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The transition to magic bullets – transition state analogue drug design

Gary B. Evans, *[ab] Vern L. Schramm [c] and Peter C. Tyler [a]

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In the absence of industry partnerships, most academic groups lack the infrastructure to rationally design and build drugs *via* methods used in industry. Instead, academia needs to work smarter using mechanism-based design. Working smarter can mean the development of new drug discovery paradigms and then demonstrating their utility and reproducibility to industry. The collaboration between Vern Schramm's group at the Albert Einstein College of Medicine, USA and Peter Tyler at the Ferrier Research Institute at The Victoria University of Wellington, NZ has refined a drug discovery process called transition state analogue design. This process has been applied to several biomedically relevant nucleoside processing enzymes. In 2017, Mundesine®, conceived using transition state analogue design, received market approval for the treatment of peripheral T-cell lymphoma in Japan. This short review looks at a brief history of transition state analogue design, the fundamentals behind the development of this process, and the success of enzyme inhibitors produced using this drug design methodology.

Introduction

Drug discovery paradigms

Paul Ehrlich, the founder of chemotherapy and one of the fathers of drug discovery, talked about taking aim at disease with chemistry.[1,2] More specifically, he discussed designing or discovering "magic bullets" that possessed a high affinity and high lethal potency in relation to the disease-causing microbe or cell, but which had a low toxicity in relation to the body. This would make it possible to treat diseases without damaging the body. While this desire to "hit" disease-causing cells as selectively as possible might seem naïve in terms of what we now know about biology; especially where we look to "hit" specific proteins (a majority of drug targets)[3] as opposed to the cells themselves, this is still a sound concept. We should also keep in mind, with regards to the fidelity of the drug to a biological target, that many drugs work well because they modulate multiple targets.[4]

In order to design "magic bullets" and to improve productivity in the drug discovery industry new technologies and paradigms are being developed all the time and virtually all have their roots in academia.[5–8] One of these new drug discovery paradigms was fragment-based drug discovery. Groundwork for fragment-based design was laid by William Jencks,[9] who was also an important contributor to the transition state (TS) analogue story.[10–12] Since the early 1980s when Jencks published his theory on fragment binding, which was then borne out by experiment, the technology stayed relatively dormant until the mid-90s when Abbott published research using this technique as a platform for SAR (structure activity relationship).[13] Finally in 2011 the first fragment-derived drug, vemurafenib, was approved, targeting a mutant form of BRAF and extending life for patients with skin cancer. Since then two other drugs venetoclax and ribociclib have been approved using this approach and fragment based drug design is a staple of both industry and academia for drug discovery and design.

Our own research into new drug discovery paradigms and the synthesis of new and better drugs has focussed on an improved understanding of how enzymes function and the physical nature of the TS. Despite what we think we already know about how enzymes work and therefore how to best take aim at them with "magic bullets" the role and implications of, for example, protein dynamics in enzyme catalysis is one of the old, yet important topics in contemporary enzymology, revealing our incomplete knowledge of catalysis. In order to design our magic bullets, we have incorporated the concepts proposed by Haldane,[14] Pauling,[15] Lumry,[16] Jencks, and Wolfenden[17] that enzymes have evolved to bind most tightly to the TS of the enzyme-catalysed-reaction. While we still lack the technology to directly "image" or sketch the TS we can "freeze time" through an indirect approach to provide a blueprint for enzyme inhibitor design.[18] Vern Schramm

[a] *The Ferrier Research Institute, Victoria University of Wellington, 69 Gracefield Rd, Lower Hutt, 5010, New Zealand. E-mail: gary.evans@vuw.ac.nz; Tel: +64 4 463 0048*
[b] *The Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Auckland, New Zealand*
[c] *Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY 10461, USA*

and his group have vastly improved the process of TS analogue design (TSAD) and have worked with Peter Tyler and his colleagues at the Ferrier Research Institute to visualize enzymatic TSs of nucleoside processing enzymes as stable molecules, and as design elements for drugs or chemical probes to further elucidate enzyme function.

Enzyme transition states

In chemistry, the TS is the highest energy point of a chemical reaction and determines the rate and fate of the reaction. The function of an enzyme, like that of any other catalyst, is to make the TS for the reaction it catalyses easier to reach.[19] Understanding the TS provides insights into the chemical mechanism. A second lure to understanding the structures of the TSs of enzyme catalysed reactions is that drugs resembling the TS of an enzyme-catalysed reaction can theoretically bind many times more tightly to the target enzyme than substrate – up to 10^{10}–10^{15} times.[20,21] Of course tight binding of a molecule to its target is only the first step in drug development. A major barrier in drug discovery involves aspects around bioavailability and pharmacodynamic and pharmacokinetic properties. Although biological effects are essential, we will see below that many TS analogues sufficiently resemble normal metabolites to also have favourable biological properties.

