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1-PENTYL-3-(4-AMINOPHENYL)PYRROLIDINE-2,5-DIONE, A SELECTIVE AROMATASE INHIBITOR: *IN VIVO* STUDIES

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1-Pentyl, 1-hexyl and 1-heptyl-3-(4-aminophenyl)pyrrolidine-2,5-dione, potent inhibitors of aromatase, lower oestrogen levels in PMSG-stimulated female rats in a comparable manner to the inhibitor aminoglutethimide (AG) used clinically for the treatment of breast cancer. Pharmacokinetic studies in the rat show $t_{1/2}$ values for the 1-hexyl compound and AG of 1.8 and 5.5 h respectively. In 4 tests for CNS-depressant activity the overall order of activity was AG > 1-heptyl = 1-hexyl ≫ 1-pentyl. The 1-pentyl compound has less tendency than AG to depress white cell and platelet counts in mice and overall is the drug candidate for further studies.

KEY WORDS: Aromatase inhibitor, 1-pentyl-3-(4-aminophenyl)pyrrolidine-2,5-dione, breast cancer.

INTRODUCTION

After surgical removal of tumour tissue, aminoglutethimide (AG) is used clinically[1] as a second line drug to reduce oestrogen levels in postmenopausal women with breast cancer, so as to remove the hormonal stimulus to renewed tumour growth in the breast or in metastases. AG, initially introduced as an anti-epileptic drug, exerts its action by competitive, reversible inhibition of peripheral aromatase, the enzyme catalysing the final step in the biosynthesis of oestrogens from adrenal androstenedione.[2] The success of AG in the clinic may also depend on its ability to reduce oestrogen sulphate conversion to oestradiol by increasing its clearance.[3]

Aminoglutethimide has several undesirable clinical features:[2,4,5] (1) it is a non selective inhibitor of aromatase and inhibits other cytochrome P-450 enzymes, notably the cholesterol side chain cleavage enzyme (CSCC) leading to decreased hydrocortisone levels; to combat this action it is administered with adjuvant hydrocortisone, (2) it possessed CNS-depressant activity as a result of its anti-epileptic action which, although temporary with continued administration of the drug, can lead to patient non-compliance due to lethargy and ataxia, (3) it is a liver cytochrome P-450 inducing agent so inducing its own metabolism and that of other drugs on chronic

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administration, (4) in a small number of patients blood dyscrasias (c. 1%) and skin rashes occur. Consequently work has been directed at producing an aromatase inhibitor which is a selective inhibitor of aromatase with a reduced effect on other steroidogenic pathway cytochrome P-450 enzymes and devoid of the undesirable CNS-depressant action of AG or other clinically apparent side effects. Several such agents have been described, some of which are in clinical trials, e.g., the non-steroidal compounds CGS 16949,[6] CGS 20267,[7] R 76713[8] (and its enantiomers), pyridoglutethimide[9] and benzofuran-2-ylphenylmethyl-imidazoles[10] and the steroidal compounds 4-hydroxyandrostenedione[11] and PED.[12]

We have described[13] 3-(4-aminophenyl)pyrrolidine-2,5-dione (WSP-3) as an aromatase inhibitor comparable in potency with AG *in vitro* with no effect on CSCC. In *in vivo* studies it lowers the level of oestradiol in pregnant mares' serum gonadotrophin (PMSG)-treated rats efficiently but less effectively than AG, has little CNS-dependent action in various tests in mice (see later)[14] and does not induce a blood dyscrasia in mice.[15] In a previous paper[16] we showed that the 1-pentyl-, 1-hexyl- and 1-heptyl derivatives of WSP-3 have enhanced potency *in vitro* compared to AG and are far more selective in their action as shown by a greater inhibitory potency ratio, aromatase:CSCC. Furthermore, *in vitro* studies showed that the 1-alkyl chains were more stable to metabolism by rat liver microsomes than found[17] in a comparable well studied AG analogue, 1-octyl pyridoglutethimide. Here we describe the *in vivo* characteristics of these three compounds in animal experiments.

