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Structural Determinants of Cationic Amphiphilic Amines Which Induce Clear Cytoplasmic Vacuoles in Cultured Cells[1] (42462)

KURT J. RORIG,*[,2] ZADOK RUBEN,† AND STEVEN N. ANDERSON‡

*Departments of *Medicinal Chemistry, †Pathology, and ‡Biological Research, G. D. Searle & Company, 4901 Searle Parkway, Skokie, Illinois 60077*

Abstract. Disobutamide (D), an antiarrhythmic cationic amphiphilic amine (CAA), was withdrawn from clinical testing when clear cytopolasmic vacuoles (CCV) were found in the rat and dog during toxicity studies. To delineate the structural determinants of amines that induce CCV, we exposed cultured rat urinary bladder carcinoma and rabbit aorta muscle cells to numerous cationic drugs and chemicals and examined cells by phase light microscopy. The cationic moiety of these CAA was responsible for the induction of CCV. The very potent inducers were compounds that had two strongly basic amine (cationic) centers. The bis tertiary amines were particularly potent inducers. Aliphatic diamines of minimal lipophilicity-induced CCV, thus showing that an "amphiphilic" structural feature, though present in many CAA drugs, is not necessary for CCV induction. The distance between the two cationic centers was irrelevant to the induction of CCV. These results support the concept that CCV are a manifestation of intracellular (e.g., intralysosomal) drug storage. These structural delineations will be useful in future drug design and for further understanding of drug–cell interactions. Based on these findings, we were able to synthesize an antiarrhythmic CAA which did not induce CCV.

Disobutamide(D),α-(*o*-chlorophenyl)-α-[2-diisopropylamino)ethyl]-γ-(1-piperidino)butyramide, is an antiarrhythmic compound (1, 2) which was withdrawn from clinical testing when clear cytoplasmic vacuoles (CCV) induced by D were found in the rat and dog during toxicity studies. CCV were present in many types of cells in the dog (3) and in the epithelium of the epididymis and choroid plexus of the rat (4). Piperamide (5), chloroquine (6), tilorone, and other tertiary amines (7) induced histologically similar CCV in epithelial cells of the rat choroid plexus. All these compounds and D are cationic amphiphilic (8) amines (CAA). The biological nature and chemical content of these CCV are unclear.

We reported the induction of similar CCV by D, in 24–72 hr, in cultured mammalian cells originating from various species (9, 10), and found rat urinary bladder carcinoma and rabbit aorta muscle cells particularly useful for investigating the dynamic nature of CCV. We also determined that the cultured rat cells incorporated ^{14}C-labeled D, and the rate of uptake paralleled the induction of CCV; thus, we speculated that CCV in cultured cells were associated with drug storage (11). If the structural determinants of CAA that induce CCV become known, drug–cell interactions may be understood better and future drugs may be designed more rationally. Thus, we undertook studies to discover these structural determinants which are reported in this paper. With the structure–activity correlates determined in this work, we were able to synthesize new cardiac antiarrhythmic agents which do not induce CCV at the high dosages used in whole animal toxicology studies.

Materials and Methods. *(A) Compounds tested (Tables I and II).* Most of the compounds we tested were synthesized in our laboratories. A few α,ω-diaminoalkanes were purchased (Aldrich Chemical Co., Milwaukee, WI) and purified by us. For all compounds, the purity and identity were established by appropriate methods such as HPLC and proton and carbon-13 NMR. The compounds were used as their hydrochlorides, acetates, or phosphates.

(B) Cell culture studies. The rat urinary bladder carcinoma cells (RBT CC-8) (12) were the gift of Dr. Bendicht U. Pauli (Rush Medical College, Chicago). The rabbit aorta muscle cells were provided by Dr. Douglas O. Fisher

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[2] To whom correspondence and reprint requests should be addressed at 337 Hager Lane, Glenview, IL 60025.