Early hints that TSAD might provide a pathway to new "magic bullets" or drugs came from nature. One example is proline racemase which catalyses the interconversion of L- and D-proline.[22] In this reaction, one enzymic base abstracts the α-proton from the substrate's chiral centre and the conjugate acid of another enzymic base protonates the substrate from the opposite side. In the late 60s and early 70s pyrrole-2-carboxylate and Δ-pyrrolidine-2-carboxylate were found to bind significantly tighter than expected based on substrate binding (Fig. 1).[23,24] Both compounds bound better than substrate by around two orders of magnitude and this tight binding was rationalised as having resulted from ring planarity which resembled the proposed TS where the α-carbon atom of the substrate assumes a near-planar configuration at some point in the reaction.[19] The actual TS structure was unknown but the tight binding of these analogues led to a TS proposal consistent with the reaction mechanism and reinforced the theories of Pauling and his successors regarding enzymes and how they functioned as catalysts.

The tight binding of TS mimics has been explained by a thermodynamic model of TS stabilization.[20] In the hypothetical binding equilibrium between the enzyme and its TS, the TS is held more tightly than the substrate by a factor equal to the rate acceleration, k_{enz}/k_{non}, and the energy of this "association" is given by $\Delta\Delta G^{\ddagger}$. Although a virtual thermodynamic equilibrium cannot exist because of the sub-bond vibrational lifetime of the TS, it is instructive to imagine capturing the system at the moment the TS is formed. This energetic interaction is approximated by the real binding equilibrium with a TS analogue, which, if it were a perfect mimic, would completely convert the TS stabilization energy, $\Delta\Delta G^{\ddagger}$, into binding energy.

Alternatively, the dynamic view of TS theory explains the tight-binding property of TS analogues by a conformational collapse of the enzyme around the chemically stable mimic. The chemically inert TS analogue converts the dynamic excursions found at the TS to a stable convergence of the enzyme conformation, resulting in the conversion of catalytic dynamics into static binding energy and this is examined in detail by Schramm.[25]

In another nature-inspired TS mimic, Sawa *et al.* published in 1967 that the natural product coformycin was a powerful inhibitor of adenosine deaminase (Fig. 2).[26] Later its 2′-deoxy analogue, pentostatin,[27] was discovered and also found to be a potent inhibitor of ADA. Pentostatin was approved by the FDA for the treatment of hairy cell leukemia.[28] The extremely tight-binding inhibitory properties of coformycin and pentostatin are attributed to the unique heterocyclic ring system that structurally is very similar to the sp^3 TS believed to be involved in the deamination process.

As well as being a potent inhibitor, coformycin was an excellent chemical probe providing mechanistic information not easily accessible by other methods. In the case of adenosine deaminase, coformycin provided crucial information for better understanding its mechanism and provided an indication of binding determinants at the active site, which are important for catalysis.[29]

The search for TS analogues grew rapidly and by 1975 almost 60 putative analogues had been described.[30] The tight binding of these compounds together with others inspired by nature and drug discovery, like zanamivir, was also rationalised on this basis that they resemble the TS of the reaction catalysed. However, in all these cases the TSs had been inferred from what was known about the reaction mechanisms of the enzyme-catalysed reaction.[31–34] It is obvious in hindsight that development of methods to determine the chemical nature of the actual TS of an enzyme catalysed reaction could provide chemists with a blueprint to synthesize

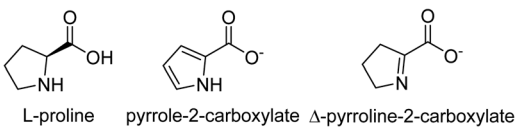


Fig. 1 The substrate of proline racemase together with putative TS analogues.

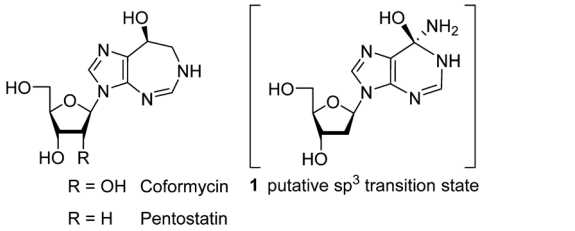


Fig. 2 The structures of coformycin and pentostatin together with the putative TS.

the ideal structure of a TS analogue. Sequential incorporation of individual TS features into candidate molecules could dissect the energetics of each enzyme-TS analogue interaction. It was proposed that this approach could offer an improvement over "ground state" or random screening drug discovery methods. Inhibitors based on TS knowledge might be expected to bind more tightly and with greater fidelity than technologies that rely on high-throughput screening, X-ray crystallography and *in silico* methods. Chemists with their increasingly deep knowledge of organic reaction mechanisms can now compute and propose a model TS structure to estimate the charge distribution and bond lengths. Quantum chemical method are now approaching the stage where it may soon become possible to rely fully on computational chemistry to provide robust models of enzymatic TS.[35]

Sketching a transition state

Experimental approaches to capturing an image of a TS offers considerable challenges as its lifetime is measured in femtoseconds – in a femotosecond light does not even travel the diameter of a bacterium.[18] Over 30 years ago the Schramm group was interested in what the differences might be between the same reaction catalysed by distinct catalysts, such as an acid-catalyzed reaction, a slow enzyme reaction, or an allosterically accelerated enzyme. In order to answer this question and starting with AMP nucleosidase, Schramm developed indirect approaches to sketch or image the TS to see if the TS varied if the reaction was catalysed by the enzyme or acid.[36] Building on the work of Melander and Saunders[37] and then Cleland,[38] these approaches combined advances in computational power with experimental and computational-quantum chemistry developed during the 2nd world war as part of the Manhattan Project.[18,36] The TSs for these reactions differed and although this research had no biological relevance toward drug design, helped the Schramm group develop the approaches needed to solve the TSs of biologically relevant enzymes.

