Biochemical Pharmacology, Vol. 20, pp. 2049–2055. Pergamon Press, 1971. Printed in Great Britain

STIMULATORY EFFECT OF 6-AMINOCHRYSENE AND CONGENERS ON ZOXAZOLAMINE METABOLISM

M. G. DONELLI and S. GARATTINI

Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy and European Organization for Research on Treatment of Cancer (E.O.R.T.C.)

(*Received* 8 *October* 1970; *accepted* 2 *January* 1971)

Abstract—6-Aminochrysene (6AC) and a series of derivatives, which are selective inhibitors of the growth of a spontaneous mammary carcinoma in mice and a rhabdomyosarcoma in rats, antagonize the muscular paralysis induced by zoxazolamine in Sprague–Dawley male rats. This effect is shown to be maximal 24 hr after treatment and decreases at longer intervals. The route of administration of both drugs does not appear to be important.

Measurements of blood and brain zoxazolamine in rats pretreated with 6AC indicate lower levels of the compound as compared with controls. Furthermore *in vitro* experiments show that the 9000 *g* liver fraction of 6AC pretreated rats metabolize zoxazolamine twice as rapidly as controls.

On the contrary, 6AC pretreatment does not reduce the duration of paralysis induced by carisoprodol, the sleeping time by pentobarbital, the convulsions or the toxicity by strychnine. Moreover, the *in vitro* metabolism of two microsomal substrates, p-NO_2 anisol and aniline, is not affected by 6AC.

These findings may well account for the strong specific inhibition of 6AC on zoxazolamine induced paralysis and are consistent with the hypothesis that 6AC resembles polycyclic hydrocarbons in its stimulating activity on hepatic microsomal enzymes.

6-AMINOCHRYSENE (6AC) and a series of derivatives are non-carcinogenic polycyclic hydrocarbons which have a selective inhibitory effect on the growth of a spontaneous mammary carcinoma in mice[1,2] and a rhabdomyosarcoma in rats.[3]

6AC is a lipid soluble compound which is poorly absorbed from the gastrointestinal tract or the peritoneal cavity (G. Franchi unpublished results). It is practically devoid of specific pharmacodynamic activities with the exception of an antagonistic effect in respect to the muscular paralysis induced by zoxazolamine. Buu-Hoi *et al.*[4] describing this effect suggested that 6AC could be an inducer of liver microsomal oxygenases responsible for the hydroxylation of zoxazolamine to a metabolite devoid of paralyzing activity. This paper gives evidence that 6AC and some congeners are powerful agents in increasing the metabolism of zoxazolamine and it suggests that its activity is similar to other polycyclic hydrocarbons.

MATERIALS AND METHODS

Groups of eight Sprague–Dawley male rats (150 ± 20 g body wt.) were used. The animals had free access to food (Diet Alal 56 of Alal, Milan) and water during the experiments. All animals were kept at a room temperature of 22° with a relative humidity of 60% in cages of Makrolon (47 × 25 × 15 cm for six rats). 6AC and its derivatives *N*(6-chrysenyl)carbamic acid, ethylester (EORTC No. 118), 6-chryseneaminomethanesulphinic acid, sodium salt (EORTC No. 334), *N*-[bis(beta-hydroxyethyl)aminoacetyl]-6-chryseneamine (EORTC No. 499) were kindly supplied to the

E.O.R.T.C. by Continental Pharma, Bruxelles and zoxazolamine by McNeil Labs. Inc., Washington, U.S.A. For the intraperitoneal injection, 6AC, its derivatives and zoxazolamine were suspended in 1% carboxymethylcellulose. For the intravenous injection, both zoxazolamine and 6AC were dissolved in a solvent containing 70% H_2O, 25% dimethylsulphoxide and 5% Tween 80 (v/v).

1. *Pharmacological endpoints*

The duration of paralysis induced by zoxazolamine and carisoprodol was evaluated by means of a rotatory bar (9 cm diameter, 2 rev./min).