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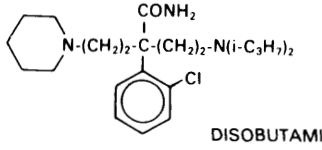
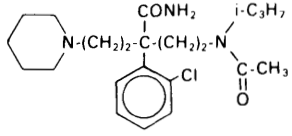
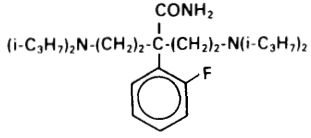
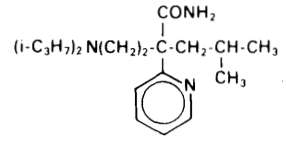
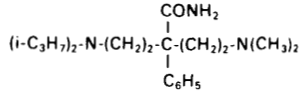
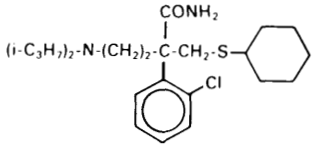
TABLE I. CATIONIC AMPHIPHILIC AMINES TESTED AND THEIR INDUCTION OF CLEAR CYTOPLASMIC VACUOLES IN CULTURED RABBIT AORTA MUSCLE AND RAT URINARY BLADDER CARCINOMA CELLS

No.	Compound structure	Rabbit cells		Rat cells		No.	Compound structure	Rabbit cells		Rat cells	
		VAC	Concn/ time	VAC	Concn/ time			VAC	Concn/ time	VAC	Concn/ time
1	 DISOBUTAMIDE	+ G	$\frac{4\times10^{-4}\,M}{24\,hr}$	+	$\frac{4\times10^{-4}\,M}{24\,hr}$	9		–		–	
2		+	$\frac{4\times10^{-4}\,M}{24\,hr}$	+	$\frac{4\times10^{-4}\,M}{24\,hr}$	10		–		–	
3		– G		+	$\frac{6\times10^{-4}\,M}{72\,hr}$	11		– G		–	

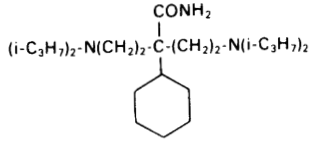
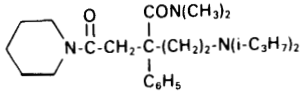
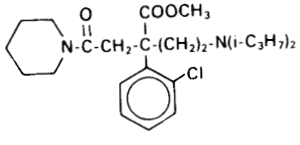
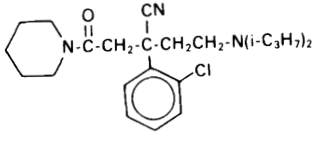
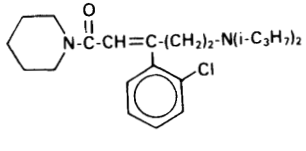
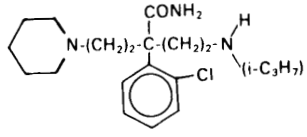
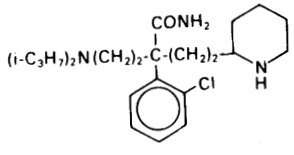
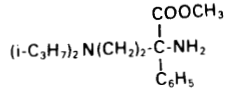
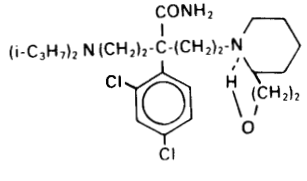
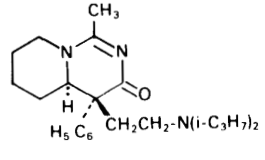
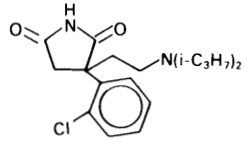
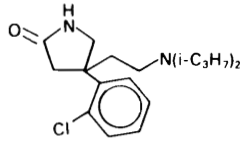
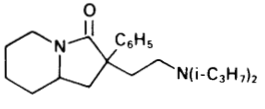
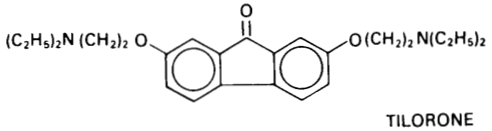
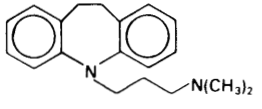
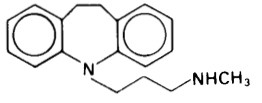
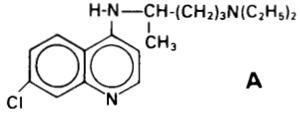
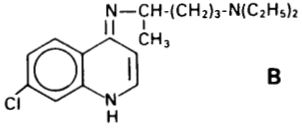
4		+	$\frac{2\times10^{-4}\,M}{24\text{ hr}}$	+	$\frac{1\times10^{-4}\,M}{24\text{ hr}}$	–	–
5		–		–		–	–
6		– G		–		– G	–
7		– G		–		– G	–
8		– G		–		–	–
12						–	–
13						– G	–
14						– G	–
15						– G	–
16						–	–