New Zealand's greatest scientist, Lord Rutherford, played a significant role in the discovery of isotopes and they are central to this process of sketching a TS.[39] Kinetic isotope effects (KIEs) inform on bond vibrational changes between the ground state and TS of a chemical reaction and are one of the most powerful experimental techniques for interrogating TS structure.[40,41] Primary KIEs occur when a bond is formed or broken to the isotopic atom during the reaction while secondary KIEs are observed when the number of bonds to the isotopic atom remains unchanged.[42] The direct comparison, equilibrium perturbation and the internal competition method are the three main methods for the measurement of KIEs.[43] Several methods, including liquid scintillation counting, mass spectrometry, nuclear magnetic resonance spectroscopy and polarimetry have been used to determine KIEs.[44–48] The Schramm group has largely focussed on radiolabels and internal competition using liquid scintillation quantitation methods to determine KIEs, but has also confirmed the approach by mass spectrometry methods.[49] Recent interpretation of the KIEs into a model of the TS involves a combined quantum mechanics and molecular mechanics approach (QM/MM), a molecular simulation method that combines QM for the chemical step and MM for the dynamics of atomic excursions.

Transition state analogues

Although KIEs and QM/MM methods provided chemical models of the TS of enzyme-catalysed reactions, as stated by Wolfenden, it would be unrealistic to suppose that an ideal TS analogue or mimic, perfectly resembling in binding properties the altered substrate in the TS, could ever be synthesized.[19] Exact mimics of the TS are not possible because of their non-equilibrium bond lengths, and the theoretical affinities have not been achieved. In saying that, the accuracy of the TS structure sketch must also be critical to realising inhibitors that approach binding constants predicted by theory.

So in general the TSAD approach requires:

1. Chemical or biochemical synthesis of substrate molecules containing isotopic labels at specific positions.

2. Determination of experimental conditions wherein intrinsic isotope effects – that is, KIEs reflecting only the chemical step – can be measured.

3. Measurement of KIEs for each position labelled in step 1.

4. Iterative computation of theoretical KIEs from quantum mechanical calculations with model structures until the KIEs match those from experiment in step 3.

Using Schramm's technology our group has come close to realising ideal or perfect TS analogues. The following provides a summary of the work to-date that led to the discovery of Immucillin-H (ImmH, also known as BCX1777, Forodesine and Mundesine®).

Nucleoside hydrolases

Following reports on the TSs formed by AMP nucleosidase (*vide supra*) Schramm's group turned to the investigation of the TS of the nucleoside hydrolase from the trypanosome *Crithidia fasciculata* and is related to the nucleoside hydrolases of trypanosomes that infect humans, including *Trypanosoma brucei* and *Trypanosoma cruzi*.[50] TS analysis began with the synthesis of individual isotopically edited versions of the substrate inosine and the KIEs were measured. Early analysis of KIE data involved bond length iteration using the BEBOVIB-IV program to model TS geometries. This work predated development of advanced QM/MM algorithms for more direct comparison of computed and measured KIEs for TS structure analysis and required modelling both the substrate, whose structure can be obtained from crystallographic, spectroscopic, or other means, and the TS. Specifically, this involved iterative model manipulation, such as fixing bond lengths and angles, and frequency calculation of all vibrational modes, with the assistance of structure software applications, such as Gaussian,[32]

which in the 1990s could be run on desktop computers for simple systems but required advanced computational capabilities for more complex systems.[20]

In the case of the nucleoside hydrolase from *C. fasciculata* the starting model for the substrate was taken from the crystal structure of inosine and the starting structure for the TS of the ribose ring portion of inosine was derived from the X-ray crystal structure coordinates for ribonolactone. Given the limitations of the BEBOVIB-IV program at that time, including the maximum number of atoms that could be employed in the model only the atoms around the reaction centre were used.[50] From this, the TS for nucleoside hydrolase was sketched within narrow limits as a structure with well-defined bond lengths and conformational properties. Shortly after this, Horenstein *et al.* published the full TS structure of the *C. fasciculata* nucleoside hydrolase[50] which had substantial oxacarbenium ion character, a protonated leaving group, the glycosidic linkage largely cleaved, and an enzyme-directed attacking O nucleophile just within bonding distance but having low bond order to its target carbon (Fig. 3). The ribose portion of the nucleoside was held in a reactive conformation *via* interactions with its hydroxyl groups.