The sleeping time induced by pentobarbital was determined by following the loss of the righting reflex.

The toxicity of strychnine was evaluated by determining the onset of convulsions and mortality.

2. In vivo *metabolism*

The *in vivo* metabolism was investigated by determining the rate of disappearance of zoxazolamine from blood and brain. Blood and tissue levels of zoxazolamine were determined according to the spectrophotometric method of Juchau *et al.*[5]

3. In vitro *metabolism*

The livers were removed immediately after sacrifice and homogenized with 4 vol. of ice-cold 1·15% KCl solution in a Potter type Teflon-homogenizer.

The homogenates were centrifuged at 9000 *g* for 20 min and the supernatant fraction was used.

Three ml of 9000 *g* supernatant equivalent to 600 mg of liver were mixed with 2 ml of solution containing NADP (1·5 μM), glucose-6 phosphate (50 μM), magnesium chloride (25 μM), nicotinamide (100 μM) potassium phosphate buffer (280 μM, pH 7·4) and the substrate represented by *p*-nitroanisol (3 μM), aniline (5 μM) or zoxazolamine (3 μM).

The mixture was incubated at 37° for 30 min in the case of p-NO_2 anisol and aniline, and for 60 min in the case of zoxazolamine.

Hydroxylation of zoxazolamine was determined by measuring the disappearance of the substrate according to the method of Juchau *et al.*[5] *O*-Demethylation of p-NO_2 anisol and parahydroxylation of aniline were determined by measuring the formation of para-nitro-phenol and para-aminophenol respectively, according to the method of Gilbert and Goldberg.[6] Protein determinations were performed according to Lowry *et al.*[7]

RESULTS

1. *The duration of zoxazolamine paralysis in rats pretreated with 6AC or derivatives*

Table 1 shows that a pretreatment with 6AC or its derivatives considerably decreases the duration of paralysis induced by zoxazolamine.

Phenobarbital, a classical inducer of microsomal enzymes,[8] given at a higher dose was less effective than 6AC or some of its congeners.

The inhibiting effect of 6AC pretreatment on zoxazolamine paralysis is maximal when zoxazolamine is administered 24 hr after 6AC and diminishes with longer pretreatment intervals. This is evident from the data reported in Table 2.

TABLE 1. DURATION OF ZOXAZOLAMINE INDUCED PARALYSIS IN RATS PRETREATED WITH 6AC AND SOME DERIVATIVES

Treatment	Dose (mg/kg/i.p.)	Duration of paralysis (min ± S.E.)	(% decrease)	P
Controls	—	238 ± 18	—	—
6AC	20	64 ± 5	73	<0·001
GECA 118	20	35 ± 2	85	<0·001
GECA 334	20	32 ± 3	86	<0·001
GECA 499	20	113 ± 14	53	<0·001
Phenobarbital	80	105 ± 17	56	<0·001

6AC and its derivatives were suspended in carboxymethylcellulose and administered intraperitoneally 24 hr before zoxazolamine.

Zoxazolamine was suspended in carboxymethylcellulose and administered intraperitoneally at the dose of 90 mg/kg.

Phenobarbital was administered intraperitoneally at the dose of 80 mg/kg daily for 2 days starting 72 hr before zoxazolamine.

TABLE 2. DURATION OF ZOXAZOLAMINE INDUCED PARALYSIS IN RATS AT DIFFERENT INTERVALS AFTER 6AC

Treatment (mg/kg/i.p.)	Time between 6AC and zoxazolamine (hr)	Duration of paralysis (min ± S.E.)	(% decrease)
Controls	—	238 ± 18	—
6AC 5	24	106 ± 3	55
	48	135 ± 6	43
6AC 20	24	56 ± 5	77
	48	81 ± 12	66
	72	166 ± 10	30
6AC 50	24	45 ± 2	81
	48	73 ± 4	70

6AC and zoxazolamine (90 mg/kg) were suspended in carboxymethylcellulose and administered intraperitoneally.