TABLE I—*Continued*

No.	Compound structure	Rabbit cells VAC	Rabbit cells Concn/time	Rat cells VAC	Rat cells Concn/time
17		–		–	
		G			
18		–		–	
		G			
19		–		–	
		G			
20	 TILORONE	+	$\frac{2\times10^{-4}\,M}{24\text{ hr}}$	+	$\frac{2\times10^{-4}\,M}{24\text{ hr}}$
		G			

No.	Compound structure	Rabbit cells VAC	Rabbit cells Concn/time	Rat cells VAC	Rat cells Concn/time
21	 IMIPRAMINE	–		–	
		G			
22	 DESIPRAMINE	–		–	
		G			
23	 A	+	$\frac{2\times10^{-5}\,M}{24\text{ hr}}$	+	$\frac{2\times10^{-5}\,M}{24\text{ hr}}$
		G			
	CHLOROQUINE				
	 B				

Note. + means CCV were present at the lowest compound concentration and at the earliest time observed; – means CCV were not present at 1×10^{-3} *M* of the compound at 72 hr incubation; G means dense cytoplasmic granules were present. Compound sources and references are: 1–4, Searle ((Ref. (1)); 5–15 and 17–19, Searle (unpublished); 16, Searle (J Med Chem **28,** 1285, 1985); 20, Richardson Merrell; 21–22, Ciba-Geigy; 23, Sterling Winthrop.

TABLE II α-ω-DIAMINES TESTED AND THEIR INDUCTION OF CLEAR CYTOPLASMIC VACUOLES IN CULTURED RABBIT AORTA MUSCLE AND RAT URINARY BLADDER CARCINOMA CELLS

No.	Compound structure	Rabbit cells		Rat cells	
		VAC	Concn/time	VAC	Concn/time
24	$(CH_3)_2$-N-$(CH_2)_2$-N$(CH_3)_2$	+	$\frac{4\times10^{-4}M}{24\,hr}$	+	$\frac{8\times10^{-4}M}{24\,hr}$
25	$(CH_3)_2$N-$(CH_2)_4$-N$(CH_3)_2$	+	$\frac{2\times10^{-4}M}{24\,hr}$	+	$\frac{4\times10^{-4}M}{24\,hr}$
26	$(CH_3)_2$N-$(CH_2)_6$-N$(CH_3)_2$	+	$\frac{2\times10^{-5}M}{24\,hr}$	+	$\frac{2\times10^{-5}M}{24\,hr}$
27	H_2N-$(CH_2)_3$-NH_2	−		−	
28	H_2N-$(CH_2)_5$-NH_2	−		−	
29	H_2N-$(CH_2)_6$-NH_2	−		−	
30	CH_3-NH-$(CH_2)_6$-NHCH_3	−		−	
31	$(C_2H_5)_2$-N-$(CH_2)_5$-NH_2	−		−	
32	$(C_2H_5)_2$-N-$(CH_2)_5$-NHC_2H_5	−		−	
33	$(CH_3)_3\overset{+}{N}$-$(CH_2)_6$-$\overset{+}{N}(CH_3)_3\cdot 2Br^-$	−		−	
34	H_2N-$(CH_2)_3$-NH-$(CH_2)_4$-NH_2	−		−	
35	H_2N$(CH_2)_3$-NH-$(CH_2)_4$-NH$(CH_2)_3$-NH_2	−		−	

Note. + means CCV were present at the lowest compound concentration and at the earliest time observed; − means CCV were not present at 1×10^{-3} *M* of the diamine at 72 hr incubation. Compound sources and references are: 24–29, Aldrich Chemical Co.; 30, Searle [Ann. **611,** 71, 78 (1958)]; 31, Searle (J Sci Ind Res. (India), **17B,** 11, 21 (1958)]; 32 Searle [J. Sci Ind Research (India), **15C,** 255–256 (1956)]; 33–35, Sigma Chemical Co.

