COMPARISON OF METABOLIC RATES OF SOME 9-ARALKYLADENINES OBTAINED USING HAMSTER HEPATIC MICROSOMES

Ding Y. Liu[1] and John W. Gorrod[2]*

[1]*Chelsea Department of Pharmacy, King's College London, University of London, Manresa Road, London SW3 6LX, UK*
[2]*Toxicology Unit, John Tabor Laboratories, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, UK*

SUMMARY

Previous investigations have revealed that N^1-oxidation is a major metabolic pathway *in vitro* for some 9-aralkyladenines (AAs) such as 9-benzyladenine (BA). However, dealkylation and other metabolic pathways are also involved. In addition, various substituents on the benzyl moiety of BA seem to have a marked effect on the metabolic rate. In order to establish the potential structure-metabolism relationship of this class of compounds, the enzyme kinetics of the substrates, which possess 2'-nitro (2NBA), 3'-nitro (3NBA), 4'nitro (4NBA), 2'-chloro (2CBA), 2'-methyl (2MBA), or 2-methoxy (2MOBA) substitution of the benzyl moiety of BA, were compared using hamster hepatic microsomes. The results show that the formation rates of the N^1-oxides are in the order 2NBA > 2CBA > BA > 3NBA and 4NBA > 2MBA and 2MOBA; the formation rates of the total metabolites except N^1-oxides are in the order 2MOBA and 2MBA > 2CBA > BA > 4NBA > 3NBA > 2NBA; however, the total biotransformation rates of the substrates are in the order 2MBA and 2MOBA > 2CBA > BA and 2NBA > 4NBA > 3NBA. The results strongly imply that the electronic, steric and other physicochemical properties are potential controlling factors for AA metabolism.

KEY WORDS

drug metabolism, N-oxidation, 9-aralkyladenines, hepatic microsome, hamster

*Author for correspondence

INTRODUCTION

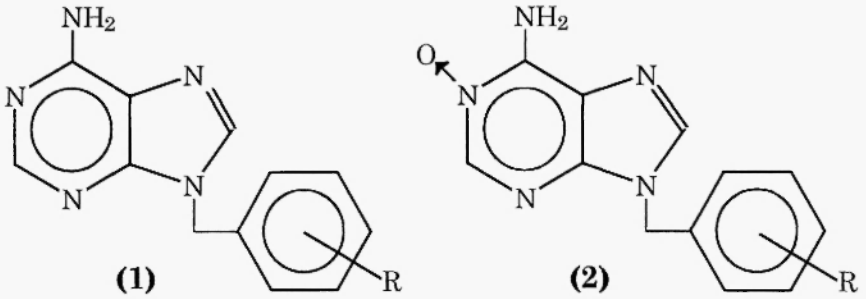
Adenine and its 9-alkyl/aralkyl derivatives (AAs) are not only biologically and pharmacologically important, but are also metabolically interesting. For example, adenine was found to be metabolised by animal hepatic microsomes to both the N^1-oxide and the N^6-hydroxylamine, the former being innocuous whilst the latter is mutagenic /1-3/. It was also found that N^1-oxides were formed from some AAs such as 9-benzyladenine (BA) and isomeric 9-(nitrobenzyl)adenines (NBAs), but not from other more hydrophilic derivatives such as adenosine, ATP or 9-methyladenine /4-8/. In addition to N^1-oxidation and dealkylation, some other unknown metabolic pathways are also involved in AA metabolism /4,7,8/. Previous investigations have revealed that the metabolic rates of some 9-aralkyladenines which possess diverse substituents at the benzyl moiety of BA vary according to the substituents /7,8/.

In order to understand and establish the potential structure-metabolism relationship, the enzyme kinetics of AA substrates which possess 2'-nitro (2NBA), 3'-nitro (3NBA), 4'-nitro (4NBA), 2'-chloro (2CBA), 2'-methyl (2MBA), or 2'-methoxy (2MOBA) substitution at the benzyl moiety of BA, were compared using hamster hepatic microsomes. It was expected that the data would be useful to aid our understanding of the factors controlling AA metabolism.

