mg/kg) solution. Blood pressure was measured with a pressure transducer (MPU-0.5, NEC San-ei) through a polyethylene cannula inserted into the left carotid artery. Heart rate was monitored with a cardiotachometer (NEC San-ei) triggered by the arterial pressure pulse. A femoral vein was cannulated with a polyethylene cannula for drug administration. Test compounds were dissolved in DMSO to form stock solutions, and appropriate dilutions in saline were prepared for injection. The solution of test compound (0.03-100 μg/mL) was administered iv in a volume of 1 mL/kg at 5-min intervals in a cumulative manner. The concentration of DMSO in the solution for injection was less than 3.4%, a concentration that did not affect the BP and HR in SHR.

Changes in BP and HR were expressed in terms of percent changes of their control values, which were obtained before the injection of a test compound into the rat. Agonists were compared for their potency to decrease BP and HR. The relative potency to decrease BP was estimated on the basis of the EC_{30} value, the mean dose that produced a 30% decrease in BP of SHR. Similarly, the relative potency to decrease HR was estimated on the basis of the ED_{10} value, the mean dose that produced a 10% decrease in HR of SHR. ED_{30} and ED_{10} values were calculated by the method of Fleming et al.[42]

Adenosine Deaminase Assays. Adenosine deaminase [EC.3.5.4.4] from calf intestinal mucosa (type III) was obtained from the Sigma Chemical Co. The enzyme experiments were done in 0.05 M phosphate buffer (pH 7.5). The assay solutions containing about 1×10^{-4} M of the compounds (**5a-e**) and 15 μg of the enzyme were incubated at 25 °C for 26 h. The absorbances at λ_{max} of these compounds were not changed.

Acknowledgment. This investigation was supported in part by Grants-in-Aid for Developmental Scientific Research and Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan.

Registry No. 1, 16321-99-6; 2, 5987-76-8; **3a**, 137896-02-7; **3b**, 137896-13-0; **4a**, 35109-88-7; **4b**, 94042-04-3; **5a**, 137896-03-8; **5b**, 99044-60-7; **5c**, 90596-73-9; **5d**, 90596-74-0; **5e**, 90596-75-1; **5e** tri-*O*-acetyl derivative, 133560-05-1; **5f**, 131242-48-3; **5g**, 100647-74-3; **5h**, 109232-63-5; **5i**, 109232-59-9; **5j**, 109232-60-2; **5k**, 109232-62-4; **5l**, 90596-71-7; **5m**, 99044-58-3; **5n**, 137896-14-1; **5o**, 137896-15-2; **5p**, 137896-16-3; **5q**, 137896-17-4; **5r**, 137896-18-5; **6**, 90596-72-8; **7**, 90596-76-2; **8**, 90596-77-3; **9**, 99044-57-2; **10**, 137896-04-9; **11**, 5987-73-5; **12**, 137896-05-0; 12 tri-*O*-acetyl derivative, 137915-41-4; **13**, 31281-86-4; **14**, 137896-06-1; **14** tri-*O*-acetyl derivative, 137896-19-6; **15**, 137915-39-0; **16**, 137915-40-3; **17**, 37151-12-5; **18**, 137896-07-2; **19**, 137896-08-3; **20**, 137896-09-4; **21**, 137896-10-7; **22**, 137896-11-8; **23**, 137896-12-9.

Substituted 4,6-Diaminoquinolines as Inhibitors of C5a Receptor Binding

Thomas J. Lanza,† Philippe L. Durette,† Thomas Rollins,‡ Salvatore Siciliano,‡ Dana N. Cianciarulo,‡ Sumire V. Kobayashi,‡ Charles G. Caldwell,† Martin S. Springer,‡ and William K. Hagmann*,†

Departments of Medicinal Chemical Research and Immunology Research, Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065. *Received December 4, 1990*

The anaphylatoxin C5a is implicated in a number of inflammatory diseases. It is a highly cationic protein with 13 of 74 amino acids being either arginine or lysine. A search focusing on positively charged molecules, particularly amine-containing functionalities, led to the discovery of substituted 4,6-diaminoquinolines 1 [*N,N'*-bis(4-amino-2-methyl-6-quinolyl)urea] and 7 [6-*N*-(2-chlorocinnamoyl)-4,6-diamino-2-methylquinoline) as inhibitors of C5a receptor binding. These two compounds inhibited the binding of radiolabeled C5a to its receptor isolated from human neutrophils with IC_{50}'s = 3.3 and 12 μg/mL, respectively. Our efforts to enhance their potencies by chemical modification revealed a narrow profile of potency for effective C5a receptor binding inhibition.

