

# 4-Aminopyrazolo[3,4-*d*]pyrimidine (4-APP) as a novel inhibitor of the RNA and DNA depurination induced by Shiga toxin 1

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## ABSTRACT

Shiga toxin 1 (Stx1) catalyses the removal of a unique and specific adenine from 28S RNA in ribosomes (RNA-*N*-glycosidase activity) and the release of multiple adenines from DNA (DNA glycosylase activity). Added adenine behaves as an uncompetitive inhibitor of the RNA-*N*-glycosidase reaction binding more tightly to the Stx1–ribosome complex than to the free enzyme. Several purine derivatives and analogues have now been assayed as inhibitors of Stx1. Most of the compounds showed only minor differences in the rank order of activity on the two enzymatic reactions catalysed by Stx1. The survey highlights the importance of the amino group in the 6-position of the pyrimidine ring of adenine. Shifting (2-aminopurine) or substituting (hypoxanthine, 6-mercaptopurine, 6-methylpurine) the group greatly decreases the inhibitory power. The presence of a second ring, besides the pyrimidine one, is strictly required. Substitution, by introducing an additional nitrogen, of the imidazole ring of adenine with triazole leads to loss of inhibitory power, while rearrangement of the nitrogen atoms of the ring from the imidazole to the pyrazole configuration greatly enhances the inhibitory power. Thus 4-aminopyrazolo[3,4-*d*]pyrimidine (4-APP), the isomer of adenine with the five-membered ring in the pyrazole configuration, is by far the most potent inhibitor of both enzymatic reactions catalysed by Stx1. This finding opens perspectives on therapeutic strategies to protect endothelial renal cells once endocytosis of Stx1 has occurred (haemolytic uraemic syndrome). In the RNA-*N*-glycosidase reaction 4-APP binds, as adenine, predominantly to the Stx1–ribosome complex (uncompetitive inhibition), while inhibition of the DNA glycosylase activity by both inhibitors is of the mixed type.

## INTRODUCTION

Shiga toxins (Stx) belong to the large family of ribosome-inactivating proteins (RIPs), mostly of plant origin, which owe

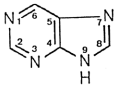
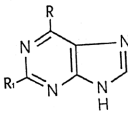
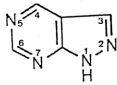
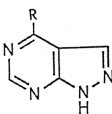

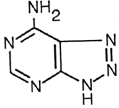
their name to the ability of irreversibly inactivating ribosomes by removing a specific adenine from the large RNA of the large ribosomal subunit (1). It is well established that adenine added to cell-free protein synthesising systems protects ribosomes from inactivation by RIPs (2–4) and that protection results from an inhibition of the RNA-*N*-glycosidase reaction of the uncompetitive type, adenine binding more tightly to the enzyme–substrate complex than to the free enzyme (5). Adenine also reduces the toxicity of Shiga toxin 1 (Stx1) internalised by Vero cells and it has been suggested that this observation might open perspectives on therapeutic strategies to protect eukaryotic cells once endocytosis of toxic RIPs has occurred (5). In mice, however, high doses of adenine are nephrotoxic (6,7) and in humans the kidney, with its high levels of Stx receptors, is the principal site of injury in haemolytic uraemic syndrome (HUS), the most threatening sequela of Stx infections (for reviews see 8–10). Besides inactivating ribosomes, Shiga toxins and plant RIPs remove *in vitro* adenine from several DNAs and a possible role of DNA as physiological substrate of RIPs has been proposed (11–14).

The present paper reports the results of a survey of several purine derivatives and analogues as inhibitors of the activity of Stx1 both on ribosomes and on DNA. Among all, 4-aminopyrazolo[3,4-*d*]pyrimidine (4-APP) emerged as the most potent inhibitor of Stx1. 4-APP, which is not nephrotoxic (6) and protects mice from the renal injury induced by adenine (7), has been largely studied in the past as a possible anti-cancer agent (15–18) and has recently been used as a tool to reduce serum cholesterol and lipoprotein levels (19,20).