MATERIALS AND METHODS

Statistical analysis was performed using the "two-tailed Student's *t*-test".

Pharmacokinetics

Groups of female rats (8–12 weeks old, 180–220 g) were injected with the test compound (25 mg/kg ip) in a mixture of carboxymethylcellulose (1%) and Tween 80 (0.1%) and blood taken by cardiac puncture at predetermined intervals. Two series of experiments were conducted: (1) short term studies with all 3 pyrrolidine-2,5-diones with sampling at 15, 30, 45, 60, 75, 90, 120 and 360 min for the 1-pentyl and 1-hexyl compounds and at 15, 30, 45, 60, 75, 90, 120 and 240 min for the 1-heptyl compound ($n = 5$). (2) longer term studies with the 1-hexyl compound and AG, with sampling at 3, 4, 5, 6 and 7 h ($n = 6$).

The results for (1) are shown in Figures 1A–1C and for (2) in Figures 2A–2B. The samples (0.5 ml plasma) were analysed by HPLC for the test compounds as previously described[16] with the following modifications: *1-pentyl*; extraction, dichloromethane (5 ml); mobile phase, acetonitrile-water (48:52); internal standard, 1-hexyl derivative. *1-hexyl*; chloroform (7 ml); acetonitrile-water (52:48); 1-heptyl derivative. *1-heptyl*; dichloromethane (5 ml); acetonitrile-water (50:50); 1-pentyl derivative. *AG*; dichloromethane (5 ml); methanol-water (38:62), flow rate, 1.3 ml/min; 1-propyl aminoglutethimide. Standards were prepared in bovine plasma and a "response" factor calculated for "area under the curve" (AUC) ratios for standard/internal standard. The concentration of drug in samples was obtained from AUC × "response" factor.