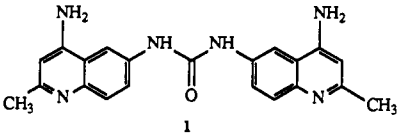
Activation of the complement system in response to immunological and nonimmunological events functions to focus and amplify the inflammatory response. In addition to the assembly of the membrane attack complex, numerous complement-derived peptides are released that interact with cellular components to propagate the inflammatory process.[1-4] These peptides called anaphylatoxins include C3a, a 77 amino acid peptide derived from C3 by the action of C3-convertases; C4a, a 77 amino acid protein produced from C4 by C1s; and C5a, a 74 amino acid protein, glycosylated in humans, cleaved from C5 by C5-convertase. Their properties include the cellular release of vasoactive amines and lysosomal enzymes, enhanced vascular permeability, and the contraction of smooth muscle. C5a also causes neutrophil chemotaxis and aggregation, stimulation of leukotriene and oxidative product release, induction of interleukin-1 transcription by macrophages, and enhanced antibody production.[5-7] For these reasons, blocking the action of C5a on its receptors may represent an intriguing therapeutic target for the treatment of diseases characterized by an excessive activity and recruitment of inflammatory cells.

The rational design of a small molecular weight antagonist to mimic the potent binding of the C5a molecule to its receptor ($K_d \approx 1 \times 10^{-10}$ M) presents a challenging problem even with considerable structural information. The three-dimensional structures of human[8] and porcine[9]

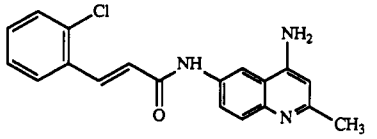
* To whom correspondence and reprint requests should be sent.
† Department of Medicinal Chemistry Research.
‡ Department of Immunology Research.

(1) Hugli, T. E. The Structural Basis for Anaphylatoxin and Chemotactic Functions of C3a, C4a, and C5a. *CRC Crit. Rev. Immunol.* **1981**, *1*, 321-366.
(2) Vogt, W. Activation of the Complement System. *Agts. Actions* **1983**, *13*, 391-397.
(3) Greer, J. Comparative Structural Anatomy of the Complement Anaphylatoxin Proteins C3a, C4a, and C5a. *Enzyme* **1986**, *36*, 150-163.
(4) Egwang, T. G.; Befus, A. D. The Role of Complement in the Induction and Regulation of Immune Responses. *Immunology* **1984**, *51*, 207-224.
(5) Weigle, W. O.; Goodman, M. G.; Morgan, E. L.; Hugli, T. E. Regulation of Immune Response by Components of the Complement Cascade and Their Activated Fragments. *Springer Semin. Immunopathol.* **1983**, *6*, 173-194.
(6) Yancey, K. B. Biological Properties of Human C5a: Selected *in vitro* and *in vivo* Studies. *Clin. Exp. Immunol.* **1988**, *71*, 207-210.
(7) Schindler, R.; Lonnemann, G.; Stanley, S.; Koch, K.-M.; Dinarello, C. A. Transcription, not Synthesis, of Interleukin-1 and Tumor Necrosis Factor by Complement. *Kidney International* **1990**, *37*, 85-93.

0022-2623/92/1835-0252$03.00/0



1



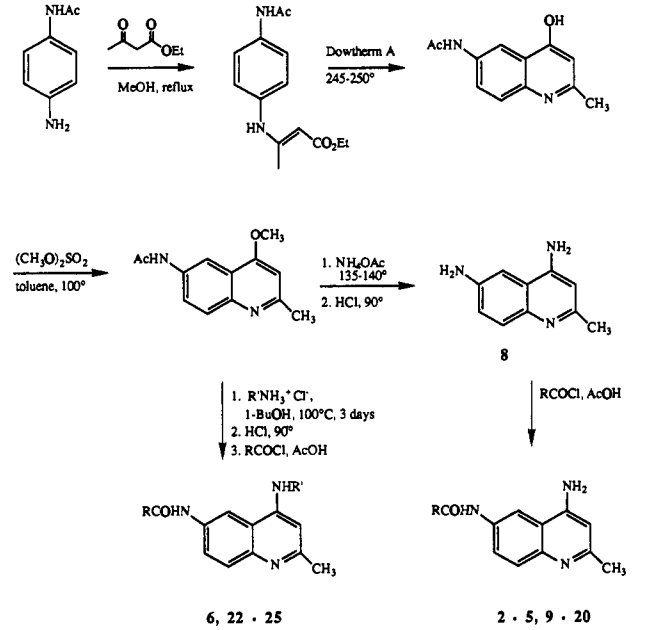
7

Figure 1. Structures of 1 and 7: inhibitors of C5a receptor binding.

