adenosine receptors belong to the extensive family of guanine nucleotide binding-protein (G-protein) coupled receptors (GPCR’s), which all consist of seven membrane spanning α-helices, that together constitute the binding site for extracellular ligands (e.g. adenosine). The heterotrimeric G-protein is composed of an α, β and γ subunit and transduces the binding of an extracellular ligand to the receptor into an intracellular response (see Figure 1.1 on page 6). This process is called signal transduction. Stimulation of this transduction mechanism is effected when an extracellular
ligand enters the binding site of the G-protein coupled receptor and thereby causes a change in the relative orientation of the transmembrane helices. These alterations then affect the conformation of the intracellular loops of the receptor that interact with the G-protein, thereby uncovering previously masked binding sites on the α-subunit where GDP is enclosed. Subsequent GDP release from the α-subunit of the G-protein allows GTP to enter the available binding site. The GTP-bound α-subunit dissociates from the βγ-subunit and either part can activate an effector system, also known as second messenger system. This is usually an enzyme such as adenylate cyclase or a phospholipase, a transport protein or an ion channel specific for $\text{Ca}^{2+}$, $\text{K}^+$ or $\text{Na}^+$. This signal transduction mechanism is turned off when the α-subunit hydrolyses the bound GTP to GDP and the three subunits recombine under formation of the inactive heterotrimeric G-protein. Recently, regulatory proteins have been identified that accelerate GTP hydrolysis and thereby return the α-subunit to its inactivated GDP-bound form. Several classes of G-proteins can be distinguished that are each associated with a distinct second messenger system. They include G$_s$, which activates adenylate cyclase; G$_i$, which inhibits adenylate cyclase and G$_q$, which activates phospholipase C.

Each of the adenosine receptor subtypes has been classically characterised by the adenylate cyclase effector system. Adenylate cyclase is an integral membrane protein and catalyses the intracellular conversion of ATP to the second messenger cAMP, which in turn affects a very wide range of cellular processes. The A$_1$ and A$_3$ receptors are coupled with G$_i$ proteins, that inhibit adenylate cyclase, leading to a decrease in cellular cAMP levels. The A$_2A$ and A$_2B$ receptors couple to G$_s$ proteins, that activate adenylate cyclase, leading to an increase in cellular
cAMP levels. At present adenosine receptors are associated with many more effector systems other than adenylate cyclase and recently accessory proteins have been discovered that influence the receptor/G-protein interaction and thus modulate the signalling reaction.28

All four adenosine receptor subtypes have recently been cloned from a variety of mammals, including humans. While there is a high sequence homology between A1, A2A and A2B receptors among mammals, the A3 subtype forms an exception. The amino acid identity between human and rat adenosine receptor subtypes is 94 \%(A1)\%, 84 \%(A2A)\%, 86 \%(A2B)\% and 72 \%(A3).14 The lesser homology exhibited for the A3 subtype is reflected by the significant differences in pharmacology between rat and human, including binding of ligands,29 tissue distribution, and diversity of structure with respect to G protein-coupling and effector systems.30

The adenosine receptor subtypes are variously expressed in effectively every organ and tissue in the body where they modulate a multiplicity of physiological processes. Adenosine receptors play an important role in the central nervous system and the cardiovascular system, in immunological and inflammatory responses, respiratory regulation and in the kidney.

**ADENOSINE IS AN AGONIST**

Adenosine is the endogenous ligand for the adenosine receptors. When this nucleoside binds to the adenosine receptor, it activates signal transduction pathways. By definition adenosine is therefore an agonist, a compound that gives a maximal response (Figure 1.2). Exposure of any GPCR to agonists for some time commonly leads to the attenuation of the agonist response.31 This phenomenon is termed desensitisation and has been demonstrated for all adenosine

![Figure 1.2](image-url)
receptors subtypes. The studies towards desensitisation of the adenosine receptors were carried out on receptors stably transfected into tissue derived cell lines and on recombinantly derived receptors. Desensitisation of the A<sub>1</sub> receptor occurs relatively slowly, with a half life of 6 to 8 hours, the A<sub>2A</sub> and A<sub>2B</sub> receptor responses are attenuated in less than an hour, while the A<sub>3</sub> receptor desensitises very rapidly, within a few minutes. More research is required towards the desensitisation mechanisms and how these events observed in cultured cells will translate in vivo.

Obviously, when adenosine receptors are to be exploited as therapeutic targets, the attenuation of the desired receptor response is undesired. A partial agonist may induce less receptor desensitisation. A partial agonist is a compound that displays a submaximal response (see Figure 1.2), whereas a compound that blocks the receptor for activation is called an antagonist. Other advantages of the therapeutic use of partial agonists may be the reduced chance of side effects and increased subtype selectivity. A compound can be a full agonist in one system and at the same time be a partial agonist in another system.

**Therapeutic Potential of Adenosine Receptor Agonists.**

Adenosine is involved in a wide variety of physiological functions by stimulating the adenosine receptors. Cloning of the four adenosine receptor subtypes and their expression in recombinant systems allowed the design and discovery of subtype selective ligands and revealed that adenosine analogues may act as agonists for the adenosine receptors. By modifying the endogenous adenosine molecule selectivity for one of the receptor subtypes can be obtained (see next section). In this section the role of the adenosine receptor subtypes in various (patho)physiological processes is described and several prospects are indicated for therapeutic intervention by selective activation of the adenosine receptor subtypes.