MATERIALS AND METHODS

Chemicals

The 9-aralkyladenines (AAs) and their N^1-oxides were prepared in our laboratories and fully characterised (Figure 1). The synthetic methods will be published elsewhere. N,N-Dimethylformamide (DMF, HPLC grade) was purchased from Aldrich Chemical Co. (Gillingham, Dorset, UK); glucose-6-phosphate (G-6-P) and nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Sigma Chemical Co. (Poole, Dorset, UK); and glucose-6-phosphate dehydrogenase (suspension grade II) was the product of Boehringer Mannheim Corporation (Lewes, East Sussex, UK). Other chemicals and solvents used were either analytical grade or HPLC grade, supplied by commercial sources.



R	Substrate (1)	Metabolite (2)
H	9-benzyladenine (BA)	9-benzyladenine-N^1-oxide (BANO)
2-NO_2	9-(2-nitrobenzyl)adenine (2NBA)	9-(2-nitrobenzyl)adenine- N^1-oxide (2NBANO)
3-NO_2	9-(3-nitrobenzyl)adenine (3NBA)	9-(3-nitrobenzyl)adenine- N^1-oxide (3NBANO)
4-NO_2	9-(4-nitrobenzyl)adenine (4NBA)	9-(4-nitrobenzyl)adenine- N^1-oxide (4NBANO)
2-Cl	9-(2-chlorobenzyl)adenine (2CBA)	9-(2-chlorobenzyl)adenine- N^1-oxide (2CBANO)
2-CH_3	9-(2-methylbenzyl)adenine (2MBA)	9-(2-methylbenzyl)adenine- N^1-oxide (2MBANO)
2-OCH_3	9-(2-methoxylbenzyl)adenine (2MOBA)	9-(2-methoxylbenzyl)adenine- N^1-oxide (2MOBANO)

Fig. 1: Structures of AA substrates and their N^1-oxides.

Preparation of microsomes and incubation procedure

Golden Syrian hamsters (male, 70-100 g) were obtained from the Biological Services Unit, King's College London. The hepatic microsomal suspensions were prepared according to the method of Schenkman and Cinti /9/ by the sedimentation of microsomes with calcium ions, as further modified by Lam *et al.* /5/. The microsomes were finally resuspended in phosphate buffer (0.2 M, pH 7.4, con-

taining 20% v/v glycerol) equal to 0.5 g original tissue/ml suspension. The suspensions were preserved at -80°C.

Incubations were carried out according to the procedure previously described /7/. To determine the metabolic rates of substrates at various concentrations, each incubate consisted of microsomal suspension (0.5 ml, equivalent to 0.25 g fresh liver tissue), NADP (1 μmol), glucose-6-phosphate (5 μmol), glucose-6-phosphate dehydrogenase (0.5 unit), magnesium chloride (10 μmol) and phosphate buffer (1.0 ml, 0.2 M, pH 7.4) in a final volume of 1.5 ml. The substrates were dissolved in DMF at various concentrations and 30 μl solution was added to each microsomal incubate. To observe the effects of variation of microsomal protein concentration on the metabolism of the substrates, various amounts of microsomal suspension was added to incubates while the substrate concentration remained constant (100 nmol/incubate). The incubation was carried out at 37°C for 30 min.

Solid phase extraction and HPLC analysis

The incubations were terminated by adding trichloroacetic acid solution (200 μl/incubate, 25% v/v in water) to the incubates. A solid phase extraction procedure established earlier by Liu and Gorrod /10/ was used for the extraction of the N^1-oxides formed during incubation and the substrates remaining after incubation. The extracts of the incubates were analysed using the reversed-phase HPLC method reported previously /10/. The only modifications of the HPLC conditions for the analysis of the incubates with 2CBA, 2MBA or 2MOBA, were that BA was used as the internal standard and the mobile phase consisted of water:methanol:diethylamine = 60:40:0.5 v/v/v. Both the amount of N^1-oxides formed during incubation and the amount of substrate which remained unchanged after incubation were determined. The total amount of metabolite, excluding N^1-oxides, was calculated by subtracting the amount of formed N^1-oxides and unmetabolised substrate remaining from the amount of substrate originally added to the incubate.

Determination of apparent Km and Vmax values for the N^1-oxidation of AAs

Having determined the N^1-oxidation rates of AAs at various substrate concentrations, the data were used to determine the apparent

Km and Vmax values by utilising the Hanes-Woolf plot of [S]/V versus [S].