## MATERIALS AND METHODS

Stx1, produced from a prototype Stx1 producer *Escherichia coli* C600 (H19J), was purified by receptor analogue affinity chromatography (21). Release from the holotoxin of the enzymatically active A1 fragment was obtained by treatment with trypsin, urea and dithiothreitol (DTT). Such release is required for the activity of Stx1 both on ribosomes (22) and on DNA (12). When Stx1 was assayed on ribosomes, the treatment was performed by the classical procedure (23). When the substrate was DNA, since the DNA glycosylase activity requires relatively larger amounts of toxin, the procedure was modified as described elsewhere (Brigotti *et al.*, manuscript submitted) using insoluble trypsin and introducing a microconcentration

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|  |   |                           | RNA- <i>N</i> -glycosidase activity<br>(% ribosomes inactivated) | DNA glycosylase activity<br>(% adenine released) |
|--|---|---------------------------|--|--|
| No addition  |   |                           | 100  | 100  |
| <div>   </div> |   |                           |  |  |
| <b>Purine skeleton</b>   |   |                           |  |  |
| Purine   |   | <b>R</b><br>H             |  |  |
| Monosubstituted derivatives  |   | <b>R<sub>1</sub></b><br>H |  |  |
| 6-Aminopurine (adenine)  |   | NH <sub>2</sub>           | 53 ± 9 (n = 4)   | 66 ± 7 (n = 3)                                   |
| 6-Methylpurine   |   | CH <sub>3</sub>           | 41 ± 3 (n = 7)   | 37 ± 6 (n = 5)                                   |
| 6-Hydroxypurine (hypoxanthine)   |   | OH                        | 71 ± 7 (n = 4)   | 61 ± 11 (n = 3)                                  |
| 6-Mercaptopurine   |   | SH                        | 83 ± 5 (n = 4)   | 98 ± 6 (n = 4)                                   |
| 2-Aminopurine  |   | H                         | 92 ± 4 (n = 3)   | 78 ± 7 (n = 3)                                   |
| Bisubstituted derivatives  |   | NH <sub>2</sub>           | 97 ± 5 (n = 3)   | 71 ± 18 (n = 6)                                  |
| 2,6-Diaminopurine  |   | NH <sub>2</sub>           |  |  |
| 2-Amino-6-hydroxypurine (guanine)  |   | OH                        | 102 ± 12 (n = 3)   | 58 ± 2 (n = 4)                                   |
| 2,6-Dihydroxypurine (xanthine)   |   | OH                        | 94 ± 9 (n = 4)   | *  |
|  |   | OH                        | 96 ± 6 (n = 4)   | 50 ± 8 (n = 3)                                   |
| <div>   </div> |   |                           |  |  |
| <b>Pyrazolo[3,4-<i>d</i>]pyrimidine skeleton</b>   |   |                           |  |  |
| Monosubstituted derivatives  |   | <b>R</b>                  |  |  |
| 4-Aminopyrazolo[3,4- <i>d</i> ]pyrimidine (4-APP)  |   | NH <sub>2</sub>           | 6 ± 4 (n = 4)  | 12 ± 4 (n = 6)                                   |
| 4-Hydroxypyrazolo[3,4- <i>d</i> ]pyrimidine (allopurinol)  |   | OH                        | 99 ± 3 (n = 3)   | 90 ± 12 (n = 4)                                  |
| <b>Other compounds</b>   |   |                           |  |  |
| 4-Aminopyrimidine  |   |                           | 89 ± 8 (n = 5)   | 80 ± 5 (n = 3)                                   |
| 8-Azaadenine   |  |                           | 96 ± 10 (n = 3)  | 112 ± 6 (n = 3)                                  |
| 1-Methyladenine  |   |                           | 100 ± 1 (n = 3)  | 75 ± 23 (n = 7)                                  |
| 1- <i>N</i> <sup>6</sup> -ethenoadenine  |   |                           | 95 ± 3 (n = 3)   | 86 ± 16 (n = 4)                                  |
| Adenosine  |   |                           | 102 ± 2 (n = 3)  | n.d.   |
| AMP  |   |                           | 97 ± 2 (n = 3)   | n.d.   |
| 2'-Deoxyadenosine  |   |                           | n.d.   | 91 ± 7 (n = 3)                                   |
| 2'-dAMP  |   |                           | n.d.   | 66 ± 4 (n = 3)                                   |

