*Biochemical Pharmacology*, Vol. 35, No. 23, pp. 4341–4343, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00


# Photoaffinity analogues of methotrexate as probes for dihydrofolate reductase structure and function

(*Received* 31 *March* 1986; *accepted* 19 *May* 1986)

Previous work from our laboratory [1] has established the efficient syntheses of lysine (APA-Lys)* and ornithine (APA-Orn) analogues of the folate antagonist drug methotrexate (MTX). Both analogues are potent inhibitors of dihydrofolate reductase (DHFR), the intracellular target for MTX and other antifolate drugs. Reaction of APA-Lys and APA-Orn with dansyl chloride furnishes the corresponding dansyl derivatives [2, 3]. These two fluorescent compounds, like their precursors, are effective inhibitors of DHFR isolated from several sources. They also exhibit an enhancement of their fluorescence and a shift in fluorescence maxima to a lower wavelength when bound to DHFR [4]. In addition, the dansyl derivative of APA-Lys competes effectively with MTX for transport into intact L1210 cells, both MTX-sensitive and -resistant, and becomes bound to intracellular DHFR [5]. These results are consistent with conclusions drawn from X-ray crystallographic analyses of inhibitor complexes of DHFR, which suggest that the $\gamma$-carboxylate of the glutamate moiety of MTX plays a minor role in the binding of MTX to DHFR [6–8].

As a complement to fluorescent probes, the technique of photoaffinity labeling has been widely used in the study of molecular interactions in biological systems (for a review, see Ref. 9). An advantageous property of photoaffinity labeling probes over chemically reactive affinity probes is that it allows the study of both competitive, reversible as well as covalent, irreversible interactions with a single ligand and its receptor. This technique was applied recently to folate-dependent enzymes with the synthesis of 2′-azidoaminopterin [10]. However, this compound was incorporated into *Escherichia coli* DHFR with an efficiency of only 0.5%. The present study describes the syntheses and inhibitory potencies of four photoaffinity analogues of MTX. These include $N^{\alpha}$-(4-amino-4-deoxy-10-methylpteroyl)-$N^{\varepsilon}$-(4′-azidosalicylyl)-L-lysine (APA-ASA-Lys), the corresponding ornithine analogue (APA-ASA-Orn) and their iodinated counterparts (APA-I-ASA-Lys and APA-I-ASA-Orn). A preliminary account of these syntheses has appeared [11, †]. In addition, data on the photoaffinity labeling of murine L1210 DHFR with APA-[$^{125}$I]ASA-Lys are described.

