Biochemical Pharmacology, Vol. 28, pp. 3589–3593

0006-2952/79/1215-3589 $02.00/0

A POSSIBLE ASSOCIATION BETWEEN MEMBRANE-FLUIDIZING PROPERTIES AND PORPHYRIN-INDUCING ACTIVITY OF DRUGS*

IAN R. NEILSON, FREDERICK T. CHAYKOWSKI, MICHAEL A. SINGER and GERALD S. MARKS

Departments of Pharmacology and Medicine, Queen's University, Kingston, Ontario, Canada

(*Received* 14 *March* 1979; *accepted* 11 *June* 1979)

Abstract—In order for a drug to induce porphyrin accumulation it must be lipophilic and possess an uncharged polar group. Similar properties are required for maximum fluidization of phospholipid bilayers, suggesting that porphyrin-inducing drugs may act by inducing transitions in membrane lipids. For this reason a series of lipophilic analogues of adamantane and benzene, which have an uncharged polar group and had been shown previously to fluidize phospholipid bilayers, were tested for porphyrin-inducing activity in chick embryo liver cells. All the analogues induced porphyrin accumulation, thus supporting the concept that porphyrin-inducing drugs might act by inducing transitions in membrane lipids.

Previous studies indicate that a wide variety of chemicals of "diverse" structure can induce δ-aminolevulinic acid (ALA) synthetase activity and porphyrin accumulation in a chick embryo hepatic cell culture system [1]. Despite their diverse structures, these chemicals have several properties in common. First, these agents possess a certain degree of lipophilicity and are resistant to metabolism to compounds of lower lipophilicity. Second, they lack a net charge but contain a polar substituent [2, 3]. It has been demonstrated recently that small molecules possessing these same features, i.e. a certain degree of lipophilicity and a polar substituent, cause a much greater disorganization of phospholipid bilayer membranes than analogues lacking these factors [4–7]. This similarity in structural requirements for both porphyrin-inducing activity and optimal "fluidization" of phospholipid bilayers suggested to us the possibility that the action of porphyrin-inducing chemicals was exerted, at least in part, on a membrane and was a consequence of an increase in membrane fluidity. As a first step in testing this idea, we selected a series of uncharged lipophilic molecules containing a polar group and known to "fluidize" a model bilayer membrane and measured their porphyrin-inducing activity. If our theory was correct, we would find porphyrin-inducing activity in this group of compounds.

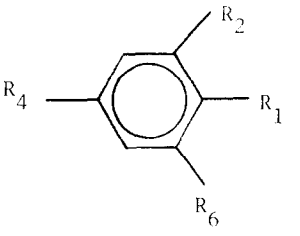
METHODS

Source of compounds. Adamantane and benzene derivatives were purchased from the Aldrich Chemical Co., Milwaukee, WI. Allyisopropylacetamide was a gift from Hoffmann La Roche, Montreal, Que. Waymouth MD 705/1 medium was purchased in powder form from the Grand Island Biological Co., Grand Island, NY. Insulin (bovine pancreas, 24 I.U./mg), L-thyroxine sodium pentahydrate (T_4), bovine serum albumin (fraction V powder), bovine serum albumin (crystallized and lyophilized, for protein standard), collagenase (type I, 460 NF units/mg), penicillin G sodium, and streptomycin sulfate were purchased from the Sigma Chemical Co., St. Louis, MO. Folin and Ciocalteau phenol reagent was purchased from Fisher Scientific, Ottawa, Ont.

Cell culture technique. The details of the cell culture technique have been described previously [8, 9]. The cells were maintained in 6-cm diameter disposable plastic Petri dishes (Falcon Plastics, Oxnard, CA) containing 5 ml of Waymouth MD 705/1 medium supplemented with 60 mg penicillin G, 100 mg streptomycin sulfate, 1.0 mg insulin and 1.0 mg T_4 per litre. After an initial incubation period of 24 hr, the medium was discarded and replaced with fresh medium. Chemicals, dissolved in 95% ethanol (10 μl), unless indicated otherwise, were added to the cell cultures, and the dishes were reincubated. Porphyrin content of cells and medium and protein content of cells were measured quantitatively 24 hr later [1]. Results are expressed as ng porphyrins/mg of protein. For the assay of ALA-synthetase, the cells were maintained in 10-cm diameter dishes containing 15 ml of the medium. Chemicals were dissolved in 95% ethanol (30 μl). ALA-synthetase activity was measured 24 hr later [10]. Results are expressed as nmoles ALA/100 mg of protein/hr.

Membrane fluidity data. The relative effectiveness of these various compounds to perturb a lipid bilayer was taken from previously published data [6, 7]. The word "fluidizing" as used in this paper refers to the capacity of a given molecule to increase phospholipid fatty acyl chain motion at a temperature below the normal transition temperature of that phospholipid.