(University of Rhode Island) and Dr. George C. Fuller (G. D. Searle & Co., Skokie). Rabbit or rat cells were plated in Lux tissue culture eight-well multiplates. Throughout the experiments, the culture medium (pH 7.2–7.4) was M-199 supplemented with 10% bovine fetal serum and 20 m*M* Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanol sulfuric acid). Incubation was in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. After initial plating and 24-hr incubation, the medium was removed and 2 ml of medium containing a test compound was added. The standard compound concentration series was 0, 1, 2, 4, 6, 8, and 10×10^{-4} *M*. If a compound caused cell death at 1×10^{-4} *M*, a similar series of concentrations at 10^{-5} *M* was used. Cells were observed with a phase light microscope *in situ* at 2, 24, 48, and 72 hr. D always was used as a positive control. Every compound was tested at least twice in each cell line.

Results. The effect of CAA compounds is presented in Table I. Compounds 2–15 were chosen for their overall chemical similarity to D. Compounds 2, 3, and 4, all strongly basic bis tertiary amines, formed CCV. Compound 4 shows that neither the halogen nor the aromatic ring of disobutamide are necessary for CCV induction. Compounds 5–9 did not induce CCV. Their common structural features are one strongly basic tertiary amine and one nonbasic amide. Compounds 10 and 11, which have only one strongly basic tertiary amine, did not induce CCV. Compounds 12–14 contain two strongly basic amino groups, but only one amino group is tertiary. An overview of the results of compounds 1–14, which are structurally similar, suggests that the lipophilic moiety of these CAA was not the determinant in CCV induction. In fact, many compounds had the same lipophilic moiety, but their induction of CCV differed.

Compound 15 is anomalous. In four trials, it was negative and in one, it induced a few CCV. Although the hydroxyethyl group reduced the pK_a by about 1.1 units, due to in-

tramolecular hydrogen bond formation between the hydroxyl and piperidino nitrogen, this moderate reduction of basicity seems insufficient to account for the absence of CCV.

Compounds 16–19 resembled D in antiarrhythmic activity, but did not induce CCV. Although compound 16 appears to have two strongly basic tertiary amine groups, titration revealed only one strongly basic center (pK_{a1} = 4.8; pK_{a2} = 10.3).

Compounds 20–23 represent drugs previously reported (6, 7). Imipramine, 21, and desipramine, 22, each of which has only one strongly basic amino function (the other amino group is aromatic; $pK_a \cong 5$), did not induce CCV. Tilorone, 20, a bis-tertiary amine, and chloroquine, 23, did induce CCV. Chloroquine has a 4-amino group that appears aromatic but, in reality, is strongly basic (pK_{a1} = 8.10; pK_{a2} = 9.9) (13). The tautomeric form, 23B, responsible for this high basicity (14), also makes this amino group a tertiary one. In summary, the results of Tables Ia and Ib show that two highly basic (measured ionization constants are pK_{a1}, 9.7–10.7, and pK_{a2}, 8.1–9.2) tertiary amino groups were required to induce CCV at the concentrations we used in our cell culture system.

Tilorone, 20, imipramine, 21, desipramine, 22, and chloroquine, 23, all induced dense cytoplasmic granules (DCG) in the rabbit cells. DCG are compatible with intralysosomal concentric lamellar polar lipidosis (8, 15), and their induction by these four CAA was not unexpected. Many of our compounds also induced DCG without CCV induction (compounds 6, 7, 8, 11, 13, 14, 15, 17, 18, and 19). We conclude that CCV and DCG induction require differing structural and physicochemical features.

In order to clarify the role of the amino functions in the induction of CCV, we examined a group of α,ω-diamines as presented in Table II. These amines had no aromatic groups and were, therefore, minimally lipophilic. We, thus, separated out the "amphiphilic" character of compounds of Table I to leave the relatively pure cationic character of compounds in Table II. Compounds 24–26 are strongly basic bis-tertiary amines which induced CCV. Compounds 27–35 are strongly basic di-, tri-, or tetraamines which lack the two tertiary amino structures. They did not induce CCV. Compounds 24–26 also showed that the distance between the two tertiary amino groups is irrelevant to CCV induction. Compound 33, with two quaternary cationic centers, probably cannot penetrate cell membranes readily and so does not induce CCV.