RESULTS AND DISCUSSION

When different concentrations of AA substrates were examined in the incubates, the total biotransformation rates of AAs by hamster microsomes were as shown in Figure 2. It seems that 2CBA, 2MBA and 2MOBA have very similar characteristics with regard to the biotransformation rates of the substrate. These three substrates are easily metabolised by hamster microsomes. Other substrates, including BA and NBAs, showed slower biotransformation rates than 2CBA, 2MBA and 2MOBA and less similarity to each other. It is apparent that the catalytic capacities of the enzymes in hamster microsomes vary greatly according to different AA substrates.

In addition, with various substrate concentrations in the incubates, the different formation rates of N^1-oxides among AAs also clearly

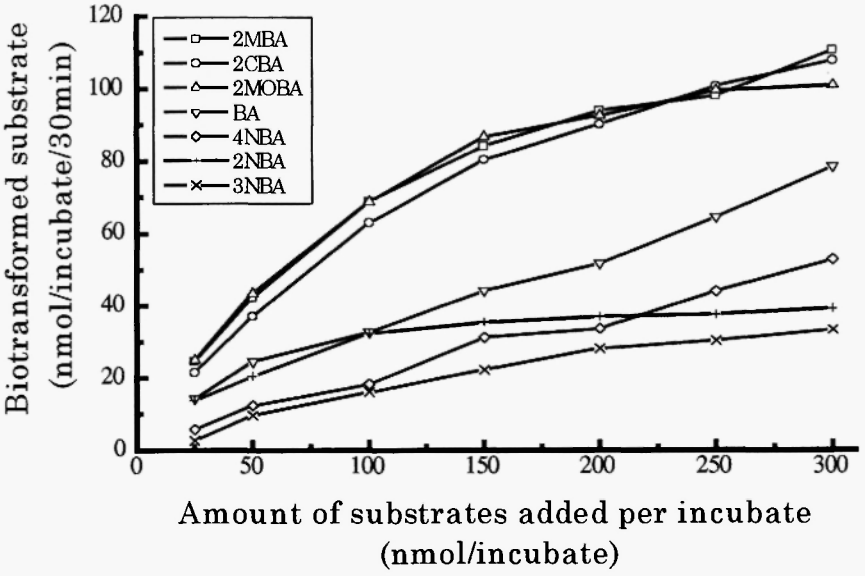


Fig. 2: Biotransformation rates of AA substrates at various concentrations. Data are the mean of duplicates.

showed that the activity of N^1-oxidase(s) towards different AA substrates were quite variable (Figure 3). The formation rates of the N^1-oxides of 2MOBA and 2MBA at various concentrations could not be established as the amounts of N^1-oxide formed during incubation were too small too be accurately determined. The results show that the order of N^1-oxide formation was 2NBA > 2CBA > BA > 3NBA and 4NBA.

The kinetics of enzymatic N^1-oxide formation from AAs were determined by Hanes-Woolf plot (Figure 4); the kinetic constants, i.e. Vmax and Km, obtained are summarised in Table 1. It is apparent that the Vmax values, which indicate the catalytic capacities of enzymes towards the substrates in question, are in the order 2NBA > 2CBA > BA > 3NBA > 4NBA. The Km values, which represent the reciprocal affinity of the enzyme for the substrate, are in the order of 2NBA > 2CBA > 3NBA > 4NBA > BA. It was observed that BA, 3NBA and 4NBA have a higher affinity to the N^1-oxidases than 2NBA and 2CBA,