* Supported by the Medical Research Council of Canada.



	Derivative	R_1	R_2	R_4	R_6
(a)	para-t-butylbenzyl alcohol	CH_2OH	H	$C(CH_3)_3$	H
(b)	para-t-butylphenol	OH	H	$C(CH_3)_3$	H
(c)	2,6-di-t-butyl-4-methylphenol	OH	$C(CH_3)_3$	CH_3	$C(CH_3)_3$

Fig. 1. Chemical structure of various adamantane analogues.

RESULTS AND DISCUSSION

Three lipophilic benzene derivatives containing polar groups were selected for this study, viz. para-t-butylbenzyl alcohol (Fig. 1a), para-t-butylphenol (Fig. 1b) and 2,6-di-t-butyl-4-methylphenol (Fig. 1c). All three of these benzene analogues cause significant fluidization in the model bilayer system [6, 7]. These three compounds were found to induce porphyrin biosynthesis in the chick embryo liver cell culture (Table 1). A full dose–response curve of porphyrin-inducing activity could not be conducted because of the toxicity of the phenolic compounds at high doses.

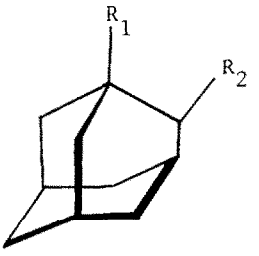
Our next studies were designed to investigate the porphyrin-inducing activity of a series of lipophilic adamantane derivatives containing a polar group. These compounds had been shown previously to fluidize phospholipid bilayers [6, 7]. Substitution of adamantane with a carbonitrile group (Fig. 2h) or a bromomethyl ketone group (Fig. 2g) yielded analogues which were potent porphyrin-inducing compounds (Table 1). A dose-related increase in porphyrin accumulation was demonstrated (Fig. 3) with 2-adamantanone (Fig. 2c), 2-adamantanol (Fig. 2b), 1-admantyl methyl ketone (Fig. 2f), 1-adamantanemethanol (Fig. 2d) and 1-admantanol (Fig. 2a; Table 1). One of the hydroxylated derivatives, 1-adamantaneethanol (Fig. 2e), was active at low doses (Table 1) but could not be tested at higher doses due to a toxic effect on the cells.

Granick [1] assumed that porphyrin accumulation represented an approximate index of ALA-synthetase activity in the cell culture. To determine whether the porphyrin accumulation observed in our studies was associated with an elevation of ALA-synthetase activity, the following experiments were performed. 1-Adamantanol (100 μg/ml) was added to the cell culture and 24 hr later ALA-synthetase activity was measured [10]. The activity of the enzyme was found

Table 1. Porphyrin-inducing activity of adamantane and benzene analogues in chick embryo liver cells*

Compound	Concentration (nmoles/ml)	Concentration (μg/ml)	Porphyrin accumulation (ng/mg protein ± S.E.M.) Treatment	Control
2,6-di-t-butyl-4-methylphenol	100	22	100.7 ± 4.6	25.7 ± 1.5
para-t-butylphenol	100	14	84.5 ± 5	25.5 ± 1
para-t-butylbenzyl alcohol	50	8	44.7 ± 7	25.5 ± 1
	150	25	93 ± 4	25.5 ± 1
	300	50	206 ± 14	25.5 ± 1
1-adamantanecarbonitrile	186	30	200.7 ± 20.3	18.1 ± 0.7
1-adamantyl bromomethyl ketone	117	30	238.8 ± 20.6	18.1 ± 0.7
1-admantanol	66	10	24.3 ± 2	25 ± 1
	197	30	42.3 ± 2.1	25 ± 1
	660	100	242.6 ± 19.2	25 ± 1
1-adamantaneethanol	55	10	44 ± 4.5	25 ± 1
	165	30	131 ± 9	25 ± 1

* All values represent the means of five determinations ± S.E.M.



	Analogue	R_1	R_2
(a)	1-adamantanol	OH	H
(b)	2-adamantanol	H	OH
(c)	2-adamantanone	H	O
(d)	1-adamantanemethanol	CH_2OH	H
(e)	1-adamantaneethanol	CH_2CH_2OH	H
(f)	1-adamantyl methyl ketone	$COCH_3$	H
(g)	1-adamantyl bromomethyl ketone	$COCH_2Br$	H
(h)	1-adamantanecarbonitrile	CN	H

Fig. 2. Chemical structure of various benzene analogues.

to be 170.9 ± 9.9 nmoles ALA/100 mg of protein/hr compared to a control value of 70.5 ± 9.7. In a similar experiment, para-t-butylbenzyl alcohol (50 μg/ml) caused an elevation of the enzyme to 110.6 ± 7.8 nmoles ALA/100 mg of protein/hr (control, 70.5 ± 9.7). Each point represents the mean of five determinations ± S.E.M. Thus, the accumulation of porphyrins is accompanied by a significant ($P < 0.05$) elevation of ALA-synthetase activity.