Since both compounds 6AC and zoxazolamine were injected intraperitoneally it is possible that 6AC impaired in some way the absorption of zoxazolamine. Therefore two experiments were performed in which either zoxazolamine or 6AC were injected intravenously. The results shown in Table 3 indicate that in both cases 6AC is effective

TABLE 3. INFLUENCE OF THE ROUTE OF ADMINISTRATION ON THE DURATION OF ZOXAZOLAMINE INDUCED PARALYSIS IN RATS PRETREATED WITH 6AC

6AC dose (mg/kg)	Zoxazolamine dose (mg/kg)	Duration of paralysis (min ± S.E.)	(% decrease)	P
—	50 i.v.	114 ± 24	—	
20 i.p.	50 i.v.	18 ± 2	84	<0·001
—	90 i.p.	232 ± 26	—	
10 i.v.	90 i.p.	63 ± 12	73	<0·001

For the intravenous injection the dose in mg/kg of both drugs was dissolved in 10 ml of a solution containing 70% H_2O, 25% dimethylsulphoxide, and 5% Tween 80 (v/v). Time between 6AC and zoxazolamine was 24 hr.

in reducing zoxazolamine paralysis when administered 24 hr before zoxazolamine, independently on the route of administration.

2. In vivo *metabolism of zoxazolamine in normal and 6AC pretreated rats*

Since an interference with the rate of metabolism of zoxazolamine may be involved in the effect described above, determinations of zoxazolamine were performed in brain and plasma of control and 6AC pretreated rats.

Table 4 shows that brain and plasma levels of zoxazolamine are significantly lower in 6AC pretreated animals than in controls. However, at the end of paralysis the brain level of zoxazolamine is the same in normal and 6AC pretreated rats thus suggesting that only a metabolic difference is involved whereas the sensitivity to zoxazolamine appears to be unchanged.

TABLE 4. BRAIN AND PLASMA LEVELS OF ZOXAZOLAMINE (90 mg/kg i.p.) IN NORMAL AND 6AC PRETREATED RATS

Treatment	Time after treatment with zoxazolamine (min)	Zoxazolamine (μg/g or ml $\pm$ S.E.) Brain	Plasma
Controls	20	115·6 $\pm$ 4·3	31·0 $\pm$ 0·9
	40	76·7 $\pm$ 9·3	30·3 $\pm$ 1·1
	60	84·2 $\pm$ 5·0	25·4 $\pm$ 1·5
	120	58·4 $\pm$ 6·0	19·8 $\pm$ 0·2
6AC	20	97·2 $\pm$ 4·4*	33·8 $\pm$ 1·3
	40	79·8 $\pm$ 5·7	25·1 $\pm$ 1·8*
	60	36·9 $\pm$ 9·5*	11·1 $\pm$ 2·1*
	120	8·8 $\pm$ 4·6*	4·1 $\pm$ 0·3*

* $P < 0{\cdot}01$.

6AC was suspended in carboxymethylcellulose and administered at the dose of 20 mg/kg i.p. 24 hr before zoxazolamine.

3. In vitro *metabolism of zoxazolamine in normal and 6AC pretreated rats*

An *in vitro* experiment gives further evidence that 6AC stimulates the hepatic metabolism of zoxazolamine.

Table 5 reports the disappearance of zoxazolamine incubated with 9000 *g* liver fraction of normal and 6AC pretreated rats. It may be seen that the livers of 6AC pretreated rats metabolize zoxazolamine twice as much as controls. In order to investigate whether 6AC, like phenobarbital,[8] increases the total amount of liver proteins, the protein content of the 9000 *g* fraction of rats pretreated with 20 and 50 mg/kg of 6AC has been determined.

Table 6 shows that the total protein content was significantly decreased in the liver of 6AC pretreated rats up to at least 48 hr after treatment.