**Figure 1.** Chemical structure of the compounds tested as inhibitors of the RNA-*N*-glycosidase activity and of the DNA glycosylase activity of Stx1. In the RNA-*N*-glycosidase assay [<sup>14</sup>C]phenylalanine incorporated by untreated ribosomes was 10 006 ± 963 d.p.m. (*n* = 11) and by ribosomes treated with 0.05 nM Stx1 1911 ± 530 d.p.m. (*n* = 11), which gives an average of 80% of ribosomes inactivated by the toxin. The actual number of ribosomes inactivated by Stx1 in each experiment was assumed to be 100% in reporting the effect of the various purine derivatives and analogues (1 mM). In the absence of Stx1 none of the compounds tested, nor KOH when used to solubilise them, affected polyphenylalanine synthesis. In the DNA glycosylase assay the adenine released by 200 nM Stx1 in the absence of inhibitors was 41 ± 13 pmol (*n* = 11) when KOH was absent and decreased to 20 ± 6 pmol (*n* = 3) and 12 ± 3 pmol (*n* = 4) in the presence of 1.7 and 3.1 mM KOH, respectively. The results with inhibitors (1 mM) are given as a percentage of the actual adenine release measured in each experiment in their absence at the appropriate KOH concentration. n.d., Not done. \*Not measurable because of the low solubility of guanine in 25 mM KOH and the resulting high concentration of K<sup>+</sup> in the DNA glycosylase assay.

step in order to reduce the amount of urea and DTT carried along with the toxin in the assays. Purine, its derivatives and analogues, and all other compounds tested as inhibitors of Stx1 were purchased from Sigma-Aldrich (St Louis, MO) and used as 5 mM solutions in water with the following exceptions, all in 25 mM KOH: 15 mM 6-mercaptopurine, 15 mM 8-azaadenine, 8 mM allopurinol and 6 mM guanine. The presence of monovalent cations interferes in the DNA glycosylase assay (see legend to Fig. 1) and in Figure 1 the adenine released by Stx1 in the presence of each compound (1 mM) is referred to controls containing the same amount of K<sup>+</sup>. Since 4-APP was

not soluble in 25 mM KOH at concentrations above 5 mM, in order to avoid the interference by K<sup>+</sup>, the compound was preferentially dissolved at 5 mM in 0.05 N HCl and back-titrated to pH 4.0 just before use (24). Back-titration was performed by addition of an equal volume of 3.75 mM Tris base. No interference by this solvent was observed in the assays.

The RNA-*N*-glycosidase activity of Stx1 was assayed by the two-step procedure previously described (25,26) which measures the extent of inactivation of ribosomes. In the first step 80S *Artemia salina* ribosomes (10 pmol in the routine assay) were preincubated (10 min at 28°C) in a 10 µl reaction mixture in

the absence and in the presence of Stx1. In the second step, 2.5 pmol of the preincubated ribosomes were withdrawn and tested for their ability to synthesise polyphenylalanine in a 100  $\mu$ l poly(U) translation system. Adenine, and other compounds tested as inhibitors of Stx1, were either absent or present in the preincubation mixture. None of them, in the absence of Stx1, affected ribosomes as deduced from their ability to synthesise polyphenylalanine.

For kinetic analysis, ribosomes ranged from 0.17 to 0.84  $\mu$ M and the preincubation time was reduced to 5 min during which the rate of inactivation was linear at all ribosome concentrations. Data are presented as double-reciprocal plots of the rate of ribosome inactivation against ribosome concentration as described by Pallanca *et al.* (5).

In a set of experiments, the effect of Stx1 and inhibitors was assayed using an unfractionated rabbit reticulocyte lysate system translating endogenous mRNA (27).  $IC_{50}$  (concentration of Stx1 causing 50% inhibition of protein synthesis) was calculated by the least-squares method applied to the linear regression between fractional activity of protein synthesis and log of Stx1 concentration.