None of the compounds in Table II induced DCG. Thus, the results tabulated in Table II provided information on two important points: (i) confirmed that the strongly basic bis-tertiary amino structure is the determinant which induces CCV; (ii) showed that the "amphiphilic" character is not necessary for induction of CCV. The results from Table II, in conjunction with those from Table I, suggested that "amphiphilic" character and the lipophilic moiety are important for the induction of DCG.

Discussion. From this study, our related work (11, 16), and the work of Ohkuma and Poole (17), we conclude that CCV represent intracellular (e.g., intralysosomal) storage depots of protonated amines.

While Ohkuma and Poole (17) observed CCV induction in mouse peritoneal macrophages by monoamines at higher concentrations, our cell systems did not show CCV induction by monoamines at concentrations which did not cause cell death. Diamines, because of their dicationic nature and consequent greatly diminished probability of doubly deprotonating to neutral molecules (11), are more permanently trapped within the intracellular acidic compartments (e.g., lysosomes) than are monoamines. Thus, diamines produce CCV at lower culture medium concentrations than do monoamines. Tertiary diamines are particularly apt to induce CCV at lower external concentrations when compared to secondary or primary diamines. The much larger hydration shells of primary and secondary amines (as compared to tertiary amines) undoubtedly impede cytoplasmic transit and membrane crossing of these amines relative to tertiary amines. Further, the $\rangle$N–H bonds of primary and secondary amines can form hydrogen bonds with the carbonyl oxygen of peptides and carbohydrates, thereby slowing their cytoplasmic transit even more in comparison to tertiary amines which lack the $\rangle$N–H bond. Lastly, primary and secondary amines can form covalently bonded carbinolamines (Schiff bases) with cytoplasmic carbonyl compounds (sugars, etc.), which tertiary amines cannot do. Whatever combination of these "retentive forces" may operate in the cell, their effects are, of course, multiplied by the

presence of two amino functions on the same molecule.

Finally, we showed that those structural features of drugs which induce CCV differ from those which induce DCG. Substantial lipophilicity, in addition to a cationic feature, are apparently important for the induction of DCG. CCV and DCG are distinct morphologically and in their content. They likely represent variants of intracellular accumulation of drug, lipid, and other cellular chemicals. Presently, we are researching the differences between the chemical content of DCG and CCV.

We chose cultured cells for this structural correlation research after establishing parallelism with whole animal studies. As in this study, compounds 1, 2, 3, and 4 were positive, and compounds 5, 6, 9, and 16 were negative for induction of CCV in the rat choroid plexus epithelium after oral administration of 300 mg/kg for 5 days (4). The present work has shown that a cell culture system is powerful and economical of time and material in the elucidation of structure–activity correlates in drug-induced intracellular storage. In comparison to whole animals, negative CCV induction in cell culture systems cannot be due to poor absorption, first-pass metabolism, rapid excretion, or animal variability. That compound 12, a monodealkylated metabolite of D, did not induce CCV confirms the discriminatory power of the cell culture system.

We thank Professor Bernard M. Wagner for many stimulating discussions and for his constant encouragement. We acknowledge Dr. Ragab El-Rashidy for the numerous pK_a determinations and Dr. Robert J. Chorvat for syntheses and purification of several compounds. We also thank Dr. David C. Dodd for editorial comments and Leah Williams for typing the manuscript.

Note added in proof. In a recent publication, there was an implied causal emphasis on the piperidine ring of disobutamide in relation to induction of CCV (H. Koizumi, M. Watanabe, H. Numata, T. Sakai, H. Morishita. Toxicol Appl Pharmacol **84**:125–148, 1986). Our work clearly shows that this emphasis is unwarranted.

1. Yonan PK, Novotney RL, Woo CM, Prodan KA, Hershenson FM. Synthesis and antiarrhythmic activity of α,α-Bis[dialkyl-amino)alkyl] phenylacetamides. J Med Chem **23**:1102–1108, 1980.
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