Table 1. Comparative antiviral effect of synthetic nucleosides on herpes simplex virus and mengovirus

	Virus rating (VR)	
	Herpes simplex virus‡	Mengo virus§
5,7-Dimethyl-3-β-D-ribofuranosyl-s-triazolo[4,3-a]pyrimidine (I)	0.1	0.1
5-Methyl-3-β-D-ribofuranosyl-s-triazolo[4,3-a]pyrimidine	< 0.1	0
5,7-Dimethyl-2-β-D-ribofuranosyl-s-triazolo[1,5-a]pyrimidine (II)	0.5	0.7
7-Methyl-2-β-D-ribofuranosyl-s-triazolo[1,5-a]pyrimidine	0.3	0.4
1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin)	1.1	1.0
*5-Methyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide	0.1	0
†3,5-Dimethyl-1-β-D-ribofuranosyl-1,2,4-triazole	0.1	0

*Ref. 11, †Ref. 12.
‡ KB-cells were infected with herpes simplex virus (100 × $CCID_{50}$‖) and exposed to different concentrations of the test compounds followed by incubation for 2 days. At the end of the period of incubation the reduction of CPE was evaluated by the "virus rating" method.
§ L-cells were infected with 100 × $CCID_{50}$‖ mengovirus and exposed to different concentrations of test compounds for 2 days. The reduction of CPE was evaluated by the "virus rating" method.
‖ Cell culture infecting dose 50 (infecting 50 per cent of the cell cultures).

Table 2. Effect of I and II on mengovirus and herpes simplex virus growth

	Mengo virus*	Herpes simplex virus†
	titer (log_{10} PFU/ml)	
Control: 0 hr	3.23	3.48
Control: 24 hr	8.08	8.51
5,7-Dimethyl-2-β-D-ribofuranosyl-s-triazolo[1,5-a]pyrimidine (II)		
200 μg/ml	7.40	8.45
500 μg/ml	6.95	8.28
5,7-Dimethyl-3-β-D-ribofuranosyl-s-triazolo[4,3-a]pyrimidine (I)		
200 μg/ml	7.85	8.46
500 μg/ml	7.28	8.43

*L-cells.
† KB-cells.
Cell monolayers (1×10^5 cells per 35-mm Petri dish) were washed with phosphate-buffered saline and incubated with either virus at a multiplicity of 10 PFU per cell. At the end of the period of adsorption the monolayers were washed 3 times with culture medium and one plate was frozen as zero-time control. The other dishes received 2 ml of complete medium containing either II or I as indicated. After 24 hr of incubation all cultures were frozen and thawed 3 times and virus titrations were performed [8].

(VR < 0.1), it gave additional support to the former hypothesis that the flexibility of the glycosidic bond is an important parameter in determining the biological activity of the drug. This compound has the 5-methyl group at the peri position pushing ribose moiety to the anti position as in I. The data for 7-methyl-2-β-D-ribofuranosyl-s-triazolo [1,5-a]pyrimidine, on the other hand, are consistent with previous considerations, since it mimics II, lacking one methyl group and not affecting the rotation about the glycosidic bond.

It was found by Witkowski *et al.* [5], that none of the ribavirin analogs substituted at position 5 did exhibit any favorable antiviral activity as compared to the parent 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin). Theoretical studies [10] ascribe this inactivity to steric reasons, which prohibit the high anti-region required as a biologically important conformation specified by the enzyme IMP dehydrogenase. Therefore, it was of interest to include 5-methyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide [11] as well as 3,5-dimethyl-1-β-D-ribofuranosyl-1,2,4-triazole [12] as reference compounds to our study test. It was concluded that methyl group at position 5 in a triazole ring hindered the rotation of the ribose moiety from ^1H and ^{13}C n.m.r. relaxation and NOE data, which precluded the formation of the active high anti-conformation and is in complete agreement to the theoretical calculations [10]; the antiviral activity of 5-methyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide has been determined as zero.

