INDUCTION AND INHIBITION OF THE *IN VITRO* N^1-OXIDATION OF 9-BENZYLADENINE AND ISOMERIC 9-(NITROBENZYL)ADENINES

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SUMMARY

The present study investigated some aspects of the enzymology of the *in vitro* N^1-oxidation of 9-benzyladenine (BA) and isomeric 9-(nitrobenzyl)adenines (NBAs) using various potential inducers and inhibitors of cytochrome P-450 (CYP). When incubated with phenobarbital-induced rabbit hepatic microsomes, the N^1-oxidation rates of BA and 9-(4-nitrobenzyl)adenine were about 6- and 2-fold higher than that of the control, respectively; while the N^1-oxidation of 9-(2-nitrobenzyl)adenine and 9-(3-nitrobenzyl)adenine was not markedly affected. In contrast, β-naphthoflavone and Arochlor 1254 showed no inductive effects towards the N^1-oxidation of any of these substrates. Using 12 typical CYP inhibitors, it was found that nifedipine (CYP3A inhibitor) and haloperidol (CYP2D inhibitor) showed significant inhibition towards the N^1-oxidation of BA and NBAs. Therefore, the N^1-oxidation of BA and NBAs is probably catalysed by CYP3A and CYP2D subfamilies. Furthermore, when 9-(4-nitrobenzyl)adenine was incubated with compounds which possessed a certain chemical similarity to the adenine substrate, various degrees of inhibition of N^1-oxidation of 9-(4-nitrobenzyl)adenine were observed. These observations allowed a preliminary indication as to the structure-metabolism relationship of 9-substituted adenine derivatives.

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KEY WORDS

N-oxidation, hepatic microsome, cytochrome P-450, 9-aralkyladenines, induction, inhibition

INTRODUCTION

As an important class of aminoazaheterocyclic compounds, the metabolism of purine and adenine derivatives is of considerable interest. With various mammalian hepatic microsomal preparations, it was previously found that metabolic N^1-oxidation occurred for some 9-aralkyl- and 9-alkyl-adenines (AAs), such as 9-benzyladenine, 9-benzhydryladenine and isomeric 9-(nitrobenzyl)adenines /1-5/. But the studies failed to find the N^1-oxide of certain other AAs such as 9-methyladenine, adenosine, ATP, ADP and 9-trityladenine. Based on the *in vitro* N^1-oxidation data of AAs, the potential relationship between N^1-oxidation and certain physicochemical characteristics of AAs has also been investigated using various methods including computer modelling techniques /1,3,6,7/.

However, the enzymic mechanism relating to AA N^1-oxidation has not yet been investigated in detail. For instance, although some data obtained support the suggestion that the microsomal cytochrome P-450 system, especially the phenobarbital inducible isoforms, is involved in the catalysis of AA N^1-oxidation /1,8/, it remains unclear which cytochrome P-450 isoforms are responsible for the catalysis. In order to establish the enzymological profile related to the *in vitro* N^1-oxidation of AAs, it was necessary to study the induction and inhibition of AA N^1-oxidation using various inducing and inhibiting reagents.

One of the important characteristics of drug metabolising enzymes is that the levels and the activities of many enzymes involved are regulatable, especially the cytochrome P-450 system. Regulation can occur at gene expression, enzyme protein translation or post-translational stages, and can be influenced not only by certain endogenous factors but also by many exogenous chemicals, such as drugs. Moreover, many chemicals can also inhibit the metabolism of other chemical(s) via competitive or non-competitive mechanisms and thus modulate the metabolic rate of the processes. The mechanisms and scope of activities of many chemicals towards drug metabolising enzymes have been well documented /9,10/. It has proved very useful

to utilise chemicals as probes to study the enzymological processes involved in the metabolism of the drug substrate in question.

The results obtained in the present investigation on the induction and inhibition of AA N^1-oxidation are presented in this report.