**Central nervous system.**

Within the central nervous system (CNS), adenosine is an important modulator of neurotransmission, and has been implicated in many physiological functions such as regulation of arousal and sleep, anxiety, cognition and memory. Thus A<sub>1</sub> receptor agonists may serve as sleep promoters and have been implicated as potential drugs in the treatment of anxiety. Adenosine regulates pain transmission particularly by activation of adenosine A<sub>1</sub> receptors at spinal, supraspinal and peripheral sites. Several known pain killers alter the extracellular availability of adenosine and subsequently modulate pain transmission. Acute exposure to capsaicin, the ‘hot’ component of chilli peppers, induces a reduced sensitivity to chemicals, heat and pressure. Capsaicine and also the opioid morphine increase endogenous adenosine...
release in the spinal cord, which is believed to account for their analgesic (pain killing) effect. When given spinally, adenosine can provide a long-lasting analgesia in both rats and humans.

Under certain pathological conditions, like trauma, ischaemia (stroke) and seizure activity, adenosine can serve a significant neuroprotective function. These traumas involve an increase in neurotransmitter release. The excessive firing of neurotransmitters is ultimately responsible for neural degeneration and destruction of nerve cells, which leads to brain damage or eventually death. The development of drugs that (indirectly) activate the A1 receptor, consequently inhibiting neurotransmitter release, may therefore be clinically useful in the treatment of ischaemia or epilepsy.

Also in chronic neurological disorders such as Alzheimer’s or Huntington’s disease activation of adenosine receptors can be of clinical importance. The most widely used drugs in Alzheimer’s disease increase the availability of acetylcholine in central cholinergic pathways by inhibiting the enzyme acetylcholinesterase. Another strategy to enhance cholinergic transmission might be to activate adenosine A2A receptors, which facilitate acetylcholine release, or to block adenosine A1 receptors, which inhibit acetylcholine release. In rodent models of Huntington’s disease both adenosine A1 receptor agonists and adenosine A2A receptor antagonists appear to attenuate the striatal lesions as well as the dystonia, the repetitive muscle contractions that cause the typical jerking movements of body parts.

Cardiovascular system
Adenosine is clinically used as an antiarythmic agent, Adenocard™ (i.v.), to restore normal heart rhythm in patients with abnormally rapid heartbeats originating in the upper chambers of the heart, so-called paroxysmal supraventricular tachycardia. The therapeutic effect is brought about by activation of the A1 receptors localised on the sinoatrial node, which interrupts the excessive electrical impulses in the sinus and atrioventricular nodes. Selective action at the site of administration can be achieved as a result of the rapid metabolism of adenosine. Development of stable adenosine agonists that can be orally administered may be useful in the management of cardiac arrhythmias.

Vasodilation of coronary vessels is achieved by activating A2A receptors and adenosine itself, marketed as Adenoscan™ (i.v.), is applied for cardiac imaging in the evaluation of coronary artery disease. Selective A2A agonists are currently in clinical trials as potent vasodilators.

Inflammatory responses
Elevated levels of adenosine have been measured in the lung fluid and the exhaled breath condensate of patients with inflammatory disorders of the airways such as asthma and chronic obstructive pulmonary disease (COPD). Adenosine has therefore been suspected to have a pathogenic role in such chronic disorders. Inhaled adenosine has the effect of causing mast
cell-dependent bronchoconstriction in asthmatic subjects, but causes bronchodilation in nonasthmatics. Degranulating mast cells that release allergic mediators like histamine, are considered to be play a crucial role in these diseases. Activation of A3 and A2B receptors has been shown to facilitate the release of allergic mediators from mast cells, while activation of A2A receptors leads to inhibition of histamine release. The inhalation of the nonselective adenosine receptor antagonist theophylline (present in tea) is widely used as an antiasthmatic therapy and its mechanism of action may involve blocking of the low affinity A2B receptor. Alternatively, selective A2A agonists are currently in clinical development as anti-inflammatory agents for the treatment of asthma and COPD.40

Cancer
Significantly elevated levels of adenosine are found in the extracellular fluid of solid tumors, suggesting a role of adenosine in tumor growth.42 Adenosine was found to exert its effects on proliferation and on cell death mainly through the A3 adenosine receptors. Exposure of various cell lines to A3 receptor selective agonists showed inhibition of cancer cell proliferation, while stimulating the growth of bone marrow cells.43 These results suggest that A3 agonists may have potential as anticancer agents and could be useful as adjuvants to chemotherapy.

Agonists of A2A receptors are proposed to be used as vasodilators of intratumoural blood vessels, facilitating the delivery of anticancer drugs into the tumour tissue. This might be particularly important for malignant brain tumours, where the limited effectiveness of chemotherapy has been attributed to insufficient drug delivery into the tumour cells.42

The diverse physiological functions of adenosine, some of which have been mentioned above, demonstrate the significant benefits of developing therapeutics for the regulation of adenosine receptors. However, the widely spread distribution of adenosine receptors in mammalian cell types, the existence of at least four distinct subtypes together with the variability of physiological responses means that exploiting this potential requires agonists and antagonists that are highly subtype and tissue type selective to be of value as therapeutics.