The sketch generated of the TS **2** provided a starting point for synthesis of a designed TS analogue. The role of the aglycon was revealed through TS analysis and relatively simple mimics synthesised, such as compounds **4** and **5** incorporating an aglycon mimic exhibited a slow onset tighter binding phase as well as binding constants 4 to 5 magnitudes tighter than substrate and significantly tighter than the iminoribitol **3** itself.[51]

Purine nucleoside phosphorylase

Purine nucleoside phosphorylase (PNP) is one of the enzymes comprising the purine salvage pathway.[52,53] PNP catalyzes the reversible phosphorolysis of purine ribo- or 2′-deoxyribonucleosides to the purine and ribose- or 2-deoxyribose 1-phosphate. Interest in PNP as a drug target arose from the observation that some children lacking PNP lacked T-cell function but retained normal B-cell function.[54,55] Rapidly dividing human T cells are susceptible to the loss of PNP, because of their high concentration of the enzyme deoxycytidine kinase (dCK). Loss of PNP causes accumulation of 2′-deoxyguanosine, converted to 2′-deoxyguanosine monophosphate (dGMP) by dCK. It accumulates as 2′-deoxyguanosine triphosphate (dGTP) in activated, but not resting T cells. This nucleotide accumulates in T cells because it does not diffuse across cell membranes and because T-cells have relatively low nucleotide phosphomonoesterase activity to metabolize dGMP. High concentrations of dGTP allosterically inhibit ribonucleoside diphosphate reductase (rNR). This inhibition prevents the conversion of cytidine diphosphate (CDP) to 2′-deoxycytidine diphosphate (dCDP); therefore, DNA synthesis is impaired in activated T cells, eventually resulting in cell death.[52,55]

As such, PNP inhibitors were proposed to have potential for the treatment of T-cell leukemias or lymphomas, in organ transplantation, in T-cell-mediated autoimmune diseases such as rheumatoid arthritis and psoriasis, and in the autoimmune disorder causing insulin-dependent diabetes.[56–58] Individuals with genetic deficiencies of PNP that retain as little as 5% of normal catalytic activity do not exhibit manifestations of T-cell deficiency and do not accumulate circulating dGuo. Therefore, nearly complete inhibition of PNP (>95%) must be achieved to increase the dGuo concentration to the level required for T-cell toxicity.[59] As early as 1972 Parks began studying the substrate specificity of PNP with structural variants of the glycone and aglycone.[58,60] Warner Lambert/Parke Davis, Ciba Geigy, Welcome Research Laboratories and several other pharmaceutical companies and Universities were interested in this target up until the 1990s however they had not achieved inhibition constants necessary to sufficiently inhibit PNP activity.[61–66] Starting from nucleoside analogues like acyclovir and its diphosphate (Fig. 4), these included compounds like 9-benzyl-8-aminoguanine, 5′-iodo-9-deazainosine, PD 119229, CI-972, and CI-1000 all of which exhibited nanomolar inhibition constants. Some of these drug candidates were progressed to studies in rats and primates.[61,67–69] BioCryst Pharmaceuticals employed crystallographic modelling techniques to design inhibitors of PNP[56,63,70] and progressed one example (Peldesine) into phase III clinical trials for the treatment of psoriasis.[52] Enzymatic studies indicated that Peldesine had a rapid off rate and could not inhibit PNP sufficiently to elevate plasma dGuo to levels necessary for T-cell suppression.[71]

In order to design a drug that completely inhibited whole body PNP, leading to the build-up of dGuo and T-cell death we needed a low nanomolar–picomolar inhibitor with a longer off-rate from the enzyme. Therefore, having refined the TSAD approach through our work on nucleoside hydrolases we took

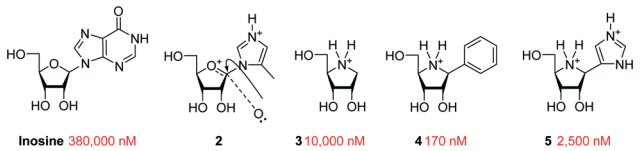


Fig. 3 Substrate of nucleoside hydrolase together with a putative TS and early TS analogues. The structure of the base unit of TS **2** is not complete due to the limitations of BEBOVIB. The inhibition constant K_i is denoted in red.

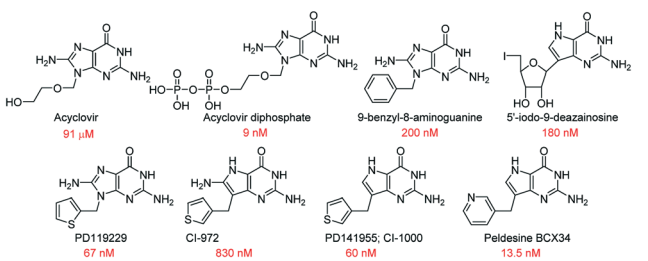


Fig. 4 PNP inhibitors together with their inhibition constants against Hs PNP. The inhibition constant K_i is denoted in red.

up the challenge to tackle the problems of finding a potent PNP inhibitor.

Bovine PNP is 87% identical in amino acid sequence to human PNP and was used for the KIE experiments. Synthesis of the labelled substrates was performed in two stages, each involving several enzymes and substrates in a single reaction mixture. In most cases, an appropriately radiolabelled glucose and/or ^{15}N-substituted adenine was converted to ATP, which was isolated by HPLC. The purified nucleotides were dephosphorylated to adenosine, and converted to inosine by adenosine deaminase.[20,44]