## *Materials and methods*

APA-Lys and APA-Orn were synthesized, purified and characterized as previously described [1, 2]. All synthetic steps were performed under subdued light. APA-Lys (50 mg, 110 $\mu$moles) was dissolved in 100 ml of 40 mM lithium carbonate buffer, pH 9.5. To this clear yellow solution was added an equimolar amount of the *N*-hydroxysuccinimidyl ester of 4-azidosalicylic acid (Pierce Chemical Co.) in 1.0 ml of silylation grade acetonitrile. The homogeneous solution was rotary evaporated to dryness after reaction for 2 hr at ambient temperature. The crude product was purified via silica TLC in iPrOH/MeOH/$NH_4OH$ (3:1:1). The product, APA-ASA-Lys (50% yield), had an $R_f$ of 0.54. Mass spectroscopy revealed a $MH^+$ of 615. HPLC was also employed to verify the purity of the photoaffinity analogues. The compounds were injected onto a Waters C-18 $\mu$Bondapak reversed-phase column (7.8 × 300 mm). The solvents used for elution were: A, 0.1% trifluoroacetic acid (TFA) in water; B, 0.1% TFA in 1-propanol. A gradient of 100% A to 50% B was used over 60 min at a flow rate of 1.0 ml/min, followed by an isocratic of 50% B. APA-ASA-Lys had a retention time of 47 min (40% B) as a single symmetrical peak. IR(KBr) 1390 $cm^{-1}$ (C—O), 1600–1700 $cm^{-1}$ (amide), 2140 $cm^{-1}$ (azide), 3100–3600 $cm^{-1}$ (C═O, N—H); UV (pH 7.0) $\lambda_{max}$ 370 nm, 302 ($\varepsilon$22,100), 274, 258. APA-ASA-Orn was prepared in a similar fashion and had an $R_f$ of 0.63 in iPrOH/MeOH/$NH_4OH$ (3:1:1). Mass spectroscopy gave a $M^+$ of 601. The compound gave a single symmetrical peak on HPLC with a retention time of 48 min (41% B). Iodination of either APA-ASA-Lys or APA-ASA-Orn was achieved using an excess of $Na^{127}I$ and chloramine T. The iodinated products APA-I-ASA-Lys and APA-I-ASA-Orn, obtained in high yields, exhibited $R_f$ values of approximately 0.6 and 0.7 respectively. Both compounds gave symmetrical peaks on HPLC with retention times of 75 min, 50% B (APA-I-ASA-Lys) and 77 min, 50% B (APA-I-ASA-Orn). APA-[$^{125}$I]ASA-Lys was prepared as follows. APA-ASA-Lys (1.9 $\mu$moles) was admixed with 1.1 mCi $Na^{125}I$ in 0.1 N NaOH (5 $\mu$l) in a volume of 150 $\mu$l dimethylformamide (DMF). Three iodobeads (Pierce) were then added and the reaction was allowed to proceed for 2 min at ambient temperature. The reaction mixture was loaded onto a silica TLC place and developed in iPrOH:MeOH:$NH_4OH$ (3:1:1) in subdued light. The $R_f$ for the radioiodinated analogue was 0.62 and the specific radioactivity was $2.97 \times 10^5$ cpm/nmole.

L1210 dihydrofolate reductase was assayed and purified by MTX-affinity chromatography as previously described [5]. The assay concentration of dihydrofolate was 50 $\mu$M; NADPH concentration was 75 $\mu$M. The concentration of DHFR was determined by MTX titration. Murine L1210 DHFR (173 nmoles) was incubated with a 2-fold molar excess of APA-[$^{125}$I]ASA-Lys in 3.3 ml of 50 mM $KPO_4$, pH 7.0, for 20 min at 4° in subdued light. The solution was then irradiated for 3 min (1800 $\mu W/cm^2$) in a quartz cuvette with a hand held longwave ultraviolet lamp. The labeled enzyme was then denatured in 6 M urea at 70° for 70 min. The material was then passed through a Sephadex G-25 column (1.4 × 36 cm) containing 50 mM $NH_4HCO_3$–6 M urea, pH 9.5, exhaustively dialyzed against distilled $H_2O$ and lyophilized. Under these conditions, [$^3$H]MTX was completely dissociated from DHFR (data not shown).

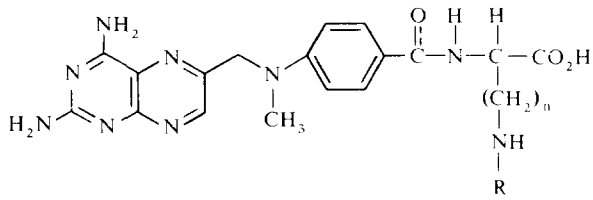
## *Results and discussion*

The concentration of inhibitor needed to inhibit DHFR activity by 50% ($IC_{50}$) was used to ascertain the inhibitory potencies of the photoaffinity analogues relative to that for MTX. As shown in Table 1, all of the compounds, including the parent compounds APA-Lys and APA-Orn, were

* Abbreviations: APA, 4 - amino - 4 - deoxy - 10 - methylpteroic acid; APA-Lys, $N^{\alpha}$(4-amino-4-deoxy-10-methylpteroyl)L-lysine; APA-Orn, $N^{\alpha}$-(4-amino-4-deoxy-10-methylpteroyl)-L-ornithine; APA-ASA-Lys, $N^{\alpha}$-(4-amino-4-deoxy-10-methylpteroyl)-$N^{\varepsilon}$-(4′-azidosalicylyl)-L-lysine; APA - ASA - Orn, $N^{\alpha}$ - (4 - amino - 4 - deoxy - 10 - methylpteroyl)-$N^{\alpha}$-(4′-azidosalicylyl)-L-ornithine; DHFR, dihydrofolate reductase (EC 1.5.1.3); and MTX, methotrexate, 4-amino-4-deoxy-10-methylpteroylglutamic acid.