We conclude that of the compounds synthesized 5,7-dimethyl-2-β-D-ribofuranosyl-s-triazolo[1,5-a]pyrimidine has the most favorable inhibitory effects on both herpes simplex virus and mengovirus. In contrast, 5,7-dimethyl-3-β-D-ribofuranosyl-s-triazolo[4,3-a]pyrimidine is ineffective in either antiviral test system, suggesting that the flexibility of the glycosidic bond is an important parameter in determining the biological activity of the drug.

Acknowledgements—This investigation was supported by grants from the Research Community of Slovenia and Krka Pharmaceutical and Chemical Works. We are indebted to

Prof. Dušan Hadži for support and encouragement. The excellent technical assistance of Miss Marija Taiber is gratefully acknowledged.

Central Institute for Tumors and Allied Diseases, Ilica 197, 41000 Zagreb BRANKO BRDAR

Krka Pharmaceutical and Chemical Works, 68000 *Novo Mesto* MIHA JAPELJ

Boris Kidrič Institute of Chemistry, Hajdrihova 19, 61000 Ljubljana, Yugoslavia JOŽE KOBE

REFERENCES

1. M. Hari, E. Ito, T. Takita, G. Koyama, T. Takenchi and H. Umezawa, *J. Antibiot. Tokyo*, Ser. A, **17**, 96 (1964).
2. J. Giziewicz, E. De Clercq, M. Luczak and D. Shugar, *Biochem. Pharmac.* **24**, 1813 (1975).
3. T. Novinson, R. Hanson, M. K. Dimmitt, L. N. Simon, R. K. Robins and D. E. O'Brien, *J. med. Chem.* **17**, 645 (1974).
4. D. L. Miles, D. W. Miles, P. Redington and H. Eyring, *J. Theor. Biol.* **67**, 499 (1977).
5. J. T. Witkowski, R. K. Robins, R. W. Sidwell, L. N. Simon, *J. med. Chem.* **15**, 1150 (1972).
6. D. G. Streeter, J. T. Witkowski, G. P. Khare, R. W. Sidwell, R. J. Bauer, R. K. Robins and L. N. Simon, *Proc. natn. Acad. Sci. U.S.A.* **70**, 1174 (1973).
7. D. L. Miles, D. W. Miles and H. Eyring, *Biochim. biophys. Acta* **518**, 17 (1978).
8. B. Brdar and E. Reich, *J. biol. Chem.* **247**, 725 (1972).
9. R. W. Sidwell and J. H. Huffman, *Appl. Microbiol.* **22**, 797 (1971).
10. D. L. Miles, D. W. Miles, P. Redington, H. Eyring, *Proc. natn. Acad. Sci. U.S.A.* **73**, 4257 (1976).
11. S. R. Naik, J. T. Witkowski and R. K. Robins, *J. Heterocyclic Chem.* **11**, 57 (1974).
12. J. Kobe and J. Cotua Valdes, *Carbohydrate Res.* **66**, 222 (1978).

Biochemical Pharmacology, Vol. 28, pp. 1686–1688.
© Pergamon Press Ltd. 1979. Printed in Great Britain.
0006-2952/79/0515–1686 $02.00/0

High benzo[a]pyrene hydroxylase activity in the marine fish *Stenotomus versicolor*

(*Received* 14 *August* 1978; *accepted* 7 *November* 1978)

Hepatic microsomal cytochrome P-450 systems in fish are generally like those in mammals, with cytochromes P-450 having similar optical and electron paramagnetic resonance (e.p.r.) spectra and catalytic functions [1, 2]. Hepatic mixed-function oxygenase activities in fish are, however, often low when compared to those in mammals [3, 4]. Fish mixed-function oxygenases, notably aryl hydrocarbon (benzo[a]pyrene; BP) hydroxylase, can be experimentally induced by treatment with known organic contaminants [5, 6], and it is also possible for such induction to occur in the environment [6].

