Biochemical Pharmacology, Vol. 27, pp. 123–125. Pergamon Press. 1978. Printed in Great Britain.

Comparative inhibition of biosynthetic processes in rat liver testicular cells by D-, L- and DL-methadone

(*Received* 28 *December* 1976; *accepted* 18 *March* 1977)

Methadone as a mixture of two optical isomers is used clinically in the maintenance program for the treatment of persons formerly dependent on heroin. Many studies *in vivo* and *in vitro* confirm that the analgesic activity of the L-isomer is up to fifty times higher than that of the D-isomer [1–5]. According to clinical reports, DL-methadone has many side effects (e.g. decreased sexual interest, impaired secondary sex organ function, impotency and constipation) [6–9] which may include reduced plasma testosterone levels in humans and in animals [10–14]. In mice it has been shown that methadone caused regressive changes in male sex accessory organs and testes [15], with increases in cAMP in the prostate [15]. Moreover, when male rats were treated with various doses of methadone shortly before mating, there was a significant increase in the neonatal mortality of the offspring [16, 17].

Even though racemic methadone is a potent narcotic which is taken in large doses over a prolonged period of time, little has been reported on the comparative cellular and biochemical effects of the racemate and of the separated optical isomers. The present paper presents comparative data on the effects of DL-, L- and D-methadone on various metabolic and biosynthetic activities in rat testicular cell suspensions *in vitro*. Spermatogenesis, with its high metabolic activity, rapid cell differentiation and heterogenous population of cells, offers a good system for the study of such drug effects *in vivo* as well as *in vitro* [18–27].

Suspensions of spermatogonial cells were prepared from two adult rat testes stripped of the tunica and chopped. The chopped tissue was suspended in Krebs–Ringer phosphate buffer, pH 7.4, with 10 mM glucose. After allowing the mixture to settle for 3 min, the cell suspension was separated from the sediment by decantation. An aliquot of the suspension (15–20 mg protein/vessel) was used for incubation [27]. After 60 min of incubation the contents of each vessel were centrifuged; the sediment was washed with cold buffer, and then homogenized in cold 10% TCA in a Potter–Elvehjem homogenizer. Fractionation and determination of the radioactivity in various materials formed in the cells were determined as previously described [27]. Statistical significance was analyzed by Student's *t*-test with the accepted level of significance being P values

Table 1. Effects of DL-methadone on L-[1-^{14}C]leucine (A), [2-^{14}C]uridine (B) and [$^{14}CH_3$]thymidine (C) metabolism and incorporation in rat testicular cell suspension*

DL-Methadone (μM)	Per cent of control (dis./min/mg protein)					
(A) L-[1-^{14}C]leucine as substrate						
	CO_2		Soluble fraction		Protein	
10	85 ± 1†		91 ± 3		69 ± 9†	
50	79 ± 5†		92 ± 2		46 ± 11†	
100	70 ± 3†		112 ± 4		30 ± 3†	
(B) [2-^{14}C]uridine as substrate						
	Soluble fraction	RNA	U	UR	UMP	UDP + UTP
10	88 ± 2	74 ± 3†	96 ± 5	94 ± 2	73 ± 2†	67 ± 6†
50	94 ± 4	66 ± 7†	104 ± 2	105 ± 2	67 ± 3†	63 ± 8†
80	97 ± 4	47 ± 1†	107 ± 2	114 ± 6	69 ± 3†	56 ± 5†
100	88 ± 2	39 ± 4†	99 ± 1	109 ± 2	52 ± 1†	35 ± 3†
(C) [$^{14}CH_3$]thymidine as substrate						
	Soluble fraction	DNA	T	TR	TMP	TDP + TTP
10	98 ± 2	93 ± 6	98 ± 2	98 ± 2	98 ± 2	98 ± 2
50	96 ± 1	72 ± 6†	112 ± 10	96 ± 1	96 ± 1	72 ± 2†
100	91 ± 1	62 ± 8†	91 ± 1	93 ± 1	91 ± 1	69 ± 1†