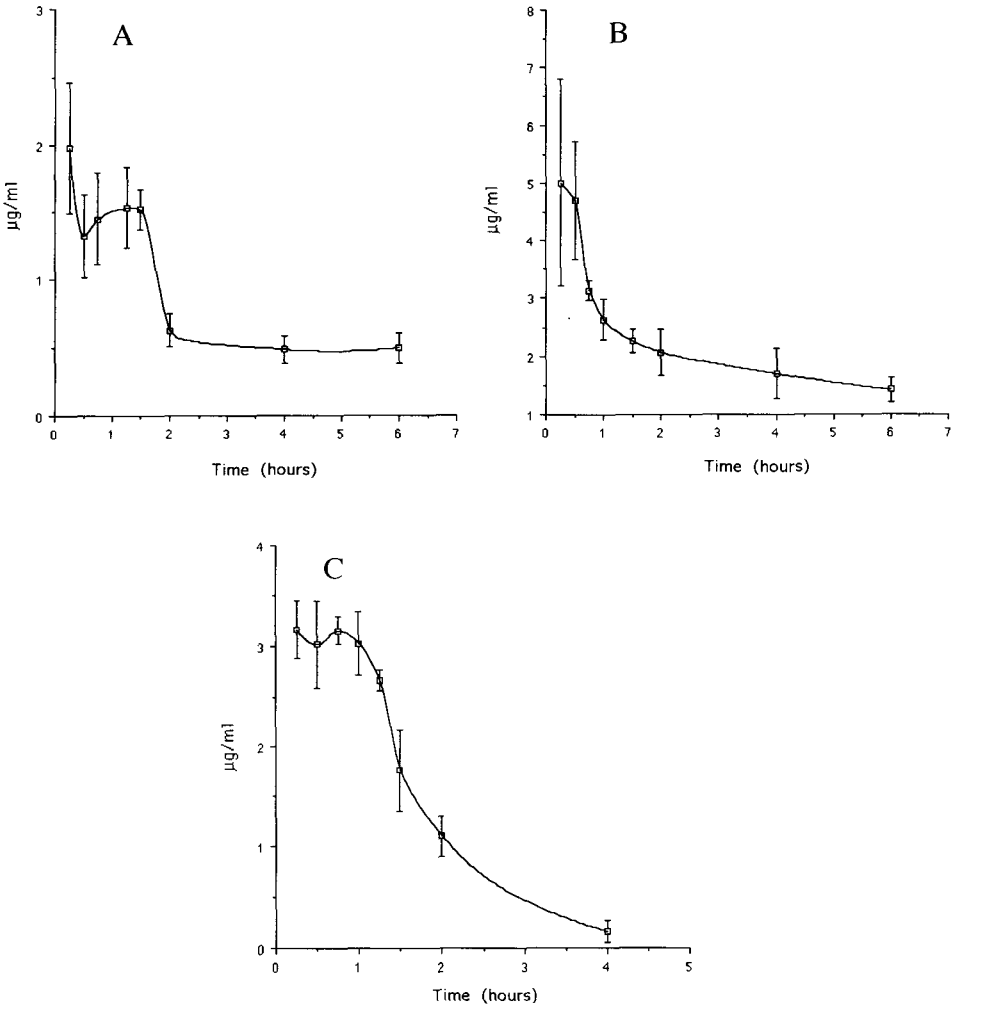

Figure 1 Plasma concentration-time profile for the compounds in female rats following i.p. administration of a single 25 mg/kg dose. (A) 1-pentyl, (B) 1-hexyl, (C) 1-heptyl. Points represent the mean ($n = 3-5$) ± S.E.M.

Reduction of Oestradiol (E_2) Levels by 1-Alkyl Compounds and AG

Groups of six female rats (200–250 g) were pre-treated with pregnant mares' serum gonadotrophin (PMSG; 100 i.u., s.c.) and on the following fifth day the test compound (25, 50 or 100 µmole.kg) was injected i.p. in the usual vehicle. Blood was obtained by cardiac puncture 3 h later from 6 rats at each dose level and the serum oestradiol determined with a radioimmunoassay kit (IDS Ltd.) The results are shown in Table 1 and are expressed as a percentage decrease in E_2 levels compared with a control group which was administered vehicle only.

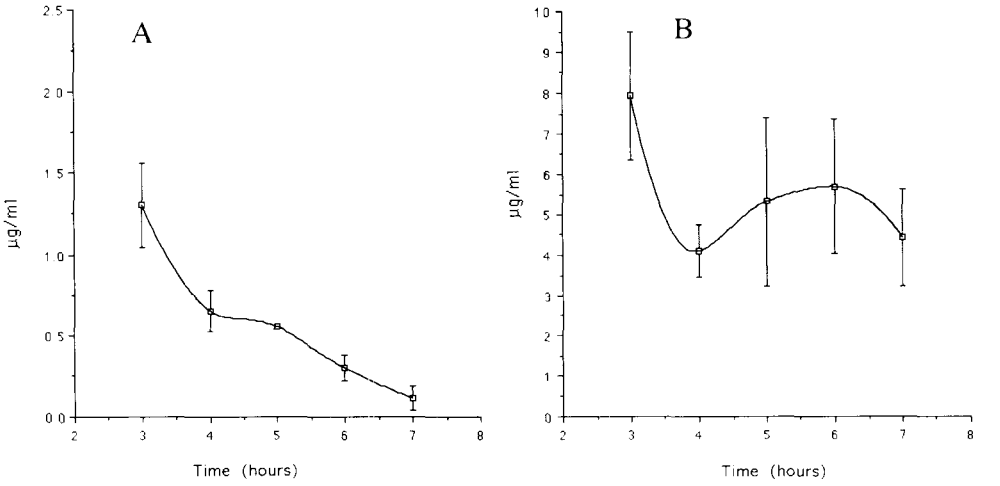

Figure 2 Plasma concentration-time profile for compounds in female rats over 3–7 h following a single i.p. dose of 25 mg/kg. (A) 1-hexyl, (B) AG. Points represent the mean ($n=6$) ± S.E.M.