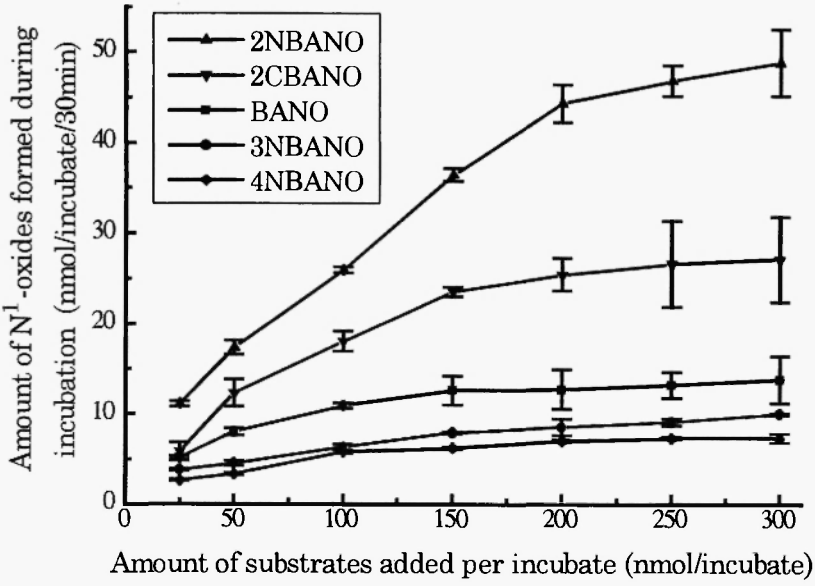


Fig. 3: N^1-Oxide formation of AAs at various substrate concentrations. Values = mean ± SD; n=3.

TABLE 1

Enzyme kinetic constants (Km and Vmax) from Hanes-Woolf plot for N^1-oxide formation from 9-aralkyladenines by the hamster hepatic microsomal incubation system

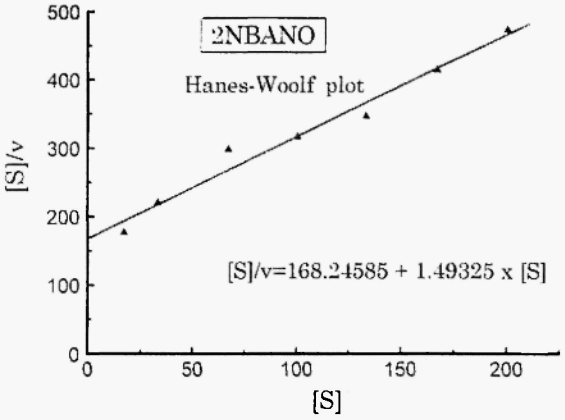
Substrate	Vmax	Km	Vmax/Km ratio ($x10^{-3}$)
2NBA	0.6697	112.67	5.94
2CBA	0.3114	62.60	4.97
BA	0.1391	33.30	4.18
3NBA	0.1047	51.52	2.03
4NBA	0.0800	47.41	1.86

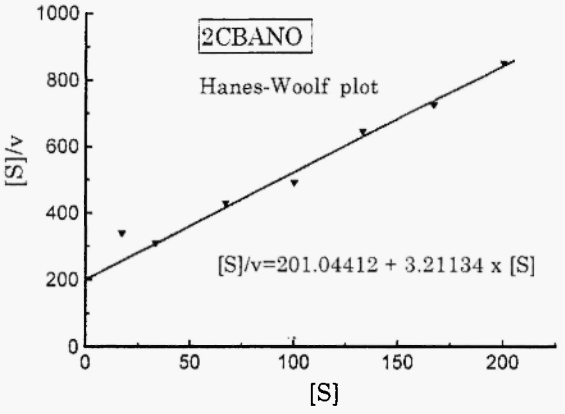
Vmax is expressed as nmol/mg protein/min, and Km as μM.

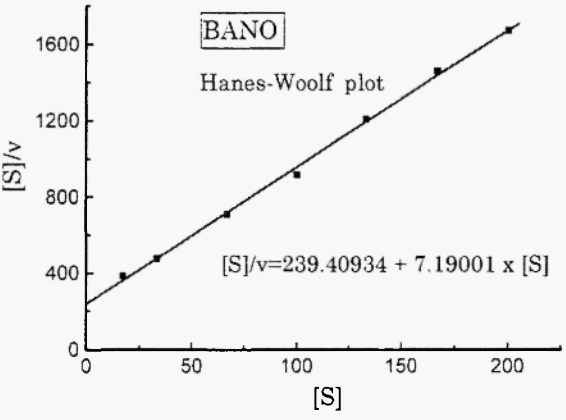
but the rates of formation of their N^1-oxides are slower than those of 2NBA and 2CBA. The velocity of an enzyme-catalysed reaction is determined not only by substrate-enzyme affinity but also by the catalytic capacity of the enzyme towards the substrate in question. The Vmax/Km ratio is a useful parameter to compare the relative catalytic activities of an enzyme towards different substrates, i.e. a higher ratio implies a stronger catalytic capacity /11/. As indicated in Table 1, the Vmax/Km ratios among the AA substrates are in the order 2NBA > 2CBA > BA > 3NBA > 4NBA (Figure 5).