The KIEs were originally studied *via* inosine phosphorolysis; however, the commitment factor of these reactions complicated the analysis (commitment factors are the ratio of forward and reverse rate constants). To minimize these shortcomings, phosphate was substituted with arsenate which reacts as a nucleophile to form ribose 1-arsenate and this is irreversibly hydrolysed to ribose and arsenate. Interpretation of KIEs for inosine arsenolysis used computer modelling and started with the crystal structure of inosine as the ground state model.[44] This was compared by bond-vibrational analysis to varied approximations of the TS until calculated KIEs best-matched experimental values. The TS occurs early in the reaction coordinate, as indicated by the significant C1′–N9 bond (1.77 Å; bond order = 0.38) that still exists to the leaving group. The 3.0 Å distance between C-1′ and the nucleophilic oxygen of arsenate indicates that essentially no bonding from the nucleophile has yet formed at the TS (Fig. 5).[20,44]

With a blueprint for the design of a TS analogue in hand we needed to consider the chemical stability of the putative TS mimic, something that would not react in the enzyme active site. To achieve this a carbon–carbon bond was introduced between the "anomeric" centre at C1′ through replacement of N9 with a carbon atom which also assured that N7 was protonated at physiological pH, another important feature of the TS. Removing the ring oxygen of the oxacarbenium ion and replacing it with a nitrogen atom mimicked the expected charge at the TS which should exploit ionic interactions in the active site.

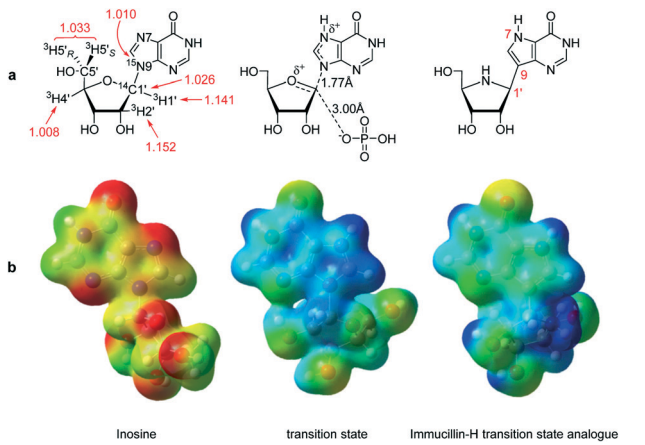


Fig. 5 (a) Structures of inosine, the TS and the proposed analogue with the KIEs shown in red. (b) Molecular electrostatic potential (MEP) surfaces for ground-state inosine, Bt (bovine taurus) PNP TS, and ImmH calculated using Gaussian03 and visualized with GaussView at a density of 0.008 electrons per bohr^3. Ball-and-stick models are superimposed with the surfaces. The colour scale indicates regions that are electron rich (red) and electron deficient (blue). Positive-charge character can clearly be seen at N7 of the TS and at the 4′-position of the ring in the analog, but these regions are electron rich in the reactant state.

ImmH aka Forodesine the active ingredient of Mundesine®

Following initial attempts to synthesise the target TS analogue of nucleoside hydrolases, the "sketch" of which bore similarities to the PNP TS blueprint, in his own laboratories Schramm approached Richard Furneaux's carbohydrate chemistry group in New Zealand where he and Peter Tyler had extensive experience synthesising iminosugars and in particular derivatives of castanospermine[72–76] and deoxymannojirimycin.[77] Tyler adapted the C-nucleoside synthesis of the deazapurines: deazainosine,[78] deazaadenosine,[79] and deazaguanosine (Fig. 6).[80] Deazainosine is structurally similar to formcyin B and all these C-nucleoside compounds had antiprotozoal activity, presumed to act by interrupting the salvage of purines or interruption of nucleic acid synthesis.[81] Our initial attempts to synthesise the target analogue followed on from Horenstein[51] which relied upon the work of Fleet who had devised an excellent synthesis of the key iminoribitol **6**.[82]

Key to the synthetic approach to the imino-C-nucleoside analogues was the synthesis of the imine **7** (Fig. 7) which had been developed by Horenstein and Schramm largely based on the work of Fleet and started from the commercially available D-gulonolactone.[82] In order to realise the blueprint or model of the PNP TS and construct a carbon–carbon bond linking the glycone mimic with the aglycone we needed to create an electrophilic centre and both the imine **7** and nitrone **8** structures would enable us to do this.

A key step in the synthesis of imine **7** involved *N*-halogenation and then dehydrohalogenation of the iminoribitol under kinetic control. This dehydrohalogenation was originally achieved using a hindered base, like lithium tetramethylpiperidide at low temperatures; however this process became problematic in large-scale synthesis requiring cooling around −100 °C or long addition times that maintained the internal reaction temperature below −70 °C.[51] The process to make this key intermediate was later refined by scientists at BioCryst starting from L-lyxonolactone[83] and using a phase

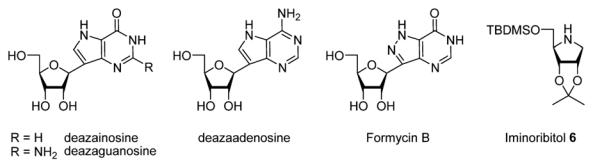


Fig. 6 Structures of deazapurines and the iminoribitol **6** a key intermediate in the synthesis of TS analogues of PNP.