The potency of adamantane compounds to fluidize membranes and to induce porphyrin accumulation was studied, using a series of six adamantane analogues with similar lipophilicity and polar groups, viz. 1-adamantanol, 2-adamantanol, 2-adamantanone, 1-adamantanemethanol, 1-adamantaneethanol, and 1-adamantyl methyl ketone (Fig. 2). Membrane-fluidizing activity, based on data reported previously [6, 7], is plotted on the abscissa (Fig. 4). Activity is

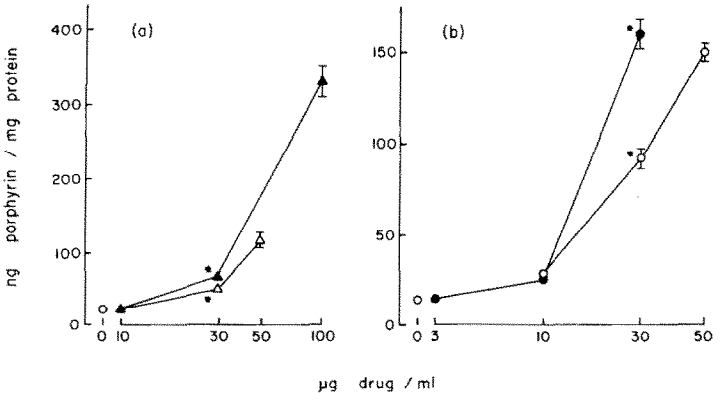


Fig. 3. Porphyrin accumulation in response to increasing doses of 2-adamantanone (▲), 2-adamantanol (△), 1-adamantyl methyl ketone (●), and 1-adamantanemethanol (○). Each point represents the mean of five determinations ± S.E.M. Asterisks denote a significant difference ($P < 0.05$) in porphyrin accumulation between dishes treated with different derivatives at the same dosage.

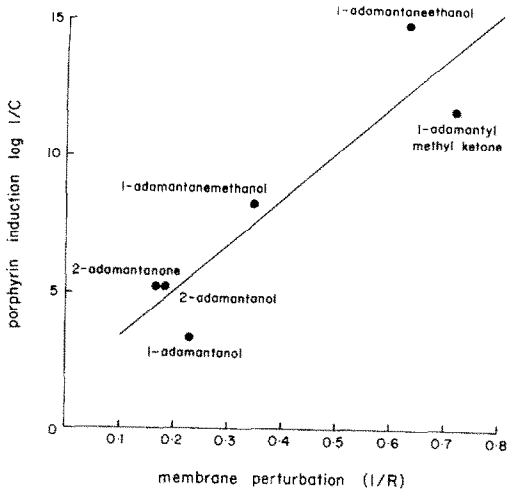


Fig. 4. Correlation of potency of compounds in perturbing membranes and in inducing porphyrin biosynthesis. Membrane-perturbing activity is expressed as the reciprocal of the value of the parameter R. R represents an empirical motion parameter that can be used to semi-quantitate phospholipid fatty acyl chain motion [6, 7]. An increase in molecular motion is characterized by a decrease in the value of R or an increase in the value of $1/R$. Porphyrin-inducing activity is expressed as the reciprocal of the millimolar concentration necessary to produce porphyrin accumulation equivalent to 3 μg/ml allylisopropylacetamide.

expressed as the reciprocal of the value of R, where R represents an empirical motion parameter that can be used to semi-quantitate phospholipid fatty acyl chain motion [6, 7]. An increase in molecular motion is characterized by a decrease in the value of R or an increase in the value of $1/R$. Porphyrin-inducing activity is plotted on the ordinate (Fig. 4). Activity is expressed as the reciprocal of the millimolar concentration needed to produce porphyrin accumulation over a 24-hr period equivalent to that produced by a standard dose of allylisopropylacetamide (3 μg/ml). As can be seen in Fig. 4, there is a correlation between the ability of compounds to fluidize liposomal membranes and the porphyrin-inducing potency of the same compounds. This correlation does not hold if 1-adamantanecarbonitrile (Fig. 2h) and 1-adamantyl bromomethyl ketone (Fig. 2g) are included in the study since these two compounds were very effective in inducing porphyrin accumulation but only moderately effective in causing membrane fluidization in the model phospholipid bilayers.