It is interesting that the N^1-oxidases showed different affinities and catalytic activities towards NBAs, with the position of substitution in the phenyl moiety having a marked effect on substrate-enzyme affinity. For example, substitution at the 2'-position tends to decrease the affinity more significantly than at the 3'- or 4'-positions. These results indicate a potential structure-metabolism relationship in the N^1-oxidation of AAs.

The formation rates of N^1-oxides not only depend on the substrate concentration, but also on the amount of N^1-oxidase(s) present in the incubation system. For further examination of differences in the enzymological processes of N^1-oxidation among BA, 2CBA and NBAs,







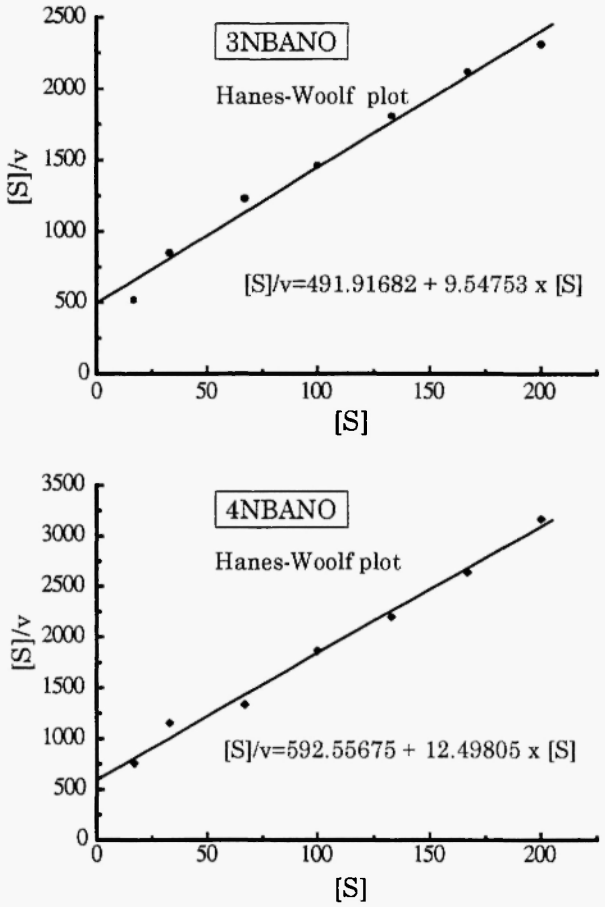


Fig. 4: Hanes-Woolf plots of N^1-oxide formation from AAs by the hamster hepatic microsomal incubation system. [S] was expressed as μM in the incubate system and V was expressed as nmol/mg protein/min.

various amounts of microsomal suspension were added to the incubates while the substrate amount was fixed at 100 nmol/incubate. The results are presented in Figure 5 which show the differences in the N^1-oxidation of various AAs. The catalytic activities of the N^1-oxidase(s) towards various substrates are in the order 2NBA > 2CBA > BA > 3NBA and 4NBA.