In order to define relationships between the chemical environment and cytochrome P-450 systems in fish, it is necessary to understand these systems in untreated or uninduced animals. In some untreated, freshwater brown trout (*Salmo trutta*), for example, the activity of hepatic microsomal BP hydroxylase was found to be much greater than that observed in mammals [7, 8]. However, the trout used in this study were all held for some period in captivity, which could introduce factors that might influence hepatic mixed-function oxygenases.

There have been no reports dealing with such high BP hydroxylase in marine fish. This communication describes some characteristics of hepatic microsomal mixed-function oxygenase activities in *Stenotomus versicolor* (scup or porgy), a marine teleost with high BP hydroxylase activity, and compares these and other features of microsomal electron transport systems to those in the mouse.

BP and 7,8-benzoflavone (7,8-BF) were obtained from the Aldrich Chemical Co, Milwaukee, WI. All other cofactors, coenzymes and substrates were obtained from the Sigma Chemical Co., St. Louis, MO.

Adult male and female scup (*S. versicolor*), about 100–200 g, were collected by angling in Great Harbor, Woods Hole, MA, or outer Hadley Harbor, Gosnold, MA, in June–August, 1975–1978. Fish were either used within 1 hr of capture, or held for periods of 2 weeeks to 8 months in 800-gal tanks at the National Marine Fisheries Service, Woods Hole, MA. Fish held were fed a diet of chopped smelt and clams, *ad lib.*, every 2 days. Tanks were equipped with flow-through water at 19 ± 1°, filtered through gravel and sand. All fish were killed outside the spawning season. Mice (*Mus musculus*) were 90-day-old adult Charles River CD-1 females of 23–26 g, maintained at the animal facilities of the Marine Biological Laboratory, Woods Hole, MA. Mice were fed Charles River diet and Purina Lab Chow, and maintained on Absorb-Dri and Bedda-Chip hardwood bedding at a density of 7/f².

Animals were killed by cervical dislocation (mice) or decapitation (scup) and excised livers were placed immediately in ice-cold 0.1 M phosphate buffer, pH 7.3. Tissues were minced and homogenized in 4 vol. of 0.1 M phosphate buffer, pH 7.3, containing 1.15% KCl and 3 mM $MgCl_2$, using a Potter–Elvehjem tissue grinder with four passes of the pestle at 1350 and four passes at 1900 rev/min. Microsomal preparations were isolated from the 9000 *g* supernatant fraction by centrifuging for 90 min at 40,000 *g* and resuspended in 3 vol. of 0.1 M phosphate buffer, pH 7.3, per g of liver.*

NADPH-cytochrome *c* (cytochrome P-450) reductase (EC 1.6.2.4) was assayed at 25° (scup) or 37° (mouse) by a modification of the method of Phillips and Langdon [9], with a reaction mixture containing 0.175 mM NADPH and 80 μM horse heart cytochrome *c* in 0.2 M potassium phosphate buffer, pH 7.7. NADH-cytochrome *c* (cytochrome b_5) reductase (EC 1.6.2.2) was assayed using the conditions for NADPH-cytochrome *c* reductase, with 0.25 mM NADH replacing 0.175 mM NADPH. Reduction of cytochrome *c* was followed at 550 nm and reference cuvettes in both cases contained reaction mixtures with no enzyme.

BP hydroxylase was assayed using 0.5-ml reaction mixtures containing an NADPH-generating system described

* Cytochrome P-450 was undetectable in the 40,000 *g* supernatant fraction. Relative specific activities of BP hydroxylase in microsomal and 40,000 *g* supernatant fractions were 5.5 and 0.1 respectively. Relative specific activities of AP demethylase were 6.3 and 0.05 respectively.

previously [10] in 0.1 M Tris, final pH 7.0 (scup) or 7.3 (mouse). Reactions were initiated by adding BP in 20 μl methanol to a final concentration of 60 μM, and incubated for 20 min at 29° (scup) or 37° (mouse). Fluorometric assay of metabolites was carried out according to modifications of the method of Nebert and Gelboin [11]. When studying 7,8-BF inhibition, both 7,8-BF and BP were added in a total of 20 μl MeOH. Aminopyrine (AP) demethylase was determined with a 1.5-ml reaction mixture containing a generating system as given above in 0.166 M HEPES, pH 7.6. Reactions, initiated by adding recrystallized AP to a final concentration of 15 mM, were incubated for 15 min at 29° (scup) or 37° (mouse), and formaldehyde was assayed according to Nash [12], as modified by Cochin and Axelrod [13]. Blank values for both BP hydroxylase and AP demethylase were determined using reaction mixtures without NADPH. Assay temperature and pH indicated for scup enzymes were optimal.