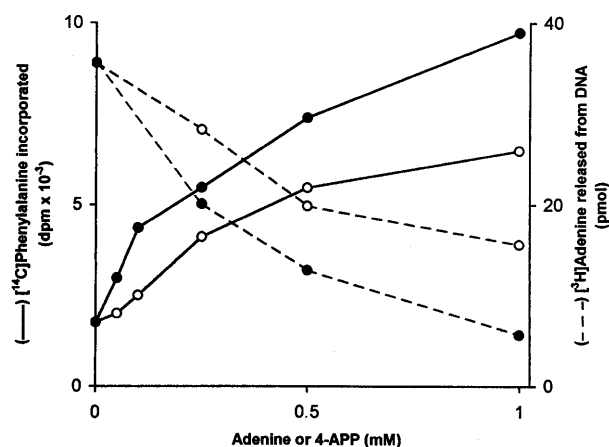
The DNA glycosylase activity of Stx1 was assayed by measuring the release of [ $^3$ H]adenine from a 2251 bp [ $^3$ H]DNA obtained by PCR amplification of the 731–2981 region of the pBR322 plasmid carried out in the presence of [8- $^3$ H]dATP (13). The assays contained, in 50  $\mu$ l of 20 mM HEPES, pH 7.5, 0.3  $\mu$ g of the substrate (corresponding to 205.5 pmol of [ $^3$ H]adenine with a specific radioactivity of 3057 d.p.m./pmol) preheated for 2 min at 100°C in order to separate the two strands of DNA (single stranded, ssDNA). Incubation was for 40 min at 30°C.

For kinetic analysis, [ $^3$ H]DNA ranged from 0.05 to 0.4  $\mu$ g and, to avoid loss of the radioactive substrate (13), 0.5  $\mu$ g of p(dT)<sub>15</sub> (Boehringer) was added. The time of incubation was 2 min and the pH was lowered to 7 in order to increase the amount of adenine released (Brigotti *et al.*, manuscript submitted) and thus the sensitivity of the assay. Adenosine residues were the substrate considered in the Lineweaver–Burk plots (28).

## RESULTS

Several base analogues were tested as inhibitors of the enzymatic activity of Stx1 on RNA in ribosomes and on ssDNA. In Figure 1 the effects of the putative inhibitors are compared at 1 mM concentration. The results show that the parent purine inhibits the RNA-*N*-glycosidase activity of Stx1 on ribosomes by 50%. Inhibition increases to 60% with adenine, in which an amino group is present in position 6, whereas other exocyclic substitutions in position 6 (methyl, hydroxyl or thiol groups) give compounds increasingly less inhibitory, in the above order, than purine itself. Shifting of the amino group of adenine from the 6 to the 2 position induces an almost complete loss of inhibitory power. Also inactive were the derivatives in which exocyclic functional groups were present both in the 2 and in the 6 position. The result is particularly interesting in the case of 2,6-diaminopurine, in which the presence of an additional amino group at position 2 abolishes the inhibitory effect of adenine.

Rearrangement of the nitrogen atoms in the five-membered ring of purine from the imidazole to the pyrazole configuration



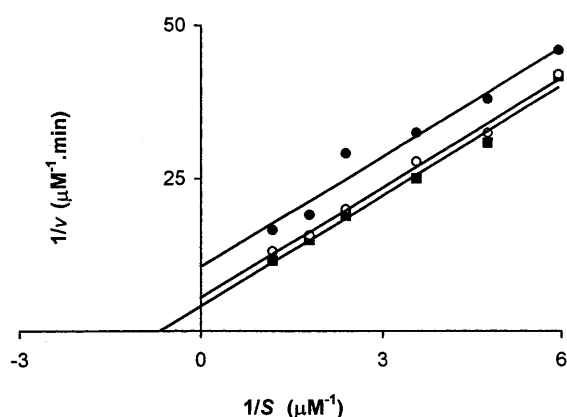
**Figure 2.** Dose-effect of adenine (open circles) and 4-APP (filled circles) on the protection of ribosomes from inactivation and on the release of adenine from DNA catalysed by Stx1. In the absence of Stx1, the [ $^{14}$ C]phenylalanine incorporated by ribosomes ( $10\,150 \pm 705$  d.p.m.,  $n = 5$ ) was not modified by either adenine or 4-APP. Incorporation in the presence of 0.05 nM Stx1 ( $1764 \pm 699$  d.p.m.,  $n = 5$ ) was gradually restored by the two compounds reaching 64 and 96% of the value obtained with untreated ribosomes at the 1 mM dose of adenine and 4-APP, respectively. Similarly, 4-APP was more effective than adenine in protecting the DNA substrate from the [ $^3$ H]adenine release induced by 200 nM Stx1 ( $38 \pm 9$  pmol,  $n = 5$ ).

greatly enhanced the inhibition induced by the presence of an amino group in the same position as in adenine. The different numbering of the cyclic atoms in purines and pyrazolo[3,4-*d*]pyrimidines should be noted (Fig. 1). Thus 4-APP was by far the most potent inhibitor of the glycosidase activity of Stx1 on ribosomes. Substitution of the amino with a hydroxyl group gave a compound devoid of activity (allopurinol).