MATERIALS AND METHODS

Chemicals

9-Benzyladenine (BA), 9-(2-nitrobenzyl)adenine (2NBA), 9-(3-nitrobenzyl)adenine (3NBA), 9-(4-nitrobenzyl)adenine (4NBA), 9-benzyladenine-N^1-oxide (BANO), 9-(2-nitrobenzyl)adenine- N^1-oxide (2NBANO), 9-(3-nitrobenzyl)adenine-N^1-oxide (3NBANO), 9-(4-nitrobenzyl)adenine-N^1-oxide (4NBANO), 2-amino-1-(4-nitrobenzyl) benzimidazole (ANBBI), 2,4-diaminopyrimidine (DAP), 2,4-diamino-5-methylpyrimidine (5MDAP), 2,4-diamino-6-methylpyrimidine (6-MDAP), 5-chloro-2,4-diaminopyrimidine (5ClDAP) and 6-chloro-2,4-diaminopyrimidine (6ClDAP) were synthesized and fully characterised in our laboratories. Pyrazole, pyrimidine, pyridazine, pyrazine, pyrazole, 4-aminopyrimidine (4AP), cimetidine and aniline were produced by Aldrich Chemical Co. (Gillingham, Dorset, UK). Phenobarbital (PB), nifedipine, tolbutamide, haloperidol, N,N-diethyldithiocarbamic acid (ADDTC), methimazole, ethylene glycol monoethyl ether (Cellosolve) were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Other commercially obtained compounds include β-naphthoflavone (BNF) from Koch-light Ltd. (Colnbrook, Buckinghamshire, UK), Arochlor 1254 (ARC) from Eli-Lilly and Company Ltd. (Indianapolis, IND, USA), 6-benzylaminopurine (6BAPU) and 4-hydroxy-1-phenyl-pyrazolo[3,4-d]pyrimidine (4HPPP) from Lancaster Synthesis (Morecombe, Lancastershire, UK).

Animal pretreatment and microsomal preparation of hepatic microsomes

Syrian hamsters (male, 80-100 g) and New Zealand white rabbits (male, approximate 2.5 kg) were provided by King's College London Biological Service Unit.

Normal hamsters were used as the source of the hepatic microsomes to study the inhibitory effects of various potential inhibitors towards AA N^1-oxidation. Rabbits were used to prepare induced hepatic microsomes. The pretreatment of the rabbits with inducers was

carried out as shown in Table 1. The hepatic microsomes were prepared using the procedure of Schenkman and Cinti /11/ as modified by Lam *et al.* /2/, based on sedimentation of the microsomal fraction of liver homogenate with calcium ions. The prepared microsomes were finally resuspended in phosphate buffer (0.2 M, pH 7.4, containing 20% glycerol) equivalent to 0.5 g fresh liver tissue per ml suspension and stored at -80°C until required. The microsomal protein content, cytochrome P-450 content, and cytochrome b_5 were measured using standard methods as described in Gibson and Skett /12/.

Incubation and analysis

Incubations were carried out in 10 ml glass tubes for 30 min at 37°C with a SS40-D shaking bath (Grant Instruments (Cambridge) Ltd., Cambridge, UK). For the induction study, the incubation system consisted of phosphate buffer (1.0 ml, 0.2 M, pH 7.4), NADP (1 μmol), glucose-6-phosphate (5 μmol), glucose-6-phosphate dehydrogenase (0.5 unit), magnesium chloride (10 μmol) and microsomal suspension (0.5 ml) in a final total volume of 1.5 ml. BA and NBAs were separately dissolved in DMF to the required concentration (10 mM) and 10 μl (containing 100 nmol of substrate) was added to each sample for incubation.

For the inhibition study, haloperidol, tolbutamide, nifedipine, 6BAP, 4HPPP and ANBBI were separately dissolved in Cellosolve at a concentration of 15 mM and 10 μl (containing 150 nmole of inhibitor) was added to the incubation system described above. Other potential inhibitors studied are water soluble and were therefore dissolved in phosphate buffer (0.2 M, pH 7.4) at a concentration of 0.3 mM and 0.5 ml (containing 150 nmol of inhibitor) was added to the incubation system described above while the total volume remained as 1.5 ml. Normal hamster microsomal preparations were used in this part of the study.