**Development of Subtype Selective Adenosine Receptor Agonists**

Adenosine receptor research over the past 20 year has shown that all adenosine receptor agonists are derivatives of the native adenosine structure.24,44 The ribose moiety appears to be essential for affinity and agonist activity, although modifications at the 3’ and 5’ position are tolerated. Increase in affinity and selectivity has been effected by modification of the ribosyl 5’-position and the 2- and N6-positions. Generally, N6-monosubstitution with a bulky cycloalkyl or arylalkyl group enhances A1 selectivity relative to the A2A and A3 subtypes as exemplified by
2-chloro-6-cyclopentyladenosine \(7\) (CCPA) in Figure 1.3. The \(N^6\)-substituents may contain heteroatoms, while stereochemistry is an also an important determinant. The 2-position can tolerate small, selectivity enhancing substituents, such as halogen or oxo groups. Removal of the 2' and 3'-hydroxyl groups leads to partial agonism with weak activity. Selectivity for the \(A_{2A}\) receptor is favoured by large substituents on the 2 position and 5'-ethylcarboxamido or 5'-azacyclic modifications on the ribosyl part. Both polar and apolar C2 linkers such as NH, O and alkynyl with lipophilic cycloalkyl or phenylalkyl groups are required for \(A_{2A}\) affinity and selectivity. An \(A_{2A}\) selective agonist frequently used as a pharmacological tool is adenosine analogue 8 known as CGS21680. For the low affinity \(A_{2B}\) receptor 5'-N-ethylcarboxamido-adenosine (NECA) 9 remains one of the most potent agonists, although it is not selective for the \(A_{2B}\) receptor. Selectivity at \(A_3\) adenosine receptors had been achieved through optimisation of substituents at the \(N^6\) and 5'-positions of adenosine. Specifically, a benzyl group on \(N^6\) seems to be preferred by \(A_3\) receptors and produced decreased potency at \(A_1\) and \(A_{2A}\) receptors. The \(A_3\) selectivity was further enhanced by the addition of 5'-N-methylcarboxamido groups as in CH-IB-MECA 10.

While adenosine analogues were usually screened for selectivity at the rat adenosine receptors, recently human recombinant adenosine receptors expressed in mammalian cell lines have become available. Marked differences in subtype selectivity between rat and human...
adenosine receptors have come to light. Agonists, like CGS21680 8 that have been used as pharmacological tools for their subtype selectivity on rat adenosine receptors, have now been shown to be less selective at human receptors as becomes evident from Figure 1.3.

In addition, with the functional evaluation of more compounds on the most recently discovered A3 receptor surprising results have been obtained concerning the functional activity of adenosine analogues. While 2-alkynyl adenosine analogues are known to be potent agonists at the human adenosine receptors, Cristalli’s group discovered that several 8-alkynyl adenosine analogues, are in fact antagonists for the A3 receptor.47 This is remarkable since adenosine derivatives with an unmodified ribose moiety have always been considered to act as (partial) agonists at the adenosine receptors. Accordingly, it has often been assumed that adenosine derivatives that activate one receptor subtype would likely activate other subtypes at concentrations at which binding is observed. Jacobson and coworkers, however, have recently shown that the known A1 selective agonist CCPA 7 is actually an antagonist of the A3 receptor.29,48 These findings require a reconsideration of the classical paradigm that adenosine analogues are always agonists of the adenosine receptors.

Lately, more sophisticated di- and trisubstituted adenosine analogues have been reported with high affinity and selectivity for the adenosine receptors. It has become increasingly difficult to predict the effect of these multiple substitutions on subtype selectivity, affinity and efficacy.246 The recently synthesised 2-triazenyladenosine derivative 11, was reported to be a potent and selective agonist for the human A1 receptor with a K_i value of 2.8 ± 0.8 nM, and a 75 fold selectivity over A2A and 214 fold over A3 receptors.46 The 2,6-disubstituted NECA derivative 12 was reported by researchers at Glaxo Wellcome as a potent and selective agonist at the A2A receptor.49 A 4’-(2-alkyltetrazoyl) adenosine derivative related to 12, GW-328267, is currently in clinical trials for inhalation therapy for asthma and COPD.40 While 2-alkynyl

![Figure 1.4](image-url)
adenosine analogues are potent agonists at the human adenosine A$_{2A}$ and A$_3$ receptors, the introduction of an additional N$^6$-substituent on 2-alkynyladenosine derivatives was shown to increase selectivity for the human A$_3$ receptor and N$^6$-methyl analogue 13 displayed a $K_i$ value of 3.4 (2.0-5.8) nM and 500 and 2500 fold selectivity over the A$_1$ and A$_{2A}$ receptors respectively.

The combination of site-directed mutagenesis, molecular modelling and the screening of known and new adenosine analogues has offered a progressive understanding of receptor-ligand interactions. This multidisciplinary approach may boost the development of potential therapeutic agents with selectivity for the adenosine receptor subtypes.

1.3 NUCLEOSIDES AS ANTI-PARASITIC AGENTS

The World Health Organisation estimates that more than two billion people are affected by tropical diseases, like malaria, African sleeping sickness, Chagas' disease and leishmaniasis. The etiological agents of these diseases are unicellular parasites belonging to the kingdom of the Protozoa. In this section a brief description of each of these parasitic diseases and their related problems concerning current chemotherapy is presented. Subsequently, the role that modified nucleosides might play as potential anti-parasitic agents, is discussed.