Table 1 Reduction in oestradiol levels in the PMSG-treated female rat on administration of certain 1-Alkyl-3-(4-aminophenyl)pyrrolidine-2,5-diones and AG

Dose (μmole/kg)	Mean reduction (%) in E_2 level			
	1-Pentyl	1-Hexyl*	1-Heptyl*	AG
25	64.1 ± 8.2	78.7 ± 9.4	ND	74.7 ± 7.5
50	74.0 ± 9.1	76.5 ± 4.8	ND	95.5 ± 1.5
100	81.7 ± 4.3	86.9 ± 4.3	88.0 ± 3.4	94.1 ± 3.2

*Used as hydrochloride salts. ND, not determined.

CNS Depressant Activity

Hypnotic action

In this test[18] the period of the loss of the righting reflex (sleeping time) was recorded in female mice receiving the compounds intraperitoneally (400 mg/kg). Below this dose none of the compounds was able to abolish the righting reflex. The results are summarised in Table 2.

Prolongation of barbitone-induced hypnosis

In this test[19] the sleeping time in female mice receiving barbitone sodium (200 mg/kg, i.p.) was determined. Either vehicle (controls) or compound was administered i.p. 20 min before barbitone injection. The results are summarised in Table 3.

Rotarod performance

This test[20] measures the time(s) for which a trained animal can balance on a rod rotating in a controlled programmed manner. On the test day the performance of female mice on the rotating rod was determined before and 30 min after either the compounds or the vehicle i.p. The results are summarised in Table 4.

Locomotor activity

This test[21] measures the spontaneous movement of pairs of female mice in an activity cage in which they have been acclimatised. Locomotor activity is assessed as the number of interruptions of infra red light beams arranged at standard points across the cage. Measurements were made 30 min after an i.p. injection of either vehicle (controls) or compound. Pairs of animals were monitored for 30 min, six pairs being used in each group. The results are summarised in Table 5.

Induction

In this test[22] the duration of action (period of absence of righting reflex) of pentobarbitone (50 mg/kg, i.p.) and zoxazolamine (120 mg/kg, i.p.) in female mice are measured. For both compounds duration of action is determined by their rates of

Table 2 Hypnotic action of test compounds (i.p.) on the righting reflex of female mice

	No. not losing righting reflex	Sleeping time (min) (mean* ± SD)
AG	0	25.3 ± 5.6
Heptyl	1	14.5 ± 6.7
Hexyl	2	8.9 ± 5.7
Pentyl	3	2.8 ± 2.4

*$n = 10$

Table 3 Effect on prolongation of the barbitone-induced sleeping time of female mice by test compounds (i.p.)

Sleeping time (min) (**mean ± SD)

		Compound		
	Control	50	100	150 mg/kg
AG	28 ± 0.5	42 ± 0.6*	66 ± 0.6*	88 ± 0.6*
(% of control)	–	(150)	(236)	(314)
Heptyl	44 ± 0.4	44 ± 0.4	65 ± 0.7*	80 ± 0.5*
(% of control)	–	(100)	(148)	(182)
Hexyl	31 ± 0.9	44 ± 0.4*	64 ± 0.3*	70 ± 0.3*
(% of control)	–	(142)	(206)	(226)
Pentyl	36 ± 0.3	40 ± 0.8	51 ± 0.7*	66 ± 0.5*
(% of control)	–	(111)	(142)	(183)

*$P < 0.05$ compared with controls. **$n = 10$.

Table 4 Effect on rotarod performance of female mice of test compounds (i.p.)

	Rotarod performance (s)			
	Control	Compound 75 mg/kg	Control	Compound 100 mg/kg
AG	698 ± 120	375 ± 80*	755 ± 61	357 ± 76*
(% of control)	–	(54)	–	(47)
Heptyl	478 ± 67	443 ± 71	820 ± 28	276 ± 75*
(% of control)	–	(93)	–	(34)
Hexyl	491 ± 69	391 ± 82	865 ± 24	293 ± 90*
(% of control)	–	(80)	–	(34)
Pentyl	662 ± 96	530 ± 115	835 ± 25	669 ± 94
(% of control)	–	(80)	–	(80)

*P < 0.05 compared with control value. Values are mean ± S.E.M., $n = 10$.