When the incubation was complete, the metabolic reactions were terminated by adding trichloroacetic acid solution (200 μl/incubate, 25% w/v in H_2O) to the incubates. The N^1-oxides formed during incubation were extracted using the solid-phase extraction method and analysed using a reversed-phase HPLC procedure established earlier /13/.

TABLE 1

Pretreatment of animals with potential enzyme inducers and the content of cytochrome P-450, cytochrome b_5 and protein in microsomal preparations

Pretreatment	Dosage regimen	Protein (mg/ml)	Cytochrome P-450 (nmol/ml)	Cytochrome b_5 (nmol/ml)	Cytochrome P-450 (nmol/mg protein)
Saline (control)	Saline 0.5 ml once daily for 3 days	5.23	3.74	2.10	0.78
Corn oil (control)	Corn oil 0.5 ml once daily for 3 days	5.20	5.12	2.10	0.89
Phenobarbital (PB)	PB 80 mg/kg in 0.5 ml saline once daily for 3 days	9.68	32 04	4.32	3.38
β-Naphthoflavone (BNF)	BNF 80 mg/kg in 0.5 ml corn oil once daily for 3 days	5.05	6.79	0.90	1.21
Arochlor 1254 (ARC)	ARC 500 mg/kg in 0.5 ml corn oil on day 1	5.28	10.57	2.67	2.22

All animals were sacrificed on the 4th day of pretreatment.

RESULTS AND DISCUSSION

Induction of N^1-oxidation

It has been previously reported that many compounds are able to induce various drug metabolising enzymes. Several extensive reviews regarding enzyme induction have been previously published /10,14-18/. It is now recognised that there are at least five distinct major inducer categories /10,17/, and phenobarbital (PB)-like inducers and 3-methylcholanthrene (3MC)-like inducers (polycyclic aromatic hydrocarbons, PAH) have been widely investigated. β-Naphthoflavone (BNF) is another characteristic inducer used as a model for the 3MC-like inducer category /19/. In addition to PB- and 3MC-like inducers, another type of inducer was also found, i.e. mixed inducers. The typical mixed inducers are certain congeners of polybrominated (PBBs) and polychlorinated biphenyls (PCBs). In these mixtures, some components show PB-type induction, while others show 3MC-type induction. Arochlor 1254 (a mixture of many isomers and congeners of PCBs) has been widely used as a mixed inducer.

The content of cytochrome P-450, cytochrome b_5 and protein in both control and induced microsomes is shown in Table 1. The results indicate a significant increase of cytochrome P-450 content following the pretreatment of animals with PB and ACR (about 8- and 2-fold, respectively), but BNF only induced a slight increase of P-450 content. On the other hand, PB pretreatment increased the microsomal protein content about 2-fold compared to control, whilst BNF and ARC pretreatment caused only a little change of protein content. These results are similar to values in the literature /20/, therefore it was concluded that successful pretreatment of animals with these inducers had occurred.

The effects of potential enzyme inducers on BA and NBA N^1-oxidation are presented in Figure 1. Although PB increased the N^1-oxidation of BA about six-fold, it showed various effects on N^1-oxidation of the NBAs. There was no marked difference on either 2NBA or 3NBA N^1-oxidation between PB-induced and control microsomes. In spite of the significant increase of 4NBA N^1-oxidation (2-fold), the induction was much weaker compared with BA. ARC and BNF pretreatment failed to exhibit any inductive effects on the N^1-oxidation of any substrate tested. Apparently, PB inducible enzymes are involved in the N^1-oxidation of BA and 4NBA, whereas, the N^1-