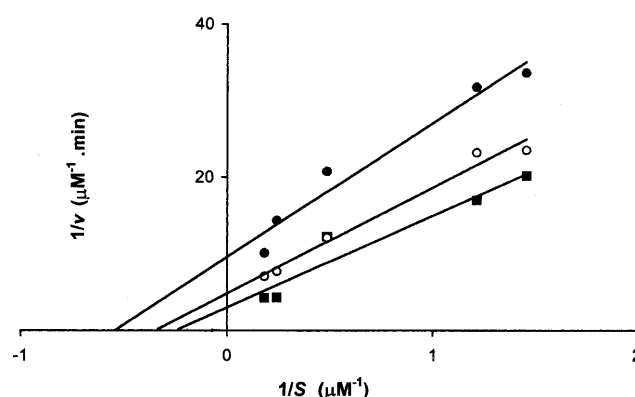
In contrast to the pyrazole enhancing effect, the absence of a five-membered heterocyclic ring in 4-aminopyrimidine or the presence in the ring of an additional nitrogen in 8-azaadenine was detrimental to the inhibitory activity. As previously reported, adenine derivatives substituted in the 1 (1-methyladenine and 1- $N^6$ -ethenoadenine) or in the 9 position (adenosine and AMP) were also inactive.

When the DNA glycosylase activity of Stx1 was measured by its ability to release adenine from ssDNA, 4-APP and adenine, in the above order, again stood out as the most potent inhibitors of the reaction (Figs 1 and 2). However, while allopurinol was again inactive (Fig. 1), bisubstitution in the purine ring did not abolish the inhibitory power, 2,6-diaminopurine and xanthine (guanine could not be tested, see legend to Fig. 1) inhibiting the reaction more than the parent purine. Thus, while the positive influence of the 6-amino group of adenine persisted, the negative influence of double exocyclic substitutions was less pronounced. Also, while the presence of deoxyribose in the 9 position of adenine led to a loss of activity in deoxyadenosine, some inhibitory power persisted in dAMP, as well as in 1-methyladenine and in 1- $N^6$ -ethenoadenine.

Figure 2 shows that, whatever the substrate (ribosomes or DNA), 4-APP is a more efficient inhibitor of Stx1 than adenine at all concentrations tested. It should be noted that the results of Figures 1 and 2 were obtained assaying Stx1 for RNA-*N*-glycosidase and DNA glycosylase activities in highly purified



**Figure 3.** Lineweaver-Burk plots of the inactivation of ribosomes by Stx1 (0.05 nM). The equations of the straight lines are:  $1/v = 6.028 \, 1/S + 4.151$  in the absence of inhibitors (squares);  $1/v = 5.995 \, 1/S + 5.474$  in the presence of adenine (open circles); and  $1/v = 5.997 \, 1/S + 10.606$  in the presence of 4-APP (filled circles). The coefficients of determination ( $r^2$ ) are  $>0.96$ . The two inhibitors were 20  $\mu\text{M}$  each. Data are means of three independent experiments.



**Figure 4.** Lineweaver-Burk plots of the release of  $[^3\text{H}]$ adenine from DNA by Stx1 (0.02  $\mu\text{M}$ ). The equations of the straight lines are:  $1/v = 11.992 \, 1/S + 2.995$  in the absence of inhibitors (squares);  $1/v = 13.849 \, 1/S + 4.828$  in the presence of adenine (open circles);  $1/v = 17.585 \, 1/S + 9.570$  in the presence of 4-APP (filled circles). The coefficients of determination ( $r^2$ ) are  $>0.92$ . The two inhibitors were 100  $\mu\text{M}$  each. Data are means of three independent experiments.

systems in which biotransformation of the purine derivatives and analogues is unlikely to occur. This approach ensures a proper attribution of the inhibitory power of the compounds tested but does not allow prediction of their behaviour in a system more similar to a cell sap. When the inhibitory effects of adenine and 4-APP were compared using an unfractionated rabbit reticulocyte lysate system translating endogenous mRNA, the same order of inhibition was observed. The  $\text{IC}_{50}$  of Stx1 (0.02 nM) was shifted to 0.08 nM by 0.8 mM adenine and by one order of magnitude (0.2 nM) by 0.8 mM 4-APP. Thus, when the lysate was treated with 0.02 nM Stx1 (the  $\text{IC}_{50}$  in the absence of Stx1 inhibitors) all ribosomes were spared from the toxin by the above concentration of 4-APP.