4. *Effect of 6AC on the activity of other drugs*

Since the administration of compounds that stimulate the activity of the liver microsomal enzymes markedly decrease the duration of action of drugs which are metabolized by this enzymatic system, the pharmacological effect or the toxicity of some of these drugs after 6AC pretreatment has been investigated.

TABLE 5. ZOXAZOLAMINE METABOLISM IN THE LIVER (9000 *g* fraction) OF 6AC PRETREATED RATS

Treatment	Zoxazolamine (μg metabolized/60 min ± S.E.)	
	per g of liver	per 100 mg of liver proteins
Controls	161 ± 22	78 ± 10
6AC	332 ± 21*	177 ± 11*

* $P < 0.01$.

330 mg of rat liver (9000 *g* fraction) were incubated at 37° with 500 μg zoxazolamine.

6AC was dissolved in 70% H_2O, 25% dimethylsulphoxide and 5% Tween 80, and administered intraperitoneally at the dose of 20 mg/kg 24 hr before the determination of the enzymatic activity.

TABLE 6. 9000 *g* LIVER PROTEIN CONCENTRATION IN RATS PRETREATED WITH 6AC

Treatment	Dose (mg/kg)	Time after treatment (hr)	Liver weight (g ± S.E.)	Protein concentration (mg/g liver ± S.E.)	P
Controls	—	—	7·6 ± 0·4	147·2 ± 4·5	—
6AC	20	24	7·7 ± 0·3	123·3 ± 3·0	<0·01
		48	7·5 ± 0·3	130·7 ± 3·5	<0·05
6AC	50	24	7·5 ± 0·4	131·5 ± 2·9	<0·05
		48	8·7 ± 0·5	124·0 ± 2·2	<0·01

6AC was suspended in carboxymethylcellulose and given i.p.

Table 7 shows that the duration of carisoprodol paralysis and pentobarbital narcosis do not decrease with a pretreatment of 20 mg/kg of 6AC. On the contrary, the convulsions and the toxicity induced by strychnine were markedly increased. To eliminate the possibility that substrates other than zoxazolamine could need a different time or dose of 6AC pretreatment to be stimulated in their biotransformation and thus inhibited in their pharmacological effect, 6AC was administered with different schedules of treatment before determining pentobarbital narcosis (see Table 8).

TABLE 7. INFLUENCE OF 6AC ON THE PHARMACOLOGICAL EFFECT OF OTHER DRUGS

	Controls	6AC	P
Duration of carisoprodol paralysis (250 mg/kg/i.p.) min ± S.E.	134 ± 14	188 ± 32	>0·05
Duration of pentobarbital narcosis (35 mg/kg/i.p.) min ± S.E.	97 ± 10	114 ± 4	>0·05
Toxicity of strychnine (1·8 mg/kg/i.p.) % convulsions	55	77	>0·05
% mortality	11	55	<0·02

6AC (20 mg/kg/i.p.) suspended in carboxymethylcellulose was administered 24 hr before the injection of carisoprodol, pentobarbital or strychnine.

TABLE 8. PENTOBARBITAL NARCOSIS IN 6AC PRETREATED RATS

Treatment	Dose (mg/kg)	Time between 6AC and pentobarbital (hr)	Animals in narcosis (%)	Sleeping time (min ± S.E.)	P
Controls	—	—	84	53 ± 1	
6AC	2 i.p.	24	87	58 ± 4	>0·05
	5 i.p.	24	75	48 ± 5	>0·05
	10 i.p.	24	87	52 ± 2	>0·05
	20 i.p.	24	100	56 ± 7	>0·05
	20 i.p.	48	100	62 ± 2	<0·05
	50 i.p.	24	100	93 ± 4	<0·01
	50 i.p.	48	100	72 ± 6	<0·02
	50 orally	48	75	72 ± 3	<0·01

6AC was suspended in carboxymethylcellulose.
Pentobarbital was administered intraperitoneally at the dose of 25 mg/kg.