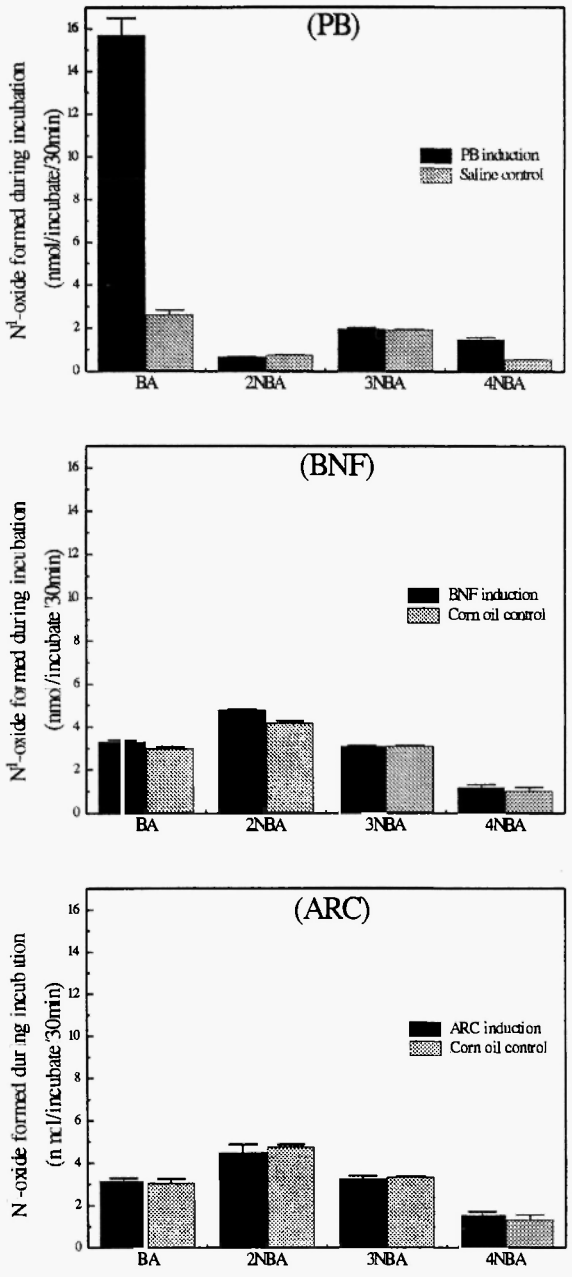


Fig. 1: Effects of potential enzyme inducers on the formation of AA N^1-oxides. Values = mean ± SD; n=3.

oxidation of 2NBA and 3NBA seem to be catalysed by different enzyme(s).

Inhibition of N^1-oxidation by some typical inhibitors

In addition to the inducibility of drug metabolising enzymes by certain xenobiotics, the catalytic activities of these enzymes can also be inhibited by many drugs/xenobiotic substances. The inhibition of drug metabolising enzymes can take place in several ways, including the destruction of pre-existing enzymes, inhibition of enzyme synthesis, competitive/non-competitive inhibition of enzyme activity, or a combination of the above, and thus decrease the metabolism of other compounds of interest.

Many compounds have previously been found to inhibit various drug metabolising enzymes /9,21,22/. Methimazole was thought to be a selective flavin-containing monooxygenase inhibitor, although it may also affect cytochrome P-450 to a lesser extent /23/. Some other compounds have been assigned as specific inhibitors of the corresponding cytochrome P-450 subfamilies, such as pyrazole (CYP2A), nifedipine (CYP3A), cimetidine (CYP3A), aniline (CYP2B), tolbutamide (CYP 2C), haloperidol (CYP2D), and diethyldithiocarbamic acid (ADDTC) (CYP2E). In the present studies, these specific inhibitors were used to probe the enzymes involved in AA N^1-oxidation.