Cytochrome P-450 was analyzed optically using a Cary 118-C difference spectrophotometer. Cuvettes contained 0.8 to 1.2 mg of microsomal protein in 1.5 ml of 0.1 M phosphate buffer, pH 7.3. CO-bubbled, dithionite-reduced microsomes occupied the sample compartment and CO-bubbled microsomes occupied the reference. Cytochrome b_5 was analyzed in similar 1.5-ml volumes, and microsomes in the sample cuvette were reduced with 0.034 mg NADH/ml. Cytochrome content was determined assuming extinction coefficients of 91 cm^{-1} mM^{-1} for cytochrome P-450 at O.D. 450–490, and 185 cm^{-1} mM^{-1} for cytochrome b_5 at O.D. 424–411 [14]. Protein was determined according to Lowry *et al.* [15].

Hepatic microsomal cytochrome P-450 in the scup and mice used here had a reduced, CO-ligated absorption maximum at 450 nm in each case. NADH-reduced cytochrome b_5 in both species had absorption maxima in the Soret region at 424 nm and minima at 411 nm. Levels of cytochrome P-450 in these mice were comparable to some [16] but lower than other [17] values reported previously for female mice. Levels of AP demethylase were also comparable [17] but BP hydroxylase activity was lower [18].

Expressed on the basis of mg of microsomal protein, the levels of cytochrome b_5, NADH- and NADPH-cytochrome *c* reductases in scup (Table 1) were about 20 per cent those in mouse, but scup cytochrome P-450 was present in comparatively greater amounts (about 50 per cent that in mouse). This level was somewhat greater than those in some other marine fish, which have about 20–30 per cent the specific content of P-450 found in mammals [3]. AP demethylase activity was 70–80 per cent lower in the scup when expressed per unit of microsomal protein (Table 1) and less than 5 per cent that in mouse when normalized to body weight, 26 ± 5 units/body vs 838 ± 297 units/g body in the mouse. Estimated turnover number in scup, 332 ± 51 units/nmole of P-450, was half that in mouse, 700 ± 200 units/nmole of P-450.

In sharp contrast to the results with AP demethylase, BP hydroxylase activity was almost ten times greater in scup than in mouse (Table 1), and the estimated turnover number was almost twenty times greater in scup, 1207 ± 100 units/nmole of P-450 as compared to 61 ± 18 units/nmole of P-450. Yet, normalized to body weight, this activity was alike in both scup and mouse (about 75–90 pmoles/min/g body wt). Thus, in spite of the differences based on microsomal protein, it would seem that the relative ability to metabolize BP was quite similar between the two species, even considering the reduced rate of activity in scup when assayed at habitat rather than optimal temperature (unpublished information).

The highly active hepatic microsomal BP hydroxylase in scup was inhibited strongly (80–90 per cent) by 10^{-4} M 7,8-BF (Table 2), while in mice this activity was only moderately inhibited by the same concentration of benzoflavone. Such

Table 2. Inhibition of scup and mouse hepatic microsomal benzo[a]pyrene hydroxylase by 7,8-benzoflavone

7,8-Benzoflavone concn (M)	% Activity remaining: Scup held (N = 15)	Scup freshly caught (N = 9)	Mouse (N = 3)
0	100*	100*	100*
1.25×10^{-7}	95 ± 5†	91 ± 12	96 ± 3
5.0×10^{-6}	70 ± 4	80 ± 21	71 ± 7
1.0×10^{-4}	17 ± 2	17 ± 2	63 ± 5

*Range of 100 per cent activities was 550–700 units · mg $protein^{-1}$ for scup, and 65–95 units · mg $protein^{-1}$ for mouse.
† All values are means ± S. D.