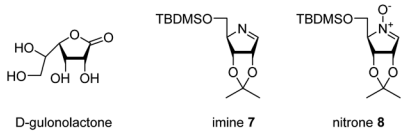
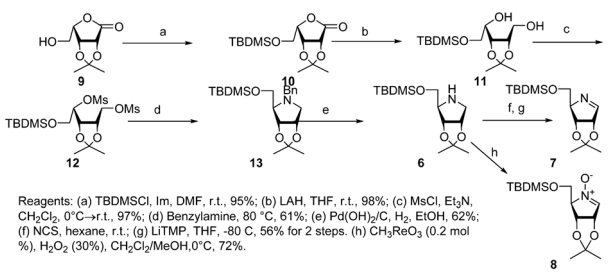


Fig. 7 Structure of D-gulonolactone and electrophilic versions of the iminoribitol.

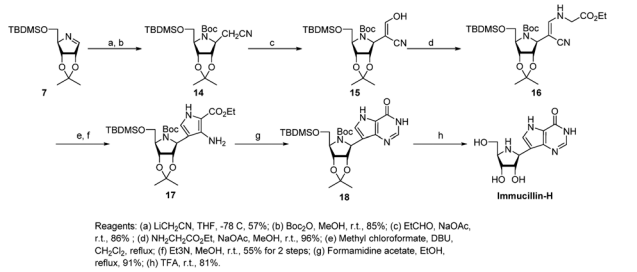
transfer method and potassium hydroxide as the base in the dehydrohalogenation step (Scheme 1).[84] As a reactive electrophile the imine could be treated with a variety of nucleophiles and addition of lithiated acetonitrile allowed the later steps to the final imino-C-nucleosides to be an adaption of the methodology developed by Klein and co-workers.[85] Where the nucleophiles proved relatively unreactive either due to steric hindrance or instability, the nitrone **8** was particularly useful and allowed us to make various homologues that could not be prepared through the imine and had the added advantage of being a white crystalline solid that could be stored indefinitely, unlike imine **7**.[86,87] The initial methodology used to prepare the nitrone **8** followed the method published by Murahashi and involved the use of selenium dioxide, hydrogen peroxide and acetone as the solvent.[88,89] Concerns over the formation of explosive acetone peroxides led us to use catalytic methylrhenium trioxide in a dichloromethane/methanol mixture and this method gave improved yields.

Once the imine was in hand conversion to the TS analogue based on the TS blueprint involved addition of lithiated acetonitrile followed by a multi-step conversion of this stub to a deazahypoxanthine moiety (Scheme 2) to afford ImmH in 20 linear steps and a 5% overall yield.[85]

In order to improve the overall yield of the ImmH production process and make it more scalable, we investigated a convergent approach to ImmH by adding a protected deazapurine to the electrophilic imine. Initially we were unsuccessful as the lithiated deazapurines tended to deprotonate substrates/solvents before addition to the imine occurred. After exploring a variety of protecting group strategies, solvents and solvent mixtures, it was found that a mixture of ether and anisole proved effective for the lithiation of **19** and subsequent addition to the imine affording protected ImmH **20** (Scheme 3). Global deprotection of the intermediate in concentrated acid proceeded in excellent yields to afford ImmH in multigram quantities.



Scheme 1 Synthesis of the imine **7** and nitrone **8**.

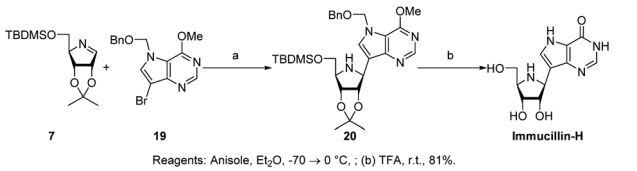


Scheme 2 Synthesis of ImmH.

The inhibition constants of ImmH and the related compound ImmG were initially assessed against bovine PNP and for ImmG exhibited slow onset tight binding and an inhibition constant of 23 pM (Fig. 8).[59] These compounds, based on TS structure, demonstrated unprecedented affinity and selectivity for bovine PNP, achieving the goals of tight-binding, slow-release kinetics.[90] Many drug discovery and development campaigns seek to result in tight binding inhibitors and in some cases, as with PNP, a slow off-rate is also important. When compared with the structurally related compounds ImmA and 4-aza-3-deaza-ImmH (Fig. 8) we can see how important the TS features of the aglycone are to affinity. With the loss of the oxygen at C6 we see a drop in affinity of 5 orders of magnitude to bovine PNP and the transposition of the nitrogen 3 to 4 leading to drop of 7 orders of magnitude.[91,92]

With bovine PNP, complete inhibition of the homotrimeric enzyme with ImmH occurred at one mole of inhibitor per mole of enzymic trimer. Binding of the TS inhibitor at one site per trimer decreased the inhibitor binding at the remaining two sites of the homotrimer. Slow inhibitor dissociation (*e.g.*, $t_{1/2}$ of 4.8 h) suggested that ImmH would have favourable pharmacologic properties and its oral bioavailability in mice was measured at 63% but was found to be lower in primates.[93,94]

Bovine PNP was crystallized in the presence of excess ImmH and phosphate to ensure symmetric occupancy of the three subunits of the trimer. Comparisons of the 1.5 Å structure to previously solved structures of PNP complexes with inosine or ribose 1-phosphate revealed important structural changes that occur as the enzyme progresses from the Michaelis complex, through the TS, to products. In particular, six hydrogen bonds were either introduced or made stronger in the PNP·ImmH·PO_4 complex compared to the PNP·inosine ·SO_4 complex. Additionally, the nucleophilic oxygen of the phosphate was brought nearly 1 Å closer to C-1′ to form an



Scheme 3 Convergent synthesis of ImmH.