Figure 2 summarises the effects of various inhibitors on the N^1-oxidation of BA and NBAs. It was previously found that the flavin-containing monooxygenase (FMO) was able to catalyse the formation of certain N-oxides /24/; the present data indicate that FMO is probably not involved in the N^1-oxidative processes of BA and NBAs, as the selective inhibitor of FMO, methimazole, did not show a strong inhibitory effect. Although methimazole caused some inhibition of N^1-oxidation, this may be due to its competitive inhibition of a cytochrome P-450 system, as this compound was previously found to be also metabolised by a cytochrome P-450 dependent enzyme system /25/.

Among the specific cytochrome P-450 inhibitors tested, nifedipine showed a very strong inhibitory effect towards all AA substrates (about 3-43% activity remaining compared with controls) in the order 3NBA > BA > 2NBA > 4NBA. As nifedipine can selectively inhibit the CYP3A subfamily /26-29/, the results indicate the involvement of CYP3A in AA N^1-oxidation. However, unlike nifedipine, another CYP3A inhibitor, cimetidine, failed to show a marked inhibition

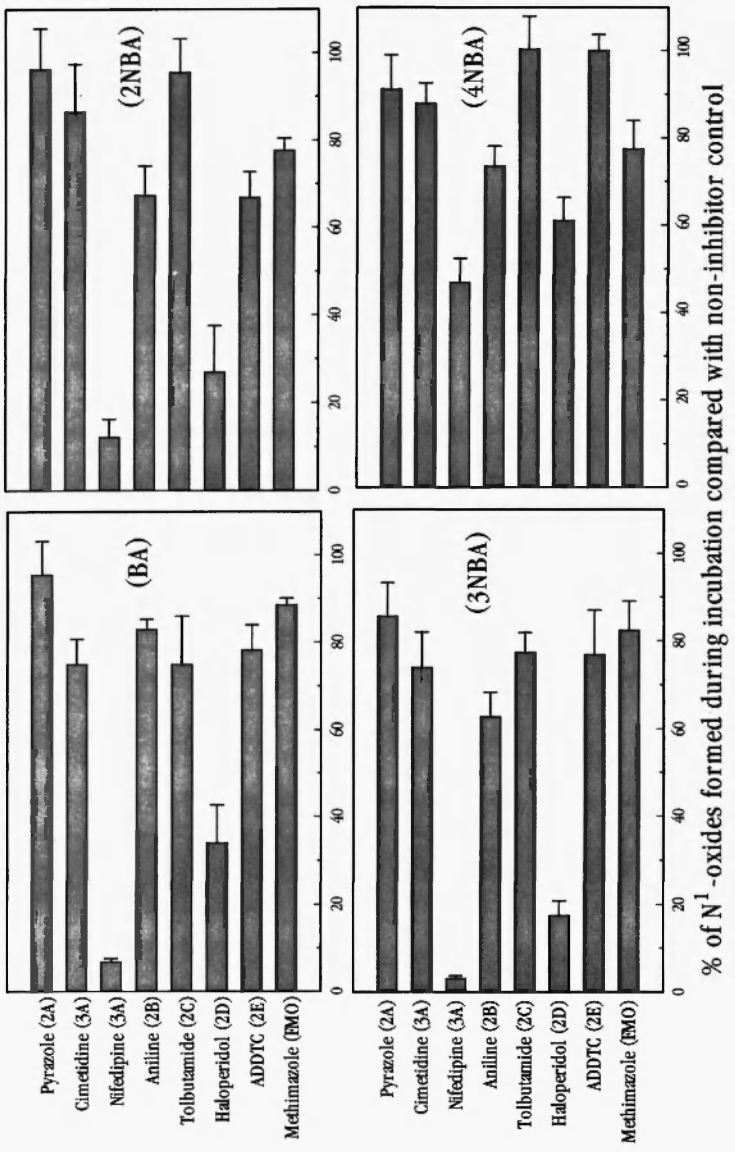


Fig. 2: Effects of various potential enzyme inhibitors on the N^1-oxidation of BA and NBAs using rabbit microsomal preparations. Values = mean ± SD; n=3.

towards these substrates. A possible reason could be that the individual isoform of the CYP3A subfamily responsible for AA N^1-oxidation is selectively inhibited by nifedipine but not by cimetidine. Another possibility is that cimetidine, being more polar than nifedipine, has a weaker enzyme interactive ability compared to the AA substrates. A previous study showed that lipophilicity of 9-alkyladenines is closely related to the occurrence of N^1-oxidation /7/.