Malaria

Approximately 40% of the world's population lives in tropical and subtropical areas where malarial parasites are endemic, and 300 to 500 million people worldwide are afflicted with the disease annually. *Plasmodium falciparum*, the agent that causes the most severe form of malaria in humans, is responsible for 1.5 to 2.7 million deaths per year, of which more than 1 million occur in children under 5 years of age. *P. falciparum* is an obligate intracellular protozoan parasite that undergoes a number of developmental stages in the human host and multiplies asexually in the red blood cell to give rise to all of the clinical symptoms of malaria: fever, with or without other indications such as headache, muscular aches and weakness, vomiting, diarrhoea, cough. Death may be due to infected red blood cells blocking blood vessels supplying the brain (cerebral malaria), or damage to other vital organs.

Malaria parasites enter their mammalian host via the bite of an infected female Anopheles mosquito (see also Box 1.1 on page 14). They make their way first, via the bloodstream, to the liver where a single parasite, 'sporozoite', invades a liver cell. Once inside, it multiplies to produce thousands of 'merozoites'. The liver cell swells and eventually bursts, releasing the merozoites into the circulation, where they set about invading the red blood cells of their host. Within the red blood cell the parasite grows via the ring stage to become a 'trophozoite'. In this
stage the parasite takes up nutrients from its host and starts growing rapidly until it reaches the 'schizont' stage and the parasite subdivides to produce 20 to 30 daughter merozoites. Then approximately 48 hours after the initial invasion, the infected red blood cell bursts, releasing the merozoites, and a new cycle begins. Its various stages and the strategy of living inside the cells of its host helps the parasite evade the host’s immune system.

Although chemotherapy and prophylaxis are available, the rapidly growing resistance against classical (and inexpensive) drugs like quinine, chloroquine and mefloquine (Lariam®) and drug toxicity articulate the acute need for more efficacious and less toxic drugs.

**African sleeping sickness**

African sleeping sickness, caused by *Trypanosoma brucei* spp, is transmitted to humans through the bite of the tsetse fly of the genus Glossina.56 When present in the insect vector the parasite is in the procyclic form, but upon introduction into the host, the trypanosomes adopt the bloodstream form, and they proliferate in the blood and lymphatic systems, before invading the central nervous system. Cerebral invasion is responsible for the disturbances in patients’ sleep patterns and other neuropsychiatric disorders. Sleeping sickness falls into two clinical categories depending on which trypanosome subspecies is responsible: *T. b. gambiense* causes a chronic disease that takes several years to progress to the second meningoencephalitic stage; *T. b. rhodesiense*, however, causes an acute form of the disease, taking just a few weeks to reach this second stage. Sleeping sickness is a daily threat to more than 60 million people in 36 countries of sub-Saharan Africa, 22 of which are among the least developed countries in the world. The estimated number of people thought to have the disease is between 300 000 and 500 000. However, only 3 to 4 million of these people are under surveillance and the 50 000

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### Box 1.1. Parasite and disease facts.

<table>
<thead>
<tr>
<th>Disease</th>
<th>People at risk</th>
<th>Parasite</th>
<th>Insect</th>
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<td></td>
<td></td>
<td>genus</td>
<td>order</td>
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<td>Malaria</td>
<td>~2 billion</td>
<td><em>Plasmodium</em> spp</td>
<td>Apicomplexa</td>
<td>mosquito (Anopheles )</td>
</tr>
<tr>
<td>African sleeping sickness</td>
<td>60 million</td>
<td><em>Trypanosoma brucei</em> spp</td>
<td>Kinetoplastida</td>
<td>tsetse fly (Glossina)</td>
</tr>
<tr>
<td>Chagas’ disease</td>
<td>40 million</td>
<td><em>Trypanosoma cruzi</em></td>
<td>Kinetoplastida</td>
<td>reduviid bug (Triatoma)</td>
</tr>
<tr>
<td>Leishmaniasis</td>
<td>350 million</td>
<td><em>Leishmania</em> spp</td>
<td>Kinetoplastida</td>
<td>sandfly (Phlebotomus)</td>
</tr>
</tbody>
</table>
deaths reported in 2001 do not reflect the reality of the situation, but simply show the absence of case detection. If untreated, both forms of the disease are fatal at the second stage, and unfortunately the treatment of African trypanosomiasis is still unsatisfactory. Eflornithine, the sole drug developed in recent times, is effective only for late-stage gambiense disease and is very expensive. However, production of eflornithine is once again commercially viable thanks to its cosmetic properties in the control of unwanted facial hair (Vaniqa™ cream). Two other drugs, pentamidine and suramin which are incapable of crossing the blood-brain barrier, are used for the treatment of early-stage gambiense and rhodesiense disease, respectively, but have serious side effects. Since its introduction in 1949 the arsenical drug melarsoprol remains the first-line drug for late-stage disease of both forms of sleeping sickness, but is very toxic and even fatal. Up to 10% of the patients die from melarsoprol induced reactive encephalopathy. Moreover, none of the African trypanocides can be given orally.