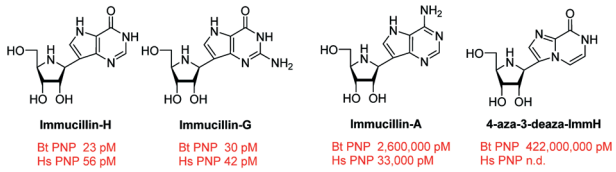


Fig. 8 Inhibition constants of some immucillins against bovine and human PNP. The inhibition constant K_i is denoted in red.

ion pair with the cationic N-4′ of ImmH. The 3.2 Å distance between the nucleophile and the anomeric carbon of ImmH is consistent with the structure for the early dissociative TS determined from KIE analysis by Kline and Schramm.[44]

In vitro studies of ImmH demonstrated that it suppressed the growth of T-cell leukemia cell lines in the presence of added dGuo. In 2000 BioCryst Pharmaceuticals Ltd licensed all PNP inhibitors produced by the Schramm–Tyler collaboration that had an IC_{50} of less than 50 nM and this included ImmH.[95,96] BioCryst demonstrated that ImmH was an effective inhibitor of PNP in mice and primates by either oral or intravenous administration, satisfying the essential requirements for bioavailability and biological efficacy.[97,98]

Interestingly, the final dissociation constants of ImmH when assayed against human PNP were higher than for bovine PNP (Fig. 8). To further investigate this difference, the human protein was expressed and purified and the TSAD process repeated. Distinct from the bovine PNP TS the distance from the leaving purine group to the 1′-carbocation is greater in human PNP and this distance needed to be incorporated into analogues specific for the human enzyme. Human PNP is characterized by a fully-developed ribocation in an S_N1-like TS, thus it was necessary to shift the cationic charge on the ring to the anomeric position.[99] With these design principles specific for human PNP, DADMe-ImmH (**22**) and DADMe-ImmG (**23**) were designed as potential mimics of the TS and synthesised as second generation analogues specific for human PNP (Fig. 9). Their dissociation constants of 8.5 and 7 pM, respectively established that the fine details of TS analysis can yield specific and powerful analogues.

The original synthesis of DADMe-ImmH (**22**) was a convergent approach starting with the 3-hydroxymethyl-4-hydroxypyrrolidine (**21**). Compound **21** was first synthesised by the Aldrich Chemical company in 1965 (ref. 100) as a mixture of stereoisomers and later stereospecifically by Ichikawa.[101] In the interim, Bols *et al.* had synthesised compound **21** and screened it and a variety of derivatives against PNP.[102] In 2001 Pedersen *et al.* published a scalable method starting from D-xylose and we adapted this chiral pool method initially before developing our own asymmetric synthesis (Scheme 4).[103]

Another component of the convergent approach was the synthesis of a series of 9-deazapurines substituted with an aldehyde group at C-9 (Scheme 5). These could be formed through the appropriately protected 9-bromo-9-deazapurine *via* a lithium halogen exchange reaction followed by quenching with DMF to yield the desired aldehydes.[86] For example, reductive amination of the aldehyde **28** with the amine **21** followed by deprotection afforded the second generation DADMe-ImmH (**22**) in good yield.

Later a synthetic method was developed that allowed scale-up and production of the DADMe-Immucillins using the Mannich reaction in a synthesis free of protecting groups (Scheme 6).[104]

DADMe-ImmH (**22**) was a more potent inhibitor of human PNP than ImmH. A single oral dose in mice caused inhibition of the target enzyme for the approximate lifetime of circulating erythrocytes.[105] Also licensed to BioCryst, DADMe-ImmH (**22**) was sub-licensed to Roche who investigated its efficacy against psoriasis in a series of phase I and II human clinical trials.[106] BioCryst successfully completed a phase II study in gout with DADMe-ImmH (**22**) given the trade name Ulodesine. It was found to be safe and generally well tolerated when added to allopurinol for up to 24 weeks. Patients at the end of therapy generated a healthy immune response to vaccination. Adverse event frequency and severity was similar among all groups and no differences were seen in the rate or severity of infections. There were no protocol-driven study drug withdrawals due to low lymphocyte counts in the placebo, 5 mg d^{-1}, and 10 mg d^{-1} groups. The efficacy of Ulodesine in reducing serum uric acid levels at 12 weeks was sustained at 24 weeks.[107]

The next two generations of the Immucillins were based on attempts at structural simplification of the azasugar moiety. Initially we made two simple acyclic analogues of Ulodesine through the Mannich reaction of diethanolamine and its one carbon homologue 3-[(2-hydroxyethyl)amino]-1-propanol with 9-deazahypoxanthine (**27**) to afford compounds **31** and **32** (Fig. 10) and these were nM inhibitors of HsPNP, significantly less potent than DADMe-ImmH (**22**).[108]