In addition, haloperidol, a CYP2D inhibitor /30-32/, also showed a significant inhibitory effect on all AA substrates tested, although less strong than nifedipine. This phenomenon suggests that the CYP2D subfamily of cytochrome P-450 is also involved in AA N^1-oxidation. However, it was recently reported that haloperidol can also be converted by human liver CYP3A4 /33,34/, therefore its inhibition of AA N^1-oxidation may be partially due to an effect on CYP3A.

Aniline was thought to be a selective inhibitor of the CYP2B subfamily (Castagnoli, personal communication), but it failed to show significant inhibition of AA N^1-oxidation, including BA and 4NBA whose N^1-oxidation was found to be inducible by PB.

Some other potential inhibitors tested, such as tolbutamide (CYP2C inhibitor) /35/ and ADDTC (CYP2E inhibitor) /36/, also showed a lesser inhibition of the N^1-oxidation of some AA substrates. This may be interpreted as these inhibitors also slightly affecting CYP3A or CYP2D subfamilies, or that a wide range of cytochrome P-450 subfamilies are actually involved in AA N^1-oxidation to various extents. However, a CYP2A inhibitor, pyrazole (Castagnoli, personal communication), showed no effect on AA N^1-oxidation. Nevertheless, the evidence is not enough to completely exclude the involvement of CYP2A in AA N^1-oxidation, as there is little published evidence to support pyrazole as a suitable standard inhibitor of the CYP2A subfamily, while several investigations have shown that pyrazole is more suitable as an inducer of the CYP2E1 isoform /37/.

Inhibition of N^1-oxidation of 4NBA by some structurally related compounds

As discussed in previous investigations, many substrate characteristics of AAs are involved in controlling their *in vitro* N^1-oxidation, such as the lipophilicity, electronic properties and stereo structure /6, 7/. However, all these factors depend on the chemical structures of the AA substrates. Therefore, it seems to be equally important to under-

stand how variation of AA structures modulates their N^1-oxidation. It has been noticed that the good substrates of AA N^1-oxidase(s) in microsomes are some 9-aralkyladenines, such as BA and NBAs, which possess several component moieties and groups. In order to deduce the contribution of these component moieties and groups in affecting the N^1-oxidation, a suitable strategy would be to observe the N^1-oxidative rate of AAs when co-incubated with compounds with structural similarities. In the present study, 4NBA was chosen as a model substrate as it showed a medium N^1-oxidative rate among the AAs studied /4/.

Figure 3 shows the inhibitory effects on 4NBA N^1-oxidation by various compounds whose structures are shown in Figure 4. It is clear that pyrimidine, one of the structural moieties of 4NBA, fused with an imidazole ring to form the purine moiety, showed no marked inhibition towards 4NBA N^1-oxidation. Similarly, pyridazine and pyrazine, the diazine isomers of pyrimidine, as well as pyrazole also failed to inhibit 4NBA N^1-oxidation. This is possibly due to either the high polarity of these compounds which prevents access to the N^1-oxidase(s), or their lack of binding to the N^1-oxidase(s).

However, 4AP (4-aminopyrimidine) produced a significant inhibition of 4NBA N^1-oxidase(s), thus providing important evidence that the amino group of AAs is probably a crucial binding site for AA N^1-oxidase(s). This evidence is also against the above assumption that isomeric diazines cannot access the enzyme(s) due to high polarity. On the other hand, DAP (2,4-diaminopyrimidine) did not show any inhibition of 4NBA N^1-oxidation. This could be interpreted as the extra amino group at the 2-position of AAs may be able to block the binding of the substrate with the N^1-oxidase(s). This postulate seems to be supported by the weak effects of 5MDAP, 5ClDAP, 6MDAP and 6ClDAP towards 4NBA N^1-oxidation.