Table 1. Hepatic microsomal electron transport components and mixed-function oxygenases in scup and mouse*

Component	Scup (N)	Mouse (N)
Liver wt/body wt (%)	1.01 ± 0.10† (58)	5.24 ± 0.29 (5)
Microsomal protein (mg/g liver)	12.4 ± 0.5 (50)	20.0 ± 0.32 (5)
Cytochrome P-450 (nmoles · mg $prot^{-1}$)	0.62 ± 0.08 (30)	1.14 ± 0.12 (5)
Cytochrome b_5 (nmoles · mg $prot^{-1}$)	0.06 ± 0.02 (11)	0.33 ± 0.03 (3)
NADPH-cyt *c* reductase‡ (units · mg $prot^{-1}$)	107 ± 5 (23)	510 ± 9 (3)
NADH-cyt *c* reductase‡ (units · mg $prot^{-1}$)	183 ± 6 (10)	913 ± 59 (3)
Aminopyrine demethylase§ (units · mg $prot^{-1}$)	206 ± 34 (35)	800 ± 151 (3)
Benzo[a]pyrene hydroxylase‖ (units · mg $prot^{-1}$)	693 ± 40 (39)	72 ± 14 (4)

*Data from ♂ and ♀ fish were pooled as no significant differences were seen in any of these parameters.
† All values are means ± S. E. M.
‡ Units are nmoles cytochrome *c* reduced/min.
§ Units are nmoles HCHO produced, normalized to 1 hr.
‖ Units are pmoles 3-OH-benzo[a]pyrene equivalents produced/min.

inhibition of scup BP hydroxylase was characteristic of those fish which had been held in aquarium systems and also of fish which were assayed immediately after capture. Although typically high, levels of BP hydroxylase of less than 200 m-moles/min/mg have been observed in both freshly caught and held fish. However, even in these cases the activity was inhibited more than 80 per cent by 10^{-4} M 7,8-BF. These results indicate clearly that chemical or other factors which might differ between the aquarium and natural environments were not involved in the appearance of 7,8-BF inhibition.

The appearance of high BP hydroxylase activity in untreated scup is not a feature linked to generally high mixed-function oxygenase activity, as AP demethylase was quite low in these animals. Likewise, the differences in BP hydroxylase activity between scup and mouse are not apparently related to levels of cytochrome reductase or cytochrome b_5. Rather, the higher levels of BP hydroxylase in scup are probably due to a complement of cytochrome P-450 which catalyzes this reaction with greater efficiency, as suggested by the differential response to 7,8-BF and the high estimated turnover number in scup.

BP hydroxylase activity that is strongly inhibited by 10^{-4} M 7,8-BF is often a characteristic of cytochrome P-450 (cytochrome P-448) induced in mammals by polynuclear aromatic hydrocarbons such as 3-methylcholanthrene (3-MC) [19]. Thus, microsomal cytochrome(s) P-450 from untreated scup resembles at least catalytically, cytochrome(s) P-448 induced in certain mammals. Unlike the cytochromes in such mammals, however, the cytochrome P-450 complement in scup had a CO-bound peak of absorption close to 450 nm, rather than 448 nm. A condition similar to that in scup has also been observed in untreated brown trout, which had high BP hydroxylase activity that was sensitive to 7,8-BF inhibition [7]. This, along with results of substrate binding [8], suggested that cytochrome P-450 in that species had certain characteristics like the mammalian cytochrome P-448. In contrast to scup or trout, BP hydroxylase from little skate (*Raja erincea*), an elasmobranch fish, was sensitive to 7,8-BF inhibition only after induction by 3-MC [1].