† R. J. Kempton, L. Judge, J. H. Freisheim and A. A. Kumar, *185th American Chemical Society National Meeting*, Seattle, WA, March 20–25, 1983, ABSTR MEDI 0085.

Table 1. Dihydrofolate reductase inhibition by analogues of methotrexate*



| Compound | N = | R = | $IC_{50}$† (nM) Enzyme source *L. casei* | L1210 |
|---|---|---|---|---|
| APA-Lys | 4 | H | 54 | 21 |
| APA-ASA-Lys | 4 | 4′-Azidosalicylyl | 68 | 29 |
| APA-I-ASA-Lys | 4 | Iodo-4′-azidosalicylyl | 85 | 44 |
| APA-Orn | 3 | H | 62 | 50 |
| APA-ASA-Orn | 3 | 4′-Azidosalicylyl | 58 | 40 |
| APA-I-ASA-Orn | 3 | Iodo-4′-azidosalicylyl | 50 | 34 |

* Dihydrofolate reductase from either *L. casei* or L1210 cells (50 nM) was incubated with 75 μM NADPH and various amounts of inhibitor in 1.0 ml of 0.05 M $KPO_4$, pH 7.0, for 2 min in the dark. The solution was made 50 μM in dihydrofolate concentration and the residual enzyme activity was measured by the decrease in absorbance at 340 nm [5].

† $IC_{50}$ is the concentration of inhibitor needed to inhibit DHFR half-maximally. The $IC_{50}$ for MTX was 25 nM.

potent inhibitors of both bacterial and mammalian DHFRs. The enzyme was assayed spectrophotometrically as previously described [5]. None of the compounds was more than 4-fold less potent than MTX. The inhibitory potency of the lysine analogues decreased somewhat as modifications were made to the $N^{\epsilon}$-amino group; however, the inhibitory potency of the ornithine analogues increased as substituents were added to the $N^{\alpha}$-amino group. This pattern was observed for either reductase. The compounds APA-Lys and APA-ASA-Lys were as potent as MTX.

Irradiation of the binary complex DHFR:APA-[$^{125}$I]ASA-Lys, followed by denaturation, desalting and exhaustive dialysis, resulted in a recovery of $1.84 \times 10^7$ cpm (62 nmoles) incorporated into the protein (173 nmoles). This represents a labeling efficiency of *ca.* 35%. In the absence of irradiation, no covalent incorporation occurred (data not shown). Incubation of the enzyme with a 100-fold molar excess of MTX prior to irradiation of the photoprobe afforded virtually complete protection against photoaffinity labeling (results not shown).

It was found that all of the compounds were good inhibitors of both *Lactobacillus casei* and murine L1210 DHFR. Previous work from our laboratories [2–5] showed that dansylated APA-Lys is also a capable inhibitor of DHFR. The fluorescein derivative of APA-Lys has also been synthesized and is 7- to 20-fold less potent than MTX as a DHFR inhibitor [12]. Clearly, the addition of bulky substituents onto the terminal amino group of either APA-Orn or APA-Lys is not detrimental to the binding of the compound to DHFR.