Chagas' disease
Chagas' disease is caused by Trypanosoma cruzi, for which many kinds of wild and domestic mammals act as hosts and hence as reservoirs of the disease. This flagellated protozoan parasite is transmitted to humans in different ways, either by the blood-sucking reduviid bug, or vinchuca, which deposits its infective faeces on the skin at the time of biting, or directly by transfusion of infected blood or by congenital transmission. T. cruzi infection has a wide distribution in Central and South America, where it is endemic in 21 countries. The disease affects 16 to 18 million people, and about 5 to 6 million of these have developed chronic incurable complications, such as cardiac lesions, digestive disorders, peripheral neurological lesions, appearing 10 to 20 years after the initial acute phase of the disease. The number of lethal cases, mostly among children, reported in 2002 was 13,000. There have been significant improvements in the control of Chagas' disease by breaking the transmission of the disease through targeting the insect vectors. Treatment with nifurtimox and benznidazole is available for acute stages of the disease only. New drugs are thus still needed, especially to overcome the chronic form of the disease.

Leishmaniasis
Over 20 different species of the genus Leishmania are known to be pathogenic for humans. They are all transmitted by the bite of an insect vector, the phlebotomine sandfly. The promastigote forms of the leishmanial parasite enter the human host where they are ingested by macrophages. There they metamorphose into amastigote forms and reproduce by binary fission. They increase in number until the cell eventually bursts, then infect other phagocytic cells and continue the cycle. The leishmaniases are divided into three general clinical patterns according to the form of the disease: cutaneous, visceral and mucocutaneous. Over two million
new cases of leishmaniasis are estimated to develop each year in 88 countries, with more than 350 million people at risk and a reported 59,000 deaths in 2002. New drugs are needed for leishmaniasis because the standard treatments can only be given parenterally, and the treatment courses are long, expensive, and may induce severe adverse reactions. Moreover, key products such as antimonials are being compromised by drug resistance.

Chemotherapy, even if not satisfactory, remains the principal instrument for the control of all these diseases. Vaccine development has proved difficult because many of these parasites have evolved intricate mechanisms for evading their host’s immune system. Nevertheless a handful of vaccine candidates against malaria and leishmaniasis has recently moved into development for clinical trials. The limited number of available drugs is simply a consequence of market economy principles: since people most at risk from tropical diseases are among the poorest in the world, pharmaceutical companies are reluctant to invest in the development of new drugs. Of the 1399 new chemical entities registered and marketed between 1975-1999, only 13 specifically concerned tropical diseases.

**NUCLEOBASE AND NUCLEOSIDE TRANSPORT**

The identification of fundamental biochemical disparities between a parasite and its host offers a promising strategy for the development of new chemotherapy against parasitic diseases. A striking metabolic difference between all protozoan parasites and their mammalian hosts is the purine biosynthetic pathway. Whereas mammalian cells can synthesise the purine ring from amino acids and other small molecules, all protozoan parasites studied to date are incapable of synthesising purines de novo. Instead, each genus of protozoan parasite has a distinct and unique complement of purine transporters and salvage enzymes that enable the parasite to scavenge preformed purines from the host. The salvage of host purines is initiated by their translocation – either across the parasite plasma membrane or, possibly, in the case of an intracellular parasite, across the parasitophorous vacuolar or host plasma membrane. Thus, nucleobase and nucleoside transporters serve a vital nutritional function for the parasite.

Nucleobase and nucleoside transporter proteins - and with the (near) completion of parasitic genome sequences, a growing number of their encoding genes - have been identified for the kinetoplastids, *Trypanosoma brucei* and *Leishmania* species, and for the apicomplexan parasites *Plasmodium falciparum* and *Toxoplasma gondii*. The protozoan nucleoside transporter genes identified to date have been classified as belonging to the extensive equilibrative nucleoside transporter family, which includes the human equilibrative nucleoside transporters. Members of this equilibrative nucleoside transporter family characteristically possess eleven transmembrane domains with a large intracellular hydrophilic loop linking
transmembrane domains 6 and 7.\textsuperscript{89} The number of biochemically distinct nucleoside transporters in these various parasites remains to be (genetically) determined in virtually all species except \textit{L. donovani}, where it has been genetically established that there are only two nucleoside transporters.\textsuperscript{90}

In general, uptake by transporters can be a basis for selective drug action against the parasite, if the host cells do not express an equivalent protein or if the host transporter is sufficiently different so as to have a much lower affinity or rate of uptake for the drug. A better understanding of the substrate recognition motifs of human and parasite permeases may offer leads for the development of new drugs that are selectively taken up by parasites and not by host cells.\textsuperscript{76} Nevertheless, if drug action is dependent on selective uptake, resistance may arise upon loss or mutation of the transporter involved in the uptake. Of course the mere uptake of nucleoside analogues or for that matter any potential drug is not sufficient for selective therapeutic effects and further studies of metabolic pathways within the parasite are required for the rational design of antimetabolites as parasite cytostatics.