In marine sediments, there are low levels of many organic compounds, including polynuclear aromatic hydrocarbons [20]. It is possible that scup are very sensitive to such compounds and that the amounts present in waters above coastal sediments were sufficient to induce synthesis of a cytochrome P-450 sensitive to 7,8-BF in the fish we examined. Similarly, the food of these fish, whether captive or not, may have contained foreign compounds sufficient to cause induction. These scup then, while undergoing no experimental treatment, may be showing the effects of induction by incidental exposure to environmental chemicals, and the high BP hydroxylase activity and sensitivity to 7,8-BF may not be characteristic of an uninduced state. We cannot yet distinguish, however, between this possibility and that of a constitutive cytochrome P-450 complement that is particularly sensitive to 7,8-BF. Varied experimental treatment of scup and examination of different populations of scup and other species from varied environments should aid in understanding this situation.

In summary, hepatic microsomal BP hydroxylase in the marine fish scup was about 10-fold more active than in mice. Levels of microsomal cytochromes, cytochrome reductases and AP demethylase were much lower in scup. The high BP hydroxylase in scup was strongly inhibited by 10^{-4} M 7,8-BF, and this did not differ between fish freshly collected and those held for months in aquaria. The data suggest that some marine fish may normally have cytochrome(s) P-450 catalytically resembling cytochrome(s) P-448 in mammals.

Acknowledgements—This research was supported in part by NSF Grant OCE76-84415(IDOE) and by Sea Grant 04-6-158-44016. We thank Albert Sherman for technical help, Charles Wheeler, N.M.F.S. Aquarium, Woods Hole, for cooperation in maintaining fish, and Albert Szent-Györgyi, Marine Biological Laboratory, Woods Hole, for providing the mice. Contribution number 4217 from the Woods Hole Oceanographic Institution.

Department of Biology,
Woods Hole Oceanographic Institution,
Woods Hole, MA 02543, *U.S.A.*

JOHN J. STEGEMAN
ROBERT L. BINDER

REFERENCES

1. J. R. Bend, M. O. James and P. M. Dansette, *Ann. N.Y. Acad. Sci.* **298**, 505 (1977).
2. M. Chevion, J. J. Stegeman, J. Peisach and W. E. Blumberg, *Life Sci.* **20**, 895 (1977).
3. R. J. Pohl, J. R. Bend, A. M. Guarino and J. R. Fouts, *Drug Metab. Dispos.* **2**, 545 (1974).
4. C. H. Walker, *Drug Metab. Rev.* **7**, 295 (1978).
5. U. Lidman, L. Förlin, O. Molander and G. Axelson, *Acta pharmac. tox.* **39**, 252 (1976).
6. J. F. Payne and W. R. Penrose, *Bull. envir. Contam. Toxic.* **14**, 112 (1975).
7. J. T. Ahokas, O. Pelkonen and N. T. Kärki, *Biochem. biophys. Res. Commun.* **63**, 635 (1975).
8. J. T. Ahokas, O. Pelkonen and N. T. Kärki, *Cancer Res.* **37**, 3737 (1977).
9. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
10. J. J. Stegeman, *J. Fish Res. Bd. Can.* **35**, 668 (1978).
11. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **243**, 6242 (1968).
12. I. Nash, *Biochem. J.* **55**, 416 (1953).
13. J. Cochin and J. Axelrod, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
14. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2379 (1964).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
16. A. P. Vandenberg, J. Noordhoek, E. M. Savenije-Chapel and E. Koopman-Kool, *Chem. Biol. Interact.* **19**, 185 (1977).
17. C. L. Litterst, B. I. Sikic, E. G. Mimnaugh, A. M. Gaurino and T. E. Gram, *Life Sci.* **22**, 1723 (1978).
18. J. E. Gielen, F. M. Goujon and D. W. Nebert, *J. biol. Chem.* **247**, 1125 (1972).
19. F. J. Weibel, J. C. Leutz, L. Diamond and H. V. Gelboin, *Archs Biochem. Biophys.* **144**, 78 (1971).
20. R. A. Hites, R. E. Laflamme and J. W. Farrington, *Science* **198**, 829 (1977).