Since the compounds are potent inhibitors of DHFR, they should also be viable photoaffinity probes for the binding site of DHFR. Initial studies utilizing APA-[$^{125}$I]ASA-Lys revealed that this probe could covalently modify L1210 DHFR with a very favorable efficiency of 35%, following complete enzyme inactivation. The apparent high degree of labeling is consistent with the theory that there are no strong interactions involving the binding site of DHFR and the γ-carboxylate region of the glutamate of MTX. This would allow free movement of the azidosalicyl moiety of APA-[$^{125}$I]ASA-Lys and thereby grant easy access of the reactive azide to potential target amino acid residues. Covalent modification upon irradiation of an aromatic azide depends on the juxtaposition of the generated nitrene moiety with a component of the polypeptide chain that will yield a productive reaction. The labeling efficiency depends on the number of stable covalent bonds formed with the highly reactive nitrene species. It is difficult to predict, with any accuracy, the degree of labeling in a reaction between a nitrene and a polypeptide chain. In addition, the labeling efficiency of a particular photoaffinity probe with different proteins can vary widely [13], with a 25% efficiency considered a high degree [14] and 10% considered quite good [13]. Experiments are currently being performed that will attempt to minimize any random labeling and identify any specifically labeled amino acids. Additionally, as indicated by the high efficiency with which APA-[$^{125}$I]ASA-Lys labels DHFR, this probe should prove of value in the study of other folate-binding proteins.

In summary, a total of four photoaffinity analogues of methotrexate were synthesized, including $N^{\alpha}$-(4-amino-4-deoxy-10-methylpteroyl)-$N^{\epsilon}$-(4′-azidosalicylyl)-L-lysine, the corresponding ornithine analogue, as well as their iodinated (azidosalicylyl) counterparts. All four compounds were quite potent inhibitors of dihydrofolate reductases isolated from *L. casei* or from murine L1210 cells. Photoactivation of the [$^{125}$I]azidosalicylyl-lysine analogue in the presence of L1210 dihydrofolate reductase resulted in a covalent labeling efficiency of the enzyme of 35%.

*Acknowledgements*—This study was supported by NIH Grants CA41461 (J. H. F.) and GM36097 (R. J. K.). E. M. P. is a Ryan Foundation predoctoral fellow. Mass spectra were performed by Dr Catherine E. Costello, M.I.T.

*Department of Biochemistry and Molecular Biology*
*University of Cincinnati*
*College of Medicine*
*Cincinnati, OH 45267-0522, U.S.A.*
ELMER M. PRICE

*Department of Physical Sciences*
*Northern Kentucky University*
*Highland Heights, KY 41076, U.S.A.*
LAURA SAMS
KATHLEEN M. HARPRING
ROBERT J. KEMPTON

*Department of Biochemistry*
*Medical College of Ohio*
*Toledo, OH 43699, U.S.A.*
JAMES H. FREISHEIM*

REFERENCES

1. R. J. Kempton, A. M. Black, G. M. Anstead, A. A. Kumar, D. T. Blankenship and J. H. Freisheim, *J. med. Chem.* **25**, 475 (1982).
2. A. A. Kumar, J. H. Freisheim, R. J. Kempton, G. J. Anstead, A. M. Black and L. Judge, *J. med. Chem.* **26**, 111 (1983).
3. A. A. Kumar, R. J. Kempton, G. M. Anstead, E. M. Price and J. H. Freisheim, *Analyt. Biochem.* **128**, 191 (1983).
4. A. A. Kumar, R. J. Kempton, G. M. Anstead and J. H. Freisheim, *Biochemistry* **22**, 390 (1983).
5. S. S. Susten, R. J. Kempton, A. M. Black and J. H. Freisheim, *Biochem. Pharmac.* **33**, 1957 (1984).
6. D. A. Matthews, R. A. Alden, J. T. Bolin, S. T. Freer, R. Hamlin, N. Xuong, J. Kraut, M. Poe, M. Williams and K. Hoogsteen, *Science* **197**, 452 (1977).
7. D. A. Matthews, R. A. Alden, J. T. Bolin, D. J. Filman, S. T. Freer, R. Hamlin, W. G. J. Hol, R. L. Kisliuk, E. J. Pastore, L. T. Plante, N. Xuong and J. Kraut, *J. biol. Chem.* **253**, 6946 (1978).
8. K. W. Volz, D. A. Matthews, R. A. Alden, S. T. Preer, C. Hansch, B. T. Kaufman and J. Kraut, *J. biol. Chem.* **257**, 2528 (1982).
9. V. Chowdhry and F. H. Westheimer, *A. Rev. Biochem.* **48**, 293 (1979).
10. P. F. Holmes, J. G. Liehr and J. Henkin, *Bioorg. Chem.* **11**, 281 (1982).
11. E. Price and J. H. Freisheim, *Fedn Proc.* **43**, 2120 (1984).
12. A. Rosowsky, J. E. Wright, H. Shapiro, P. Beardsley and H. Lazarus, *J. biol. Chem.* **257**, 14162 (1982).
13. R. L. Potter and B. E. Haley, *Meth. Enzym.* **91**, 613 (1983).
14. D. Cavalla and N. H. Neff, *Biochem. Pharmac.* **34**, 2821 (1985).