* Testicular cell suspension (15–20 mg protein/vessel) was incubated for 60 min at 37° in 3 ml Krebs–Ringer phosphate buffer, pH 7.4, with 10 mM glucose and 0.33 μCi L-[1-^{14}C]leucine (3.33 μCi/μmole), 0.33 μCi [2-^{14}C]uridine (20 μCi/μmole) or 1 μCi [$^{14}CH_3$]thymidine (59 μCi/μmole) used as labeled substrate. Control values in dis./min/mg of protein were: (A) CO_2 2628 ± 86; soluble fraction 2358 ± 82; protein 681 ± 6; (B) soluble fraction 1005 ± 78; RNA 126 ± 5; U 341 ± 26; UR 335 ± 29; UMP 127 ± 9; UDP + UTP 204 ± 19; μl O_2/mg protein 5.3 ± 0.2; (C) soluble fraction 1778 ± 38; DNA 39 ± 3; T 101 ± 3; TR 1539 ± 28; TMP 80 ± 4; TDP + TTP 57 ± 4; and μl O_2/mg of protein 4.0 ± 0.02. Oxygen uptake was not affected by these concentrations of methadone.

† $P < 0.02$, compared with matched controls; means ± S. D., N = 3.

Table 2. Relative inhibition of incorporation of radioactivity in testicular cell suspension assays by separate and combined isomers of methadone

Methadone	Concn (μM)	Per cent of the inhibition given by the D- + L-mixture: [^{14}C]leucine into protein	[^{14}C]uridine into RNA	[^{14}C]thymidine into DNA
L-	50	61.5 ± 6.3*	95.2 ± 2.2*	48 ± 22*
D-	50	60.1 ± 5.1*	91.5 ± 2.9*	57 ± 19*
D- + L-	25 + 25	100 ± 4.9 (N = 9)	100 ± 1* (N = 6)	100 ± 19 (N = 3)
L-	100	90.8 ± 4.0*	81.6 ± 1.9*	
D-	100	60.9 ± 5.3*	92.7 ± 4.2*	
D- + L-	50 + 50	100 ± 2.3 (N = 6)	100 ± 3.4 (N = 3)	

* Indicates a significant difference ($P < 0.02$) between the inhibition given by this single isomer and that given by the D- + L-methadone. Since the figures in this table are for relative inhibition, a figure of less than 100 means less inhibition than shown by the D- + L-mixture.

of less than 0.02. The data in the tables were obtained from experiments with eight different testicular cell preparations.

Table 1 shows that racemic methadone inhibits in a dose-related fashion the decarboxylation of L-[1-^{14}C]-leucine and its incorporation into the protein of testicular cells. The inhibition was highly significant even at 10 μM methadone. The oxygen uptake and radioactivity in the low molecular cellular fraction were not affected, indicating that defective transport of the radioactive substrate into the intracellular pool was not the cause of the diminished incorporation of radioactivity into the protein (Table 1).

Similar dose-related inhibitory effects of racemic methadone were apparent on both RNA and DNA synthesis, as measured by the incorporation of labeled [2-^{14}C]-uridine and [$^{14}CH_3$]thymidine, respectively, although the inhibition of DNA synthesis seemed to be less than that of RNA synthesis (Table 1). Chromatographic separation of the radioactive components in the acid-soluble low molecular fractions showed dose-related, significant decreases in the amount of label in the uridine nucleotides and in TDP and TTP (Table 1).

Some experiments *in vivo* [1, 2] indicate an apparent synergistic interaction between the two isomers of methadone, resulting in a greater than expected toxicity for the receiving mixture. The relative inhibitory effects of the separate isomers on testicular cell metabolism was, therefore, compared with that of a one-to-one mixture of the isomers (Table 2). In each experiment, parallel assays were run with the same testicular cell suspension in the presence of equimolar concentrations of the separate or combined isomers and under control conditions, that is with no methadone present. The inhibitions obtained with the DL-mixtures in these experiments were similar in kind and extent to those indicated in Table 1. In these parallel experiments, however, the DL-mixture was always significantly more inhibitory than either of the separate isomers at the same molar concentration (Table 2). There was no significant difference between the inhibition given by the L-isomer as compared with the D-isomer at 50 μM, but at 100 μM, the inhibitions given by these two isomers were significantly different from one another.