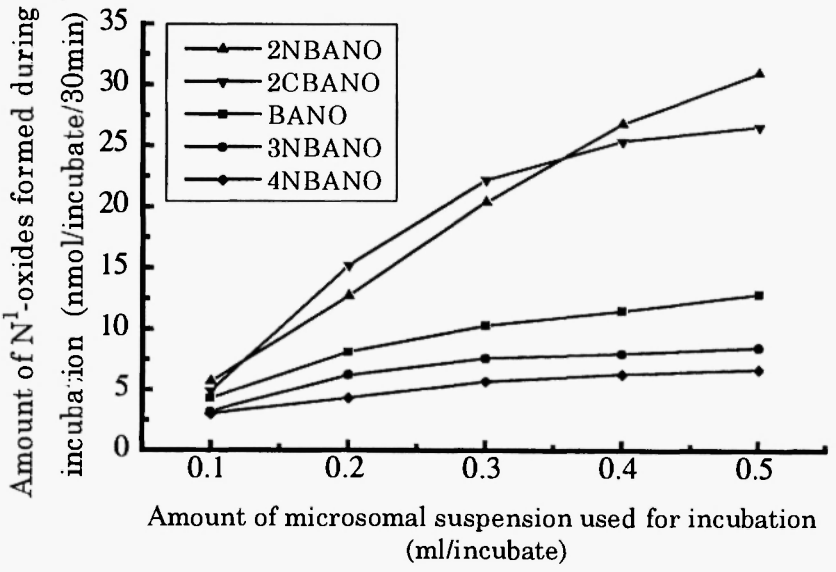


Fig. 5: Formation rates of N^1-oxides with various amounts of hamster microsomal suspension used for incubation. Microsomal suspension: 1.0 ml equivalent to 0.5 g of fresh tissue; AA substrates: 100 nmol/incubate. The data are the means of duplicates.

With the same hamster hepatic microsomes, same substrate concentration (100 nmol/incubate) and same incubation conditions, it was found that the formation rates of N^1-oxides during incubation varied significantly among the AAs studied (Figure 6A). The formation rates of N^1-oxides increased in the order 2NBANO > 2CBANO > BANO > 3NBANO and 4NBANO > 2MBANO and 2MOBANO. The formation rate of 2NBANO was about 25-fold higher than that of 2MBANO and 2MOBANO. But the formation rates were not markedly different between 3NBANO and 4NBANO, or between 2MBANO and 2MOBANO.

As mentioned earlier the N^1-oxide is not the only metabolite of the AAs investigated /3,6,7/. Certain other metabolites are also formed during incubation, such as the dealkylated metabolite, adenine, and other uncharacterised metabolites. The total amounts of these metabolites other than the N^1-oxides are shown in Figure 6B. The formation of

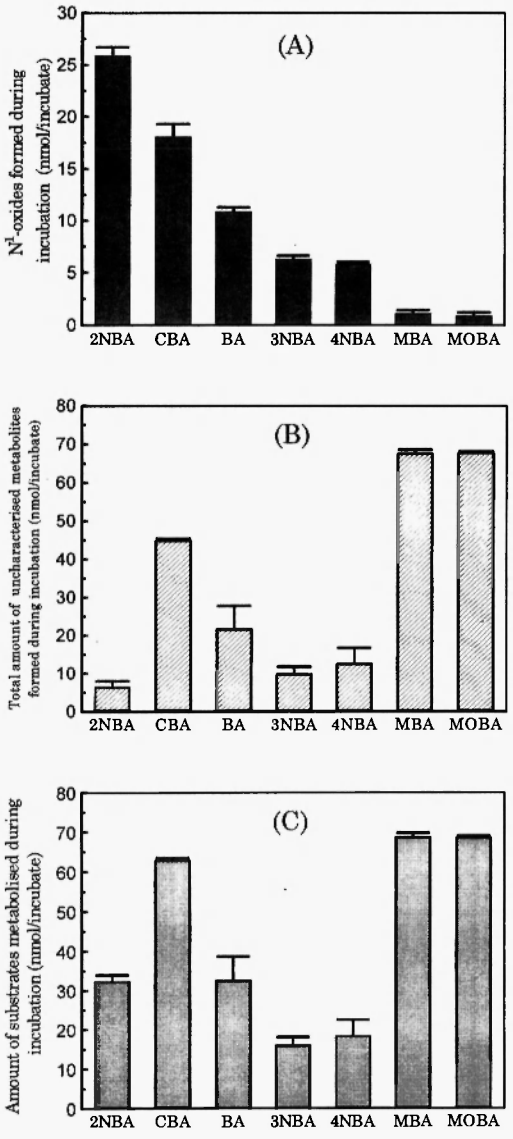


Fig. 6: Comparison of the metabolism of AAs by hamster hepatic microsomes. AAs (100 nmol/incubate) at 37°C for 30 min. Values = mean ± SD; n=3. **A**: Comparison of the formation rates of N^1-oxides; **B**: Comparison of the formation rates of total other uncharacterised metabolites; **C**: Comparison of the total biotransformation rates of substrates.

these other metabolites among AAs are in the order 2MBA and 2MOBA > 2CBA > BA > 3NBA > 2NBA. On the other hand, the total transformation rates of substrates also varied greatly among the AAs studied (Figure 6C). The total transformation seems not to parallel the formation rate of N^1-oxides and are in the following order: 2MBA, 2MOBA and 2CBA > 2NBA and BA > 3NBA and 4NBA.