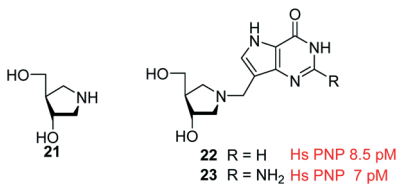
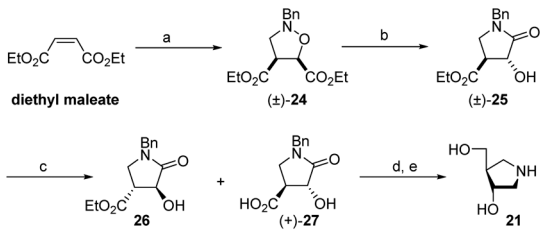
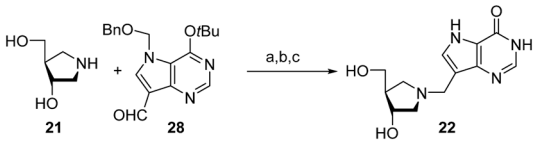


Fig. 9 Structure of iminoribitol and TS analogues DADMe-ImmH and DADMe-ImmG. The inhibition constant K_i is denoted in red.



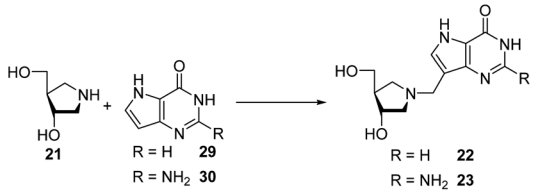
Reagents: (a) BnNOH, CH_2O, EtOH, reflux, 97%; (b) Zn, AcOH, r.t., 90%; (c) Novazyme 435, pH 7.5, 27 oC, 32% of X; (d) $BF_3.Et_2O$, $NaBH_4$, THF, r.t., 91%; (e) H_2, Pd/C, EtOH, r.t., 100%.

Scheme 4 Asymmetric synthesis of Ichikawa amine **21**.



Reagents: $NaCNBH_3$, MeOH, r.t.; (b) TFA, CH_2Cl_2, r.t., 63% for 2 steps; (c) H_2, $Pd(OH)_2/C$, EtOH, r.t.

Scheme 5 Synthesis of DADMe-ImmH *via* reductive amination.



Reagents: (a) Formaldehyde, NaOAc, EtOH, H_2O, 80°C

Scheme 6 Synthesis of DADMe-ImmH and DADMe-ImmG *via* the Mannich reaction.

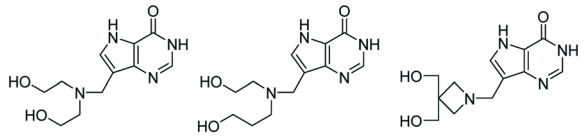


Fig. 10 Next generation PNP inhibitors. The inhibition constant K_i is denoted in red.

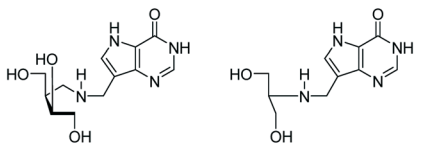


Fig. 11 Acyclic PNP inhibitors. The inhibition constant K_i is denoted in red.

While looking to reduce the ring size of the DADMe-immucillins we investigated the synthesis of azetidine **33** (ref. 109) and the aziridine homologue and found that where we looked to incorporate an aziridine ring system we observed a ring opened acyclic variant as a mixture of diastereomers which showed pM inhibition of PNP (Fig. 11). Following synthesis of the different stereoisomers, one of these, DATMe-ImmH (**34**) was found to inhibit PNP with a similar potency to that of DADMe-ImmH (**22**). In light of this result we set about making a number of further acyclic inhibitors and found that the achiral SerMe-ImmH (**35**) was the most potent PNP inhibitor of all.[110]

Clinical studies

Human patient clinical trials with ImmH were initiated by BioCryst Pharmaceuticals Inc. under the tradename Forodesine (initially as BCX-1777). Intravenous and oral formulations were administered to patients suffering from various T-cell and B-cell lymphomas and leukemias, as well as from solid tumours.[111] Further phase I and II trials of ImmH were carried out in patients with cutaneous T-cell lymphoma (CTCL). Oral Forodesine at a dose of 200 mg daily was shown to be feasible and provided partial efficacy in patients who had already failed three or more systemic therapies, with some durable responses.[112] In 2006 BioCryst sub-licensed ImmH to Mundipharma AG[113] and it has been approved in Japan as Mundesine® for use against recurrent or resistant peripheral T cell lymphoma, a heterogeneous group of clinically aggressive, rare cancers associated with poor outcomes.[114]

Conclusions

Academic research groups have made important contributions to the discovery of new medicines. In order to compete with pharmaceuticals companies, due to the comparatively limited infrastructure available to academic groups, they need to work smarter in order to have an impact. TSAD is just one such smart drug design method and has been developed into a practical approach for the development of novel drug candidates. Linus Pauling first posited the idea that enzymes have evolved to recognise the TS - the rarest of high energy states in a reaction. TS analogues rely on protein dynamic structure which has evolved to discover the TS with high probability, relative to all other dynamic structural states. The TS analogue stabilises a favoured conformational form of a thermodynamically fixed state in complex with the analogue which are released on the minute time scale. The ability to routinely establish a TS structure and develop analogues for a variety of different enzymes is now providing a constant stream of biologically magic bullets.

Conflicts of interest

There are no conflicts to declare.

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