**NUCLEOSIDE TRANSPORTERS IN TRYPANOSOMES**

Several nucleobase and nucleoside transport systems that are in fact proton symporters have been characterised in \textit{Trypanosoma brucei} cells.\textsuperscript{70,72,91} The P1 type system, encoded by the \textit{TbNT} gene family,\textsuperscript{73,74} mediates the uptake of purine nucleosides, and is detected in both the bloodstream form and procyclic form of the parasite life cycle.\textsuperscript{69} The P2 transporter, encoded by the gene \textit{TbATL},\textsuperscript{75} recognises adenosine, adenine and several important antitrypanosomal drugs and is detected only in the bloodstream form of the parasite.\textsuperscript{69} In addition, five nucleobase transporters have been found to date. The hypoxanthine transporters H1, H2, H3 and H4 and the uracil transporter U1. The H1 nucleobase transporter is expressed in procyclic \textit{trypanosomes} and transports hypoxanthine, adenine, xanthine, and guanine.\textsuperscript{72} In bloodstream form trypanosomes, there are two purine nucleobase transporters: H2, which is insensitive to inhibition by guanosine, and H3 which transports guanosine and is also inhibited by this nucleoside.\textsuperscript{70} While the genes coding for the \textit{T. brucei} purine nucleobase transporters of the H1, H2 and H3 family have not been identified to date, recently De Koning and co-workers identified and cloned a gene, \textit{TbNBTI}, that encodes a new, high affinity hypoxanthine transporter, designated H4.\textsuperscript{77} This permease is expressed in \textit{T. b. brucei} procyclics and mediates the transport of hypoxanthine, adenine, xanthine, guanine and, unlike the H1 transporter, also guanosine. In a recent paper Landfear’s group described the cloning and functional expression of a novel nucleobase transporter gene, \textit{TbNT8.1}, which is closely related to the \textit{TbNBT1} gene.\textsuperscript{78} Both leishmanial and trypanosomal nucleoside transporters have a much
higher affinity for their nucleoside or nucleobase ligands than do mammalian nucleoside transporters.\textsuperscript{80,92}

In a systematic survey De Koning and Jarvis assessed the substrate recognition motifs for the P1 and P2 adenosine transporters, which are summarised in Figure 1.5.\textsuperscript{92} For the P1 transporter the presence of a ribose moiety is essential for binding and transport, considering that purine nucleosides are actively transported, whereas purine and pyrimidine nucleobases do not affect this transporter. Both the 3’ and 5’ hydroxyl groups are involved in interactions with the transporter, but a 2’-hydroxyl group is not required. A C-6 substituent, like the 6-amino group in adenosine, is probably not involved in binding to the P1 permease, since guanosine and inosine, with a 6-keto functionality, are also effectively transported. In the purine ring N3 and N7 are essential as hydrogen bond acceptors.

For the P2 transporter the presence of a ribosyl group is not a critical requirement, since adenine displays an even higher affinity than adenosine. The region most essential for binding to the P2 transporter is formed by the N1-C6-N\textsuperscript{6}H amidine moiety, where N1 acts as a potential hydrogen bond acceptor and the 6-amino group as a possible hydrogen bond donor. Cooperation between these bonds results from the withdrawal of electron density from the amine group through the formation of a hydrogen bond to N1. In addition, Fairlamb and co-workers have suggested that the presence of a large and lipophilic residue on N\textsuperscript{6} is favourable for binding to the P2 permease.\textsuperscript{94} Also N9 was identified as essential for high-affinity binding, although it is not involved in hydrogen bonding. Rather, the N9 lone pair of electrons would be mostly fed into the π-system of the pyrimidine ring, thereby creating a partial positive charge on N9 and making the π-system more electron rich. Thus, electrostatic interactions with N9 and π-π interactions with the pyrimidine ring were inferred as vital elements for substrate or permeant binding.

\textbf{Figure 1.5.} Substrate recognition motifs for the T. brucei P1 and P2 transporters.; adapted from ref. 92 and 93.
Nucleoside uptake by Plasmodia species

The symptoms of malaria are caused by plasmodium parasites invading the red blood cells. The enclosure of the plasmodium parasite in a parasitophorous vacuole within the infected red blood cell requires that the uptake of nutrients from the human host into the parasite cytosol occurs across multiple membranes. Nutrients generally must be transported across the red blood cell membrane, the parasitophorous vacuolar membrane, and the parasite plasma membrane. After malaria infection the parasitised red blood cell undergoes marked alterations in its basic membrane transport properties. These nutrient permeation pathways involve various complex and novel elements including transporters, channels, ducts, and the tubovesicular membranes, an interconnected network extending from the parasitophorous vacuolar membrane to the periphery of the infected erythrocyte. The altered transport capabilities of the infected red blood cell, known as the new permeation pathways, appear 10 to 20 hours after invasion and are partly attributed to a non-saturable, anion selective channel on the red blood cell membrane that transports nucleosides, polyols, amino acids, sugars and also exhibits significant permeability to cations. In addition, the unusual capacity to mediate the transport of unnatural L-nucleosides underlines the broad substrate selectivity of the new permeation pathways. Also the tubovesicular membrane network has been implicated in nucleoside transport within the infected red blood cell.

The eventual transport of nucleosides across the parasite plasma membrane into the parasite cytosol was shown to be mediated by a saturable nucleoside permease. Two groups
independently described the cloning and functional characterisation of this equilibrative nucleoside transporter from *P. falciparum*. The transporter designated PfNT1 exhibits broad substrate selectivity for purine and pyrimidine nucleosides and unnatural L-adenosine but not nucleobases. The permease designated PfENT1, that has an identical amino acid sequence except for position 385, which contains Leu instead of Phe, also effectively transports nucleobases such as adenine, guanine and hypoxanthine and nucleoside analogues used as anti-viral and carcinostatic drugs. Functionally, the similar affinities of adenine and adenosine suggest that the purine system may play a major role in substrate recognition by PfENT1, unlike the situation with the mammalian equilibrative nucleoside transporters, which appear not to transport nucleobases. The PfNT1 transporter was localised on the parasite plasma membrane and is expressed throughout the intraerythrocytic phase of the parasite’s life cycle, but is upregulated in the early trophozoite stage, before the onset of nuclear division.