The metabolism of AAs is summarised and compared in Table 2. The results strongly imply a certain structure-metabolism relationship among AAs. For example, substitution with a methyl or methoxy group to give 2MBA and 2MOBA produce substrates that have the highest total substrate transformation and highest amount of uncharacterised metabolite formation, but only a minor amount of N^1-oxide formation. In contrast, substitution with an electron-withdrawing group makes N^1-oxide formation dominant. Additionally, both the total transformation of substrate and formation of other metabolites from NBAs are lower than observed with 2MBA and 2MOBA, especially 2NBA. These facts reveal the importance of AA substrate structure as an important factor quantitatively influencing the metabolic pathways. Further investigation of this aspect will be useful for understanding the relevant factors controlling AA metabolism.

TABLE 2

Comparison of metabolism of 9-aralkyladenines*

Substrate	Transformation of substrate (%)	Formation of N^1-oxide (%)	Formation of other metabolites (%)
BA	32.7	10.9	21.8
2NBA	32.4	25.9	6.5
3NBA	16.2	6.3	9.9
4NBA	18.5	5.8	12.7
2CBA	63.0	18.1	44.9
2MBA	68.8	1.1	67.7
2MOBA	68.7	0.9	67.8

* 100 nmol/incubate at 37°C for 30 min.

ACKNOWLEDGEMENTS

D.Y. Liu is grateful for the scholarships from the K.C. Wong Education Foundation (Hong Kong), the Royal Society of Great Britain and C.V.C.P. (UK), which allowed this investigation to be carried out.

REFERENCES

1. Clement B, Kunze T. Hepatic microsomal N-hydroxylation of adenine to 6-N-hydroxylaminopurine. Biochem Pharmacol 1990; 39: 925-933.
2. Clement B, Kunze T. Microsomal N-oxygenation of adenine to adenine 1-N-oxide. Arch Pharmacol 1993; 326: 25-27.
3. Gorrod JW, Ioannides C, Lam SP, Neville S. Mutagenicity testing of 9-N-substituted adenines and their N-oxidation products. Environ Health Perspect 1993; 101 (Suppl 3): 21-26.
4. Lam SP, Devinsky F, Gorrod JW. Biological N-oxidation of adenine and 9-alkyl derivatives. Eur J Drug Metab Pharmacokinet 1987; 12: 239-243.
5. Lam SP, Barlow DJ, Gorrod JW. Conformational analysis of 9-substituted adenines in relation to their microsomal N^1-oxidation. J Pharm Pharmacol 1989; 41: 373-378.
6. Lam SP, Gorrod JW. Screening for microsomal N^1-oxidation of 9-substituted adenines. Prog Pharmacol Clin Pharmacol 1991; 8: 193-201.
7. Liu DY, Gorrod JW. Metabolic N^1-oxidation of 9-benzyladenine and isomeric 9-(nitrobenzyl)adenines by hamster hepatic microsomes. Drug Metab Drug Interact 1997; 13: 195-214.
8. Liu DY, Liu ZD, El-Ghomari K, Gorrod JW. Metabolism of 9-(2-chlorobenzyl)-, 9-(2-methylbenzyl)- and 9-(2-methoxybenzyl)-adenines by hamster hepatic microsomes. Eur J Drug Metab Pharmacokinet 1997; 22: 367-373.
9. Schenkman JB, Cinti DL. Preparation of microsomes with calcium. Meth Enzymol 1978; 52: 83-89.
10. Liu DY, Gorrod JW. Solid phase extraction and HPLC determination of 9-benzyladenine and isomeric 9-(nitrobenzyl)adenines and their metabolic N^1-oxides present in microsomal incubates. J Pharm Biomed Anal 1997; 15: 1741-1750.
11. Palmer T. Kinetics of single substrate enzyme catalysed reactions. In: Palmer T, ed. Understanding Enzymes. Chichester: Ellis Horwood Ltd., 1985; 119-141.