When 6BAPU (6-benzylaminopurine, a positional isomer of BA) was tested, it showed significant inhibition of 4NBA N^1-oxidation. This is also evidence that a 6-substituent of the purine moiety is probably necessary for N^1-oxidation. Again, this evidence implies that an aralkyl/alkyl substituent at the 9-position of AAs may not be a crucial requirement for substrate-enzyme binding, although it will affect the catalytic ability of N^1-oxidase(s) towards the substrates, as demonstrated by previous investigations /1-5/.

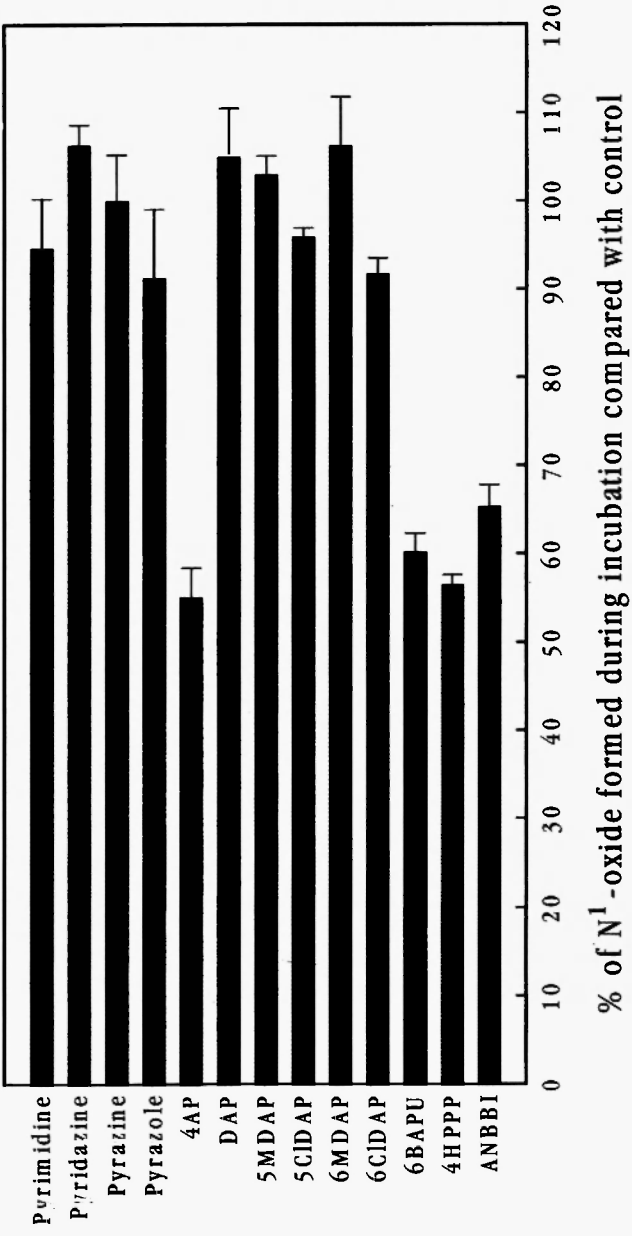
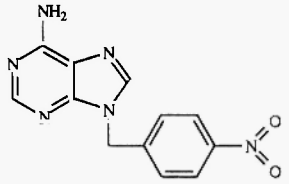


Fig 3: Effects of various structurally similar compounds on the N^1-oxidation of 9-(4-nitrobenzyl)adenine (4NBA) using hamster microsomal preparations. Values = mean ± SD; n=3.