Analysis of the *P. falciparum* genome has not revealed the presence of homologous sequences and implies that Pf(E)NT1 is the sole representative of the equilibrative nucleoside transporter family in the parasite. In addition, homologues of other nucleoside and nucleobase transporter families have not been found to date. These findings indicate that Pf(E)NT1 may be the only mechanism for nucleoside and nucleobase uptake into the parasite. The reliance of *Plasmodium* species on purine salvage and the unique transport properties of Pf(E)NT1 suggest that this transporter protein might be a viable target for the development of novel anti-malarial drugs.

**ADENOSINE ANALOGUES AS POTENTIAL ANTIPROTOZOAL DRUGS**

As argued in the previous section, parasite transporters may be practical targets for antiprotozoal chemotherapy by inhibiting nutrient transport and hence depriving the parasite of building blocks essential for its development. Alternatively, these permeases can play a role in the selective internalisation of cytotoxic agents, that target vital processes within the unicellular parasite. To date, attention has largely focused on the cytotoxic drugs approach and several, involving adenosine analogues, are discussed below.

A promising strategy for the development of new anti-trypanosomal drugs comprises the obstruction of parasite glycolysis. Unlike the insect form, the bloodstream form of *T. brucei* lacks a functional citric acid cycle and mitochondrial oxidative phosphorylation and depends solely on glycolysis for energy production. Disrupting carbohydrate catabolism in bloodstream form *T. brucei* significantly hampers parasite proliferation. In Kinetoplastida, the first seven glycolytic enzymes are enclosed in peroxisome-like organelles called glycosomes, in contrast to the situation in other organisms where the glycolytic enzymes are present in the cytosol. Any selective inhibitor developed against a *T. brucei* enzyme may also be effective on the
Table 1.1. Effect of adenosine analogues on glycolytic enzyme inhibition and parasite growth.\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>GAPDH inhibition (IC\textsubscript{50})</th>
<th>PGK inhibition (IC\textsubscript{50})</th>
<th>Growth inhibition (ED\textsubscript{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(T.\text{brucei})</td>
<td>(T.\text{cruzi})</td>
<td>(T.\text{brucei})</td>
</tr>
<tr>
<td>14</td>
<td>7 (\mu\text{M})</td>
<td>7 (\mu\text{M})</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>100 (\mu\text{M})</td>
<td>75 (\mu\text{M})</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>inactive</td>
<td>inactive</td>
<td>30 (\mu\text{M})</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data taken from references 106 and 108. \textsuperscript{b} bloodstream form. \textsuperscript{c} mammalian stage.

The corresponding enzyme of other trypanosomatids, and vice versa. Therefore most of the glycolytic enzymes are possibly also good drug targets in \(T.\text{cruzi}\) and \textit{Leishmania} species, despite the larger contribution of mitochondrial processes in the mammalian stages of these parasites.\textsuperscript{103}

Gelb and coworkers reported on the design, synthesis and screening of substituted adenosine analogues as inhibitors of trypanosomatid glycolytic enzymes.\textsuperscript{104-108} Inhibitors of \(T.\text{brucei}\) and \(T.\text{cruzi}\) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and \(T.\text{brucei}\) phosphoglycerate kinase (PGK) were identified. Although many compounds were synthesised and evaluated for their enzyme inhibitory activity, only the best enzyme inhibitors were subsequently tested for \textit{in vitro} growth inhibition of trypanosomatids. Some examples are given in Table 1.1, that showed anti-trypanosomal activity in the low micromolar range. Remarkably, the best antitypanosomal compound was not the best GAPDH-inhibitor. Alternatively, this compound might act on a target other than or in addition to the GAPDH enzyme.

An alternative, effective method of selectively killing protozoa proved to be the inhibition of the parasite polyamine biosynthetic pathway.\textsuperscript{109-111} In fact, D,L-\(\alpha\)-difluoromethylornithine 17 (DFMO, eflointhine, or Ornidy\textsuperscript{TM} manufactured by Aventis) is the drug of choice for treatment of late-stage western African human trypanosomiasis caused by \(T.\text{b. gambiense}\),
although the drug is not effective against acute *T. b. rhodesiense* infections. Eflornithine is an irreversible inhibitor of ornithine decarboxylase (ODC), the first enzyme in the polyamine biosynthetic pathway.

![Chemical structure of eflornithine](image)

**Figure 1.7.** Inhibitors of enzymes involved in polyamine biosynthesis.

Another key enzyme in the regulation and synthesis of polyamines is S-adenosylmethionine decarboxylase (AdoMetDC). Sufrin and coworkers recently published a study towards the antitrypanosomal activity of a known irreversible inhibitor or S-adenosylmethionine decarboxylase, the adenosine analogue 18 (MDL73811). Several derivatives of 18 were synthesised and antitrypanosomal evaluation *in vitro* identified the 2',3'-diacetylated analogue 19 as a potent trypanocide, which displayed ten-fold higher IC$_{50}$ values than parent compound 18 (Table 1.2). Remarkably the AdoMetDC enzyme inhibitory effect of 19 was a ten-fold lower. The reason for this discrepancy is not exactly understood.