* Address all correspondence to: Dr. James H. Freisheim, Department of Biochemistry, Medical College of Ohio, C.S. 10008, 3000 Arlington Ave., Toledo, OH 43699.

*Biochemical Pharmacology*, Vol. 35, No. 23, pp. 4343–4345, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00


# Participation of microsomal electron transport systems in nicotine metabolism by livers of guinea pigs

(*Received* 10 *February* 1986; *accepted* 19 *May* 1986)

It has been suggested that nicotine is metabolized predominantly in liver microsomes by cytochrome P-450 and FAD-containing monooxygenase [1–4]. These two enzymes have important roles in drug, insecticide and other xenobiotic metabolism [5, 6]. Since it has been shown that many kinds of substrates oxidized by FAD-containing monooxygenase are also metabolized by cytochrome P-450, the definition of the relative contribution of these two enzymes to the metabolism of common substrates is necessary [5, 6]. Recently, we showed that phenobarbital (PB)-inducible cytochrome P-450 (PB-P-450) in rat and guinea pig livers catalyzes nicotine oxidation in reconstituted systems and microsomes [7, 8]. In addition, constitutive forms of cytochrome P-450 have been assumed to participate in microsomal nicotine oxidation since PB-P-450 is scarcely detectable in liver microsomes of untreated animals [8]. Many kinds of cytochrome P-450 have been purified from microsomes of animal livers, whereas only one type of NADPH–cytochrome P-450 reductase, the other component of microsomal electron transport systems, has been found to be present in liver microsomes. By using antibody against NADPH–cytochrome P-450 reductase, therefore, we have investigated the contribution of microsomal electron transport systems to nicotine oxidation in livers of untreated and PB-treated guinea pigs.

*Materials and methods*

Male Hartley guinea pigs (30–42 days of age) were injected intraperitoneally each day for 5 days with PB (60 mg/kg). Microsomal fractions were prepared as previously described [8]. NADPH–Cytochrome P-450 reductase was purified by the method of Yasukochi and Masters [9] with some modifications. The purified enzymes showed a single protein band when submitted to polyacrylamide gel electrophoresis by the method of Laemmli [10]. Cytochrome P-450 and $b_5$ were not detected in the purified reductase fractions.

Antibody against NADPH–cytochrome P-450 reductase was produced in rabbits as previously described for PB-P-450 [8] and purified by fractionation with ammonium sulfate and chromatography with DE-52. The purified immunoglobulin fractions were designated as anti-NADPH–cytochrome P-450 reductase. The Ouchterlony double diffusion test showed that anti-NADPH–cytochrome P-450 reductase cross-reacted with the purified reductase and with the microsomal components, and formed a single precipitation line. Control immunoglobulin G was prepared by the same method using blood obtained from non-immunized rabbits. When inhibition of liver microsomal nicotine oxidase activity by anti-NADPH–cytochrome P-450 reductase was studied, microsomes were first mixed with the antibody in 20 mM potassium phosphate buffer (pH 7.4) for 10 min at room temperature; then $MgCl_2$, NADPH and phosphate buffer were added, followed by an additional 5-min incubation at 37°. After this preincubation, the reaction was started at 37° by the addition of nicotine.

Nicotine oxidase activity was determined spectrophotometrically at 259 nm as previously described [8]. NADPH–Cytochrome P-450 reductase activity was assayed by its ability to catalyze cytochrome *c* reduction in 100 mM potassium phosphate buffer (pH 7.4) at 25° [11]. Di-