Table 5 Effect on locomotor activity of female mice by the test compounds (i.p.)

	Locomotor activity (light beam interruptions/30 min)			
	Control	Compound 75 mg/kg	Control	Compound 100 mg/kg
AG	279 ± 46	144 ± 31	104 ± 13	24 ± 5*
(% of control)	–	(52)	–	(23)
Pentyl	252 ± 41	192 ± 58	127 ± 2	87 ± 4*
(% of control)	–	(76)	–	(69)
Hexyl	216 ± 38	160 ± 42	99 ± 7	28 ± 2*
(% of control)	–	(74)	–	(28)
Heptyl	447 ± 50	272 ± 49	107 ± 2	31 ± 2*
(% of control)	–	(61)	–	(29)

*P < 0.05 compared with controls. Values are mean ± S.E.M., $n = 6$ pairs.

Table 6 Induction of pentobarbitone and zoxazolamine metabolism by 1-alkyl-3(4′aminophenyl)-pyrrolidine-2,5-diones and AG

	Duration (min) of absence of righting reflex					
	Pentobarbitone			Zoxazolamine		
		Dose (mg/kg)			Dose (mg/kg)	
Compound	Control	50	100 ($n = 5$)	Control	50	100 ($n = 10$)
AG	16.8 ± 2.1	7.2 ± 1.3*	4.0 ± 1.2*	35.3 ± 1.0	9.2 ± 0.7*	5.9 ± 1.2*
1-Pentyl	33.3 ± 2.1	30.5 ± 1.2	29.6 ± 2.2	35.3 ± 1.0	26.3 ± 2.7*	17.4 ± 1.2*
1-Hexyl	33.3 ± 2.1	30.0 ± 2.6	21.6 ± 1.0*	42.7 ± 0.4	35.4 ± 0.6*	27.8 ± 2.1*
1-Heptyl	33.3 ± 2.1	19.0 ± 1.1*	14.1 ± 1.0*	42.7 ± 0.4	27.4 ± 5.6*	23.2 ± 3.9*

*P < 0.05 compared with control value. Values are mean ± S.E.M.

Table 7 Haemotoxicity effects of 1-pentyl-3-(4'-amino-phenyl)-pyrrolidinedione and AG

	Control	AG	1-Pentyl
Wbc $10^3/mm^3$	8.4±0.6	4.3±0.5*	6.5±0.6
Platelets $10^3/mm^3$	2070±110	1690±160*	1805±220

*$P<0.05$. Values are mean±S.E.M., $n=10$.

metabolism. AG and the other compounds were given i.p. daily for 4 days. On day 5, the animals received either pentobarbitone or zoxazolamine. The results are summarised in Table 6.

Haemotoxicity

AG and the pentyl compound were suspended in 0.1% carboxymethyl cellulose and administered orally to groups of female mice ($n=5$) at a daily dose of 50 mg/kg for 3 weeks. On the day following cessation of dosing, the blood picture of each animal was determined from duplicate samples. The results are summarised in Table 7.

RESULTS AND DISCUSSION

The metabolism of AG has been most extensively characterized in humans and in the rat. In the rat, the pattern of metabolism differs qualitatively and quantitatively to that in man. The most notable difference concerns the extent of N-acetylation. The rat appears to first acetylate aminoglutethimide and then further metabolize it,[23] whereas in man, it appears that AG is metabolized by several pathways other than by acetylation.[24,25] The rat is therefore not an ideal model for the study of the elimination of arylamine compounds in humans. However, as a readily available laboratory animal whose oestrogen levels respond well to priming with gonadotrophins, it provides a convenient system for studying the relationship between circulating inhibitor and hormone concentrations.