<table>
<thead>
<tr>
<th></th>
<th>AdoMetDC (IC$_{50}$)</th>
<th><em>T. b. brucei</em> (IC$_{50}$)</th>
<th><em>T. b. rhodesiense</em> (IC$_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.078 μM</td>
<td>0.1 μM</td>
<td>0.04 μM</td>
</tr>
<tr>
<td></td>
<td>0.014 μM</td>
<td>0.02 μM</td>
<td>0.054 μM</td>
</tr>
</tbody>
</table>

Table 1.2. In *vitro* inhibition of AdoMetDC and antitrypanosomal activity of adenosine analogues 18 and 19.$^a$

$^a$ Data taken from reference 112. $^b$ AdoMetDC from L1210 murine leukemia cells. $^c$ EATRO 110 strain. $^d$ KETRI 243 strain, melarsoprol and diamidine resistant. $^e$ KETRI 269 strain and $^f$ KETRI 243 As 10-3 strain both highly arsenical resistant.

In order to identify adenosine analogues as potential drugs against malaria the group of Link synthesised many nucleoside analogues via a combinatorial approach. While generally $N^6$-monosubstituted adenosine analogues showed insignificant antimalarial activity with IC$_{50}$ values not below 10 mM, the screening of a library of 5'-N-amido-5'-deoxy-N$^6$-disubstituted adenosine analogues revealed several compounds with reasonable activity against the multidrug resistant *Plasmodium falciparum* strain Dd2. Some of the most active compounds are shown in...
Figure 1.8. With respect to the different modification patterns of their adenosine analogue libraries, the authors concluded that not a single molecular target is recognised, but that potential targets may include a variety of nucleotide dependent enzymes, the parasite's nucleoside uptake machinery, and unrelated cell functions.

Cyclin-dependent kinases (CDK's) are essential for the regulation of the eukaryotic cell cycle, and several enzymes of this family have been identified in *P. falciparum*. These enzymes probably have a crucial role in parasite growth and differentiation. Significant differences exist between plasmodial and human CDK's, suggesting that these enzymes might also represent attractive targets for novel antiparasitic agents. Kinases have been targeted in anticancer chemotherapy and several purine-derived kinase inhibitors were synthesised. A large library of these purine derivatives has been screened for activity against *P. falciparum* and several purines with moderate to poor activity against mammalian CDK1/cyclin B activity showed submicromolar activity against the chloroquine resistant *P. falciparum* strain FCR-3. For example, adenine derivatives 22 and 23 demonstrated a minor inhibitory effect on the purified mammalian CDK1/cyclin B enzyme system with *IC*<sub>50</sub> values higher than 25 μM, while

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**Figure 1.8.** Antiplasmodial activity of N<sup>6</sup>,S'-disubstituted adenosine analogues; data taken from reference 114.

**Figure 1.9.** Antimalarial activity of purine derivatives; data taken from reference 119.
they showed activity against *P. falciparum* in submicromolar concentrations (Figure 1.9). Unfortunately, their inhibition data on plasmodial protein kinase activity were not provided.

### 1.4 Outline of the Thesis

The main theme of this thesis consists of the development of new, fast sorting methodology to produce adenosine analogues as selective agonists for the adenosine receptors and as compounds with antiprotozoal activity. In Chapter 2 the development of the first reported library of nucleoside monomers entirely prepared on a solid support is described. In this case the nucleoside is attached to the solid support by an ester linkage between the nucleoside 5'-hydroxyl group and a carboxyl functionalised polystyrene resin. Functionalisation of the purine ring was effected by nitration on solid support. The developed strategy was illustrated by the construction of a small combinatorial library of 2,N<sup>6</sup>-disubstituted adenosine analogues. To expand the solid phase methodology to the modification of the ribosyl moiety a sequence was developed involving the safety-catch principle, described in Chapter 3. A safety-catch linker remains inert during the solid supported diversification steps and can be ‘switched on’ at will, to allow for cleavage of the substrate from the resin. Thus, two small libraries were synthesised, composed of 5',N<sup>6</sup>-disubstituted and 2,5',N<sup>6</sup>-trisubstituted carboxamidoadenosine analogues. Chapter 4 deals with the construction of conformationally restricted adenosine analogues, making use of macrocyclisations involving the nitro substitution reactions that were so fruitfully applied in the solid supported syntheses described in the preceding chapters. The conformationally restricted adenosine derivatives were biologically evaluated at the adenosine receptors. The antiprotozoal evaluation of the synthesised nucleoside libraries is described in Chapter 5. The versatile purine nitration reaction constitutes the key step in the synthetic strategies described in this thesis. In Chapter 6 the mechanism of this purine nitration reaction is elucidated by evaluation of extensive NMR measurements. The observation of CIDNP effects in the 15N-NMR spectra established the involvement of radicals in this reaction.

### 1.5 References

Introduction

25. For structural information, see the GPCR database: http://www.gpcr.org/
86. For a review on membrane transport in the malaria-infected red blood cells, see: Kim, K. Physiol. Rev. 2001, 81, 495-537.
100. For a review on polyamine biosynthetic enzymes as drug targets in parasitic protozoa, see: Heby, O.; Roberts, S.C.; Ullman Biochem. Soc. Trans. 2003, 31, 415-419.