It can be seen that 6AC in none of the reported experimental conditions reduces pentobarbital narcosis, while at the dose of 50 mg/kg injected either intraperitoneally or orally, 24 or 48 hr before pentobarbital, results in a significant increase in the duration of narcosis.

Other data, not reported here in detail, show that EORTC 118 and EORTC 334 at a dose of 20 mg/kg i.p. given 24 hr before pentobarbital (25 mg/kg i.p.) do not change the sleeping time induced by this barbiturate either.

5. *Effect of 6AC on the* in vitro *metabolism of* p-*nitroanisol and aniline*

Table 9 shows the results obtained in an *in vitro* experiment, where the 9000 *g* liver fraction was incubated with two substrates *p*-nitroanisol and aniline which are commonly used for testing the activity of the liver microsomal enzymes. It can be seen that in 6AC pretreated rats there is practically no change in the production of the respective metabolites as compared to control animals.

TABLE 9. LIVER METABOLIC ACTIVITY (9000 *g* fraction) OF RATS PRETREATED WITH 6AC

	p-NO_2 Anisol (mμM metabolized/30 min ± S.E.)		Aniline (mμM metabolized/30 min ± S.E.)	
Treatment	per g liver	per 100 mg proteins	per g liver	per 100 mg proteins
Controls	647·6 ± 17	316·4 ± 12·1	980·9 ± 59·8	479·3 ± 31·3
6AC	734·4 ± 34·1	391·8 ± 19·9	1045·5 ± 50·6	552·7 ± 31·9

6AC was dissolved in 70% H_2O, 25% dimethylsuphoxide and 5% Tween 80 and administered intravenously at the dose of 20 mg/kg.
3 μM of p-NO_2 anisol and 5 μM of aniline were incubated at 37° with 600 mg liver (9000 *g* fraction) in the conditions described under methods.

DISCUSSION

The results of the present investigation confirm the finding of Buu-Hoi *et al.*[4] that 6AC given 24 hr before zoxazolamine decreases the paralyzing activity of this compound. The effect is maximal when the time interval between 6AC and zoxazolamine

is 24 hr and it tends to decrease at longer intervals. The activity of 6AC is already evident at 5 mg/kg and it is proportional to the administered dose. The route of administration (intraperitoneal or intravenous) of the two drugs has no importance in the appearance of this effect. The pharmacological observations are justified by the levels of zoxazolamine in brain and plasma which at comparable times are considerably lower in 6AC pretreated rats than in control animals. Furthermore at the end of the paralysis the brain levels of zoxazolamine were similar in both groups suggesting that the sensitivity to zoxazolamine was not changed by 6AC pretreatment.

The more rapid disappearance of brain and plasma zoxazolamine in 6AC pretreated rats relative to controls is explained by the higher rate of metabolism of this agent when incubated with fortified 9000 *g* liver fraction of 6AC pretreated rats. This effect is present when the rate of metabolism is expressed per gram of liver or per 100 mg of liver proteins.

In contrast to these findings 6AC pretreatment does not reduce the duration of paralysis induced by carisoprodol, the sleeping time by pentobarbital, the convulsions or the toxicity by strychnine. In fact, at given schedules of treatment 6AC prolongs the sleeping time induced by pentobarbital. Furthermore *in vitro* *O*-demethylation of *p*-nitroanisol or *p*-hydroxylation of aniline are not affected by 6AC.

The results obtained are consistent with the hypothesis that 6AC resembles in its inducing activity for liver microsomal enzymes hydrocarbons more than phenobarbital.[8–10] Whether this effect of 6AC is linked to its chemotherapeutic action on mammary carcinoma and rhabdomyosarcoma[1–3] remains to be established.

Acknowledgements—This work was supported by contracts from Euratom No. 079–69–1 BIAC and from C.N.R. No. 115/1134/0 5033. The authors are grateful to Miss Teresa Polese for her skilful technical assistance.

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