In a previous paper (5) it was established that inhibition by adenine of the RNA-*N*-glycosidase activity of Stx1 on ribosomes is of the uncompetitive type, i.e. that adenine binds more tightly to the enzyme-substrate complex than to the free enzyme. The reciprocal of the general equation which describes the effect of an inhibitor on the rate of an enzymatic reaction is

$$1/v = K_m/V (1 + [I]/K_{ic}) \, 1/S + (1 + [I]/K_{iu}) \, 1/V$$

where  $V$  is the maximal velocity,  $K_m$  the Michaelis constant,  $[I]$  the inhibitor concentration,  $K_{ic}$  the dissociation constant of the inhibitor-enzyme complex and  $K_{iu}$  the dissociation constant of the inhibitor-enzyme-substrate complex. In pure uncompetitive inhibition  $K_{ic}$  approaches infinity and the term  $[I]/K_{ic}$  drops out from the equation. Plotting the data according to Lineweaver-Burk (reciprocal of initial velocity of the reaction,  $1/v$ , against reciprocal of substrate concentration,  $1/S$ ) gives, in the absence and in the presence of the inhibitor, a family of parallel lines, in which all lines have the same slope while the ordinate intercepts increase by the factor  $(1 + [I]/K_{iu})$ . From such increase a value of  $K_{iu}$  of 66  $\mu\text{M}$  for the binding of adenine to the Stx1-ribosome complex was obtained in the previous paper (5). The results reported in Figure 3 confirm this value ( $K_{iu} = 63 \, \mu\text{M}$ ) and show that the behaviour of 4-APP on the RNA-*N*-glycosidase reaction is not dissimilar from that of adenine. The calculated  $K_{iu}$  for 4-APP

was 13  $\mu\text{M}$ , in accordance with the higher inhibitory power of the adenine isomer.

In mixed inhibition the inhibitor binds both to the free enzyme and to the enzyme-substrate complex, i.e. both  $K_{iu}$  and  $K_{ic}$  have definite values. In this type of inhibition the ordinate intercepts and the slopes of the Lineweaver-Burk plots both vary with the inhibitor concentration, the ordinate intercept increasing, as above, by the factor  $(1 + [I]/K_{iu})$  and the slope by the factor  $(1 + [I]/K_{ic})$ . As shown in Figure 4, the behaviour of both adenine and 4-APP as inhibitors of the DNA glycosylase reaction is in agreement with a mixed inhibition mechanism. From the increase in the slopes and in the ordinate intercepts, the values of  $K_{ic}$  and of  $K_{iu}$  reported in Table 1 were obtained.

**Table 1.**  $K_{ic}$  and  $K_{iu}$  of adenine and 4-APP as inhibitors of the enzymatic activity of Stx1 on ribosomes and DNA

| Inhibitor | $K_{ic}$ ( $\mu\text{M}$ ) | $K_{iu}$ ( $\mu\text{M}$ ) Substrate |     |
|-----------|----------------------------|--------------------------------------|-----|
|           |                            | Ribosome                             | DNA |
| Adenine   | 646                        | 63                                   | 163 |
| 4-APP     | 214                        | 13                                   | 46  |

The constants were obtained from the double reciprocal plots of Figures 3 and 4.