Protein synthesis has been shown to occur in all stages of spermatogenesis except in mature spermatozoa, while most RNA and DNA biosynthesis occurs in the elongated spermatids and spermatogonia respectively [25, 26, 28]. Since RNA synthesis and protein synthesis in the testis are essential for steroidogenesis [29], the direct effects of methadone on these biosynthetic processes may be related to the reported decrease of testosterone levels and related sexual problems [6–14].

The dose of DL-methadone which brought about significant inhibitions in macromolecular cellular biosynthetic processes in these experiments *in vitro* is of the same order of magnitude as the concentrations of methadone and its metabolites found post-mortem in human blood; even higher levels are found in bile, urine and some tissues [30]. In similar experiments *in vitro*, 25–50 times these doses of morphine were required to produce comparable inhibitions on protein and/or nucleic acid synthesis [31].

The exact mechanism by which methadone affects intracellular metabolism is not yet known. Present results indicate an interference with the synthesis of nucleic acids, especially in the formation of phosphorylated nucleotides. The decreased protein synthesis may be a direct effect and/or a result of the decreased RNA. At concentrations up to 100 μM of DL-methadone and under comparable experimental conditions, ATP levels were practically unaffected.

The results obtained in the experiments *in vitro* comparing the L- and D-isomers with the racemic mixtures are consistent with the reports of studies *in vivo*, indicating that DL-methadone is more toxic than the separate isomers [1, 2]. Moreover, they indicate that the D-isomer is comparable to the L-isomer in its intracellular toxic effects, although it is clearly less active in the pharmacological action on central nervous system (CNS) receptor sites [1–5]. These findings, therefore, strongly suggest the introduction for pharmacological use of only the active *l*-isomer. It should have as good as or better pharmacological activity than the racemic mixture [32], but with far less intracellular toxic effects at effective clinical dose levels. This may be particularly important during pregnancy [33], in order to minimize some of the toxic effects on the fetus of methadone maintenance therapy [34–37].

Judson *et al.* [32] recently reported that L-methadone was no better than the *dl*-isomer at reducing clinical side effects. Their concern, however, was with short-term, subjectively obvious effects rather than the type of possible cellular damage suggested by our results.

D-Methadone, which is without CNS effects, might be explored as a cytostatic agent since it is now known to have inhibitory effects on cellular biosynthetic processes in spermatogenic cells (Tables 1 and 2), and atrophic effects on the prostate, seminal vesicles and testes of animals [15].

Acknowledgements—We thank E. Sutherland for technical assistance, and Dr. R. J. Hosley from the Lilly Res. Lab., Indianapolis, IN, U.S.A., for samples of D- and L-methadone HCl. The DL-methadone HCl was purchased from

May & Baker Ltd., Canada. This work was supported by a grant from the Federal Health and Welfare Department.

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Biochemical Pharmacology, Vol. 27, pp. 125–127. Pergamon Press, 1978. Printed in Great Britain.

Clofibrate-induced alterations in zinc, iron and copper metabolism*

(*Received* 4 *February* 1977; *accepted* 19 *April* 1977)

Clofibrate (*p*-chlorophenoxyisobutyrate) is generally considered an antihyperlipidemic agent; however, it has also been demonstrated recently to alter serum protein patterns [1]. Since a number of serum proteins bind or transport trace metals, we studied the effects of various levels of dietary clofibrate on plasma zinc, iron and copper and some of the proteins associated with these elements. We also measured the liver and muscle content of zinc, iron and copper to assess if clofibrate altered their concentration.

METHODS

Clofibrate was obtained from Dr. George Brice (Ayerst Laboratories, New York, U.S.A.). Male, Fisher–Dunning rats weighing 175–255 g (Microbiological Associates, Walkersville, MD, U.S.A.) were housed in a room maintained at 22–24° and lighted from 6:00 a.m. to 6:00 p.m. The rats

* In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.