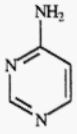
9-(4-nitrobenzyl)adenine
(4NBA)

pyrimidine

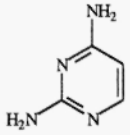
pyridazine

pyrazine

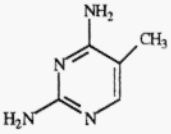
pyrazole



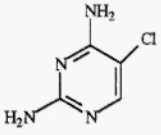
4-aminopyrimidine
(4AP)



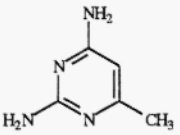
2,4-diaminopyrimidine
(DAP)



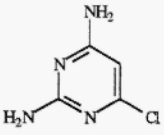
2,4-diamino-5-methylpyrimidine
(5MDAP)



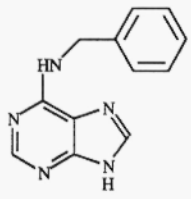
5-chloro-2,4-diaminopyrimidine
(5MDAP)



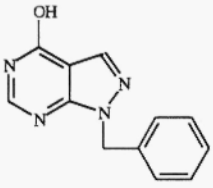
2,4-diamino-6-methylpyrimidine
(6MDAP)



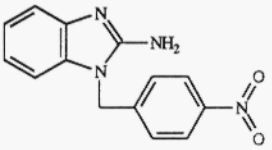
6-chloro-2,4-diaminopyrimidine
(6MDAP)



6-benzylaminopurine
(6BAPU)



4-hydroxy-1-phenylpyrazolo-
[3,4-d]pyrymidine
(4HPPP)



2-amino-1-(4-nitrobenzyl)-
benzimidazole
(ANBBI)

Fig. 4: The structures of 9-(4-nitrobenzyl)adenine (4NBA) and structurally related compounds.

It seems that the positions of the nitrogen atoms in the imidazole moiety is not an important factor for AA N^1-oxidation, as 4HPPP, i.e. 4-hydroxy-1-phenylpyrazolo-[3,4-d]pyrimidine, showed a significant inhibition of 4NBA N^1-oxidation. However, the present data are not adequate to deduce any correlation between the imidazole moiety structure and N^1-oxidation. The importance of another component moiety of AAs, i.e. the 9-substituent, has been confirmed earlier /4,5/. Again, the significant inhibition of 4NBA N^1-oxidation by 2-amino-1-(4-nitrobenzyl)benzimidazole (ANBBI), further implies an important role of the 9-substituent of AAs. Without any functional group on the benzene moiety of ANBBI, it still inhibited 4NBA N^1-oxidation, indicating that the aminoimidazole and 4-nitrobenzyl moieties probably do not prevent enzyme-substrate binding.

The above data provide some information about AA structure-metabolism relationships. Further studies using more structural analogues of AA, such as 9-(4-nitrobenzyl)purine, as potential metabolic inhibitors, may allow us to produce a more complete picture of AA structure-metabolism relationships.

CONCLUSIONS

The results suggest that similar enzymic processes are involved in N^1-oxidation of the AA substrates tested, i.e. the N^1-oxidation of BA and NBAs may be catalysed by the same group of enzymes, but an individual enzyme in this group may show different catalytic abilities to different substrates. Therefore, the N^1-oxidation of different substrates showed different responses to inducers and inhibitors.

Based on the results obtained, it is concluded that the N^1-oxidation of BA and 4NBA, but not 2NBA and 3NBA, are catalysed by PB-inducible enzymes. BNF or Arochlor 1254 showed no marked induction of any AA substrates. Both CYP3A and CYP2D subfamilies are shown to be involved in the N^1-oxidation of AAs, but more evidence is required to exclude the involvement of CYP2A and CYP2B in the N^1-oxidation of AAs. Further investigations on this aspect would be useful to fully establish the enzymes involved in the N^1-oxidation of AAs. Hopefully this will be a subject for future research.

The results suggest that the 6-amino group of AAs is a key site for substrate-enzyme binding, as discussed by Liu *et al.* /7/, leading to N^1-oxidation other than N^6-hydroxylation. Similarly, it is possible that a

suitable 9-substituent, such as benzyl in BA or isomeric nitrobenzyl in NBAs, may provide another binding site for the N^1-oxidase(s).

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