The objective of this investigation was to determine whether uncharged lipophilic adamantane and benzene derivatives containing a polar group, and *known* to fluidize phospholipid bilayers, would induce porphyrin accumulation in chick embryo liver cells. We have tested eleven analogues of adamantane and benzene with these properties and have shown that all eleven induce porphyrin accumulation. Charged benzene and adamantane analogues, as expected, were found to be inactive in inducing porphyrin accumulation. These compounds, however, are effective in fluidizing a phospholipid bilayer, indicating that not all fluidizing agents will possess porphyrin-inducing activity. Since the composition of the phospholipid bilayer used to study membrane fluidization is only a rough approximation of a hepatocyte membrane, one would not expect to obtain a strict correlation between membrane fluidization and porphyrin-inducing activity. However, our observations support an association between these two properties and suggest that the action of porphyrin-inducing drugs is exerted, at least in part, on a membrane.

A logical question is how a membrane site of action can be related to existing theories of porphyrin induction by exogenous agents. According to current ideas, there appear to be two mechanisms for the induction by chemicals of hepatic δ-aminolevulinic acid (ALA) synthetase: (1) a direct action on the nucleus to increase the amount of an induction-specific RNA for ALA-synthetase; and (2) an action to deplete a "regulating heme pool" by one of the following: (a) producing a partial block in heme biosynthesis; (b) causing destruction of the heme; or (c) increasing the synthesis of apocytochrome P-450 with increased utilization of heme for synthesis of cytochrome P-450. It has been suggested that some chemicals which induce hepatic ALA-synthetase do so by the first mechanism while others do so by the second mechanism. The most efficacious of the chemicals are considered to act by both mechanisms [11–13].

Several membranes, for example those of the nucleus and the mitochondrion, are involved in the above two mechanisms. The mitochondrial membrane could represent the membrane site of action for the following reasons. Coproporphyrinogen III is transported from its site of synthesis in cytoplasm into mitochondria for conversion to heme. Elder [14] has pointed out that it is unlikely that an anion as large as coproporphyrinogen III enters the mitochondrion passively. It is possible that a fluidizing action of a chemical on mitochondrial membranes might hinder the transport of coproporphyrinogen III into mitochondria. This action, in turn, would lead to diminished mitochondrial heme synthesis, a diminished "regulating heme pool", enhanced ALA-synthetase activity and porphyrin accumulation. A mitochondrial membrane site of action could explain the lack of activity of charged compounds which would have difficulty crossing the plasma membrane in order to gain access to this intracellular site of action. It must be emphasized that this is only one possible membrane site of action and we do not intend to imply that this is the only possible site.

REFERENCES

1. S. Granick, *J. biol. Chem.* **241**, 1359 (1966).
2. G. S. Marks, V. Krupa, F. Murphy, H. Taub and R. A. Blattel, *Ann. N.Y. Acad. Sci.* **244**, 472 (1975).
3. G. S. Marks, in *Handbook of Experimental Pharmacology* (Eds. F. De Matteis and W. N. Aldridge), Vol. 44, p. 201. Springer, New York (1978).
4. M. K. Jain, N. Y. Yu and L. V. Wray, *Nature, Lond.* **255**, 494 (1975).
5. M. K. Jain, N. Yen-Min Wu, T. K. Morgan, Jr., M. S. Briggs and R. K. Murray, Jr., *Chem. Phys. Lipids* **17**, 71 (1976).

6. F. T. Chaykowski, J. K. S. Wan and M. A. Singer, *Chem. Phys. Lipids* **23**, 111 (1979).
7. M. Singer and J. Wan, *Biochem. Pharmac.* **26**, 2259 (1977).
8. P. W. F. Fischer, R. O. Morgan, V. Krupa and G. S. Marks, *Biochem. Pharmac.* **25**, 2609 (1976).
9. R. O. Morgan, P. W. F. Fischer, J. K. Stephens and G. S. Marks, *Biochem. Pharmac.* **25**, 2609 (1976).
10. D. L. J. Tyrrell and G. S. Marks, *Biochem. Pharmac.* **21**, 2077 (1972).
11. F. De Matteis and A. H. Gibbs, *Biochem. J.* **146**, 285 (1975).
12. J. D. Maxwell and U. A. Meyer, in *Handbook of Experimental Pharmacology* (Eds. F. De Matteis and W. N. Aldridge), Vol. 44, p. 239. Springer, New York (1978).
13. P. R. Sinclair and S. Granick, *Ann. N.Y. Acad. Sci.* **244**, 509 (1975).
14. G. H. Elder, in *Handbook of Experimental Pharmacology* (Eds. F. De Matteis and W. N. Aldridge), Vol. 44, p. 157. Springer, New York (1978).