## DISCUSSION

X-ray crystallography of Shiga toxin (from *Shigella dysenteriae*), which differs from Stx1 by a single amino acid (29), has visualised (30) in the A subunit a cleft very similar to that present in the A chain of the plant RIP ricin (31). Seven of the invariant residues between Shiga toxin and ricin are found to be present in the cleft which is assumed to contain the active site (30). Computer-assisted searches of the best fitting of adenine and other small ring compounds to the putative ricin A active site and crystallographic studies of the ricin-ligand complexes have been performed (32,33). The interaction energy from hydrogen bonding is a major determinant of ricin A substrate

specificity. In the complex with adenine two key hydrogen bonds are those between the carbonyl oxygens of Gly121 and Val81 and the 6-exocyclic amino group of adenine. Val81 corresponds to Val78 in Shiga toxin, while Gly121 is replaced with the conserved Ser112 (30). This double bonding may explain the importance of an amino group in this particular position for the inhibitory activity of the compounds listed in Figure 1. Three further hydrogen bonds occur in the ricin-adenine complex (33), two between N3 of the pyrimidine ring and the side chain of the invariant Arg180 (Arg170 in Shiga toxin) and one between the hydrogen of N7 of the imidazole ring and the backbone oxygen of Gly121. This last bond cannot form in the tautomeric form of adenine in which hydrogen is missing in N7. The loss of inhibitory power in adenosine and AMP (Fig. 1) might be due to ribose fixing adenine in the less reactive tautomeric form (33) and/or to a hindrance of binding for simple steric reasons (34).

Studies performed with the formycin base (33), an aminopyrazolopyrimidine derivative which differs from 4-APP only for the different mode of attachment of the pyrazole to the pyrimidine ring, show an additional hydrogen bond between the invariant Tyr123 of ricin A (Tyr114 in Shiga toxin) and the pyrazole cyclic nitrogen (N2 in Fig. 1) which replaces the CH in position 8 of the purine ring. The same hydrogen bond can form with 4-APP and may account for its higher inhibitory power.

It should be kept in mind that the binding of RIPs to the large ribosomal rRNA involves specific interactions not only with the target adenine but also with nearby bases, particularly the two adjacent guanines (35). Moreover, recognition of the rRNA or DNA substrate might involve interactions occurring at sites remote from the catalytic adenine recognition site. The predominant uncompetitive type of inhibition by free adenine and 4-APP might be explained if these multiple interactions induce conformational changes which favour fitting into the active cleft of free bases in respect to extended rRNA or DNA substrates.

Irrespective of structural predictions, our results clearly indicate in 4-APP a good inhibitor of the activity of Stx1 both on ribosomes and DNA. The couple Stx1-APP is very similar to the couple xanthine oxidase-allopurinol. Both enzymes are strongly inhibited by the compound in which the five-membered ring of the purine derivative recognised by the enzymes (hypoxanthine for xanthine oxidase, adenine for Stx1) is rearranged from the imidazole to the pyrazole configuration (allopurinol for xanthine oxidase, 4-APP for Stx1). The action of allopurinol in the prevention of free radical damage induced by xanthine oxidase during the reperfusion of an ischemic organ is well documented (36). A similar protective effect of 4-APP against damage induced by Stx1 may be envisaged.

The role of Shiga toxins produced by enterohaemorrhagic *E.coli* strains in the pathogenesis of haemorrhagic colitis and of its life-threatening sequela HUS is well documented (8–10). Shiga toxins bind with high affinity to the oligosaccharide moiety (globotriose, Gb<sub>3</sub>) of glycolipid receptors present on absorptive villus epithelial cells in the gut and on the endothelial lining of several organs, particularly the kidney. Post-diarrhoeal HUS is primarily a microvascular disease which occurs as a complication in 5–10% of patients when Shiga toxins produced in the gut reach the bloodstream and cause renal

endothelial cell injury. Various therapeutic strategies directed against the toxin to be used at different stages of the disease are in progress. The oral administration of Synsorb Pk (Gb<sub>3</sub> coupled to silica particles), devised by Armstrong *et al.* (37), has been proposed and is presently under trial (38,39) as early therapeutic intervention in order to bind the major portion of free Stx in the gut lumen and limit its further systemic absorption. This treatment is obviously ineffective against Shiga toxins already absorbed and targeted to the Gb<sub>3</sub>-rich endothelial cells of the kidney. In this case parenterally administered water soluble receptor analogues, capable of blocking Gb<sub>3</sub> binding sites on the toxin, or, alternatively, passive immunisation with anti-Stx antibodies, might prove valuable (10). These therapeutic strategies depend upon early commencement of the treatment and are ineffective once the toxin has been internalised into renal target cells. The identification of 4-APP, a well characterised drug, as strong inhibitor of the enzymic activities of Stx1 may open perspectives for treatment of the late stages of the disease.

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