Pharmacokinetic studies were conducted on the 1-pentyl, 1-hexyl and 1-heptyl compounds in female rats with i.p. injection. In the short term studies, with blood sampling carried out frequently after initial dosing and for 2–3 h (Figure 1A–1C), drug levels decreased rapidly over the 3 h period and were down to less than half the initial levels within 2 h. Longer term studies on the 1-hexyl and, by comparison, AG to remove the distribution factor from the analysis showed that the drug plasma levels were about 6 times higher for AG than the 1-hexyl compound after 3 h and the plasma half lives were 5.5 and 1.8 h respectively (Figure 2A–2B). Previous values in these laboratories for the half life of AG vary from 2.8 to 6.5 h.

These results suggest that whereas the 1-hexyl compound is more readily metabolised *in vivo* in the rat than AG, in contrast to the liver microsomal studies where N-acetylation is absent, sufficiently high levels of the drug are available to implement its advantages of higher potency and selectivity than AG. Furthermore

the 1-hexyl compound is more stable *in vivo* than 1-octylpyridoglutethimide which is not detectable[1,2] in rat plasma after 2 h (i.v.) or after initial 15 min sampling (i.p.).

All three 1-alkyl derivatives showed the ability to lower oestrogen levels in PMSG-treated female rats and compared well with AG (Table 1). The 1-hexyl compound was marginally the most potent of the three derivatives and although less effective than AG at the lower doses they all produced a useful pharmacological effect.

The test for hypnotic action showed that all three compounds were significantly ($P < 0.05$) less CNS depressant than AG, the order of activity being AG > 1-heptyl > 1-hexyl > 1-pentyl in the ratio 9:5.2:3.2:1 respectively (Table 2).

In the prolongation of barbitone effect test the 1-heptyl and 1-pentyl showed no significant activity at 50 mg/kg (Table 3). At the higher dose all three compounds had an effect which was less marked than AG. At 100 and 150 mg/kg the 1-heptyl compound appeared to be the most active of the three compounds.

In the rotarod performance test, the 1-pentyl compound was not active at either dose used, whereas the 1-heptyl and 1-hexyl had the same order of activity as AG (Table 4).

In the test for locomotor activity, none of the compounds tested at 75 mg/kg induced significant effects (Table 5). At a dose of 100 mg/kg the 1-hexyl, 1-heptyl and AG had about the same activity which was reduced for the 1-pentyl compound.

In the four tests conducted to compare the CNS depressant activity of the three compounds with AG the 1-pentyl compound showed significantly lowered activity in all the tests whereas the 1-hexyl and 1-heptyl showed a similar but reduced activity in half the tests. The overall order of CNS depressant activity for the four compounds is AG > 1-heptyl ≡ 1-hexyl ≫ 1-pentyl.

AG is an inducing agent and increases the rate of metabolism of other drugs as well as its own. Studies on the effect of the three compounds and AG on the righting reflex of female mice dosed with either pentobarbitone or zoxazolamine (Table 6) show that AG produces a marked reduction in response to the two "probe" compounds indicating a strong inducing action. Although the other compounds have a shortening effect on the responses, it is very much less and appears generally at only a higher dose than that of AG. Of the three compounds the pentyl appears to have the lowest potential for inducing activity.

In breast cancer patients, AG treatment has given rise to a relatively high incidence (c. 1%) of blood dyscrasias. Using the mouse, we have found whereas there were no changes in either red cell count or haemoglobin concentration, AG (but not the pentyl compound) caused a significant ($P < 0.05$) reduction in white cell and platelet count (Table 7). Thus the pentyl compound appears to have a lower potential than AG for inducing blood dyscrasias.

All three compounds studied could be considered as prospective candidate aromatase inhibitors for the treatment of breast cancer in postmenopausal women since: (1) they are potent inhibitors of aromatase with selectivity towards this enzyme and little associated CSCC activity which would obviate adjuvant hydrocortisone administration necessary with AG, (2) they effectively lower plasma oestrogen levels in PMSG-treated rats due to satisfactory metabolic profiles, and (3) they have less CNS depressant activity than AG, a feature which is pronounced for the 1-pentyl compound. However, the 1-pentyl compound stands out from the others because of its lower CNS depressant activity and lower potential for blood dyscrasias.

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