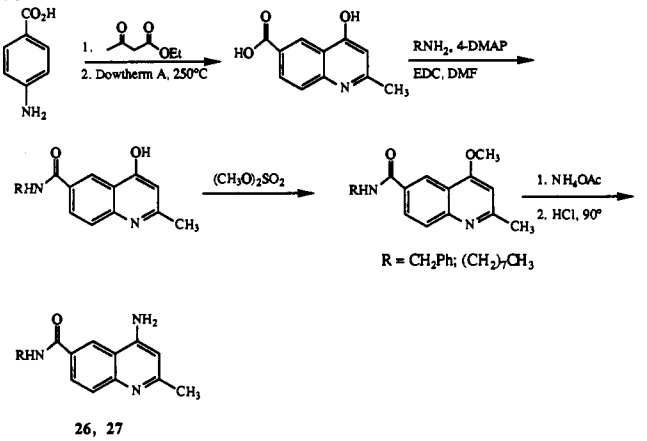
C5a have been assigned by NMR spectroscopy and analogy to the crystal structure of C3a. Deletion of the C-terminal pentapeptide to produce C5a(1–69) eliminated agonist activity but did not entirely ablate the ability of the molecule to compete with C5a for binding to the receptor on neutrophils.[10] Neutralizing antibodies to the C5a molecule have been prepared, and results implicate the region between Lys_{20} and Arg_{37} as being particularly important for receptor binding.[11] Tryptic digest of $C5a_{desArg74}$ afforded peptidyl fragments 20–37 and 47–62, with diminished receptor binding activity ($\sim 4.4 \times 10^{-4}$ M). Important amino acid residues were also identified by site-directed mutagenesis.[12] Variants in the carboxyl terminus and in the disulfide-linked core region gave significant decreases in receptor binding and neutrophil activation.

Conceptual models of multiple binding sites for C5a have been proposed.[13] There appears to be an "internal" recognition site in the globular domain and an "activation" site within the C-terminal region. Cyanogen bromide cleavage of porcine $C5a_{desArg74}$ gave two fragments: CN-I ($C5a_{1-17}$) and CN-II ($C5a_{18-73}$).[14] The N-terminus fragment CN-I was devoid of agonist activity while the C-terminus fragment CN-II was a weakly active agonist of neutrophil degranulation and spasmogenic activity of guinea pig ileum and parenchymal lung strips. This further implicated the C-terminus region as possessing the activation or agonist site. Considerable progress in identifying key interactions in the C-terminus region and reducing these to octapeptides possessing nanomolar affinities for the receptor

Scheme I



Scheme II



have been reported by researchers at Abbott.[15]

Of the 74 amino acids that comprise the human C5a molecule, 13 are either arginine or lysine residues, rendering C5a a highly cationic species. Cationic polypeptides have been investigated as antagonists of C5a activities.[16] Protamine and poly-L-Arg blocked C5a-mediated histamine release in basophils and prevented chemiluminescence and β-glucuronidase release in neutrophils. Very recently, the sequence of the human C5a receptor has been deduced from its cDNA sequence and found to share many features associated with the rhodopsin superfamily of GTP-linked binding proteins.[17] Interestingly, there are seven aspartic acid residues in the first extracellular region of this receptor. Not unexpectedly, the anionic properties of the C5a receptor complement the cationic features of its ligand. The basic nature of the C5a molecule led us to concentrate our efforts toward discovering selective C5a

(8) Zuiderweg, E. R. P.; Nettesheim, D. G.; Mollison, K. W.; Carter, G. W. Tertiary Structure of Human Complement Component C5a in Solution from Nuclear Magnetic Resonance Data. *Biochemistry* **1989**, *28*, 172–185.

(9) Williamson, M. P.; Madison, V. S. Three-Dimensional Structure of Porcine $C5a_{desArg}$ from ^1H Nuclear Magnetic Resonance Data. *Biochemistry* **1990**, *29*, 2895–2905.

(10) Chenoweth, D. E.; Hugli, T. E. Demonstration of Specific C5a Receptor on Intact Human Polymorphonuclear Leukocytes. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3943–3947.

(11) Johnson, R. J.; Tamerius, J. D.; Chenoweth, D. E. Identification of an Antigenic Epitope and Receptor Binding Domain of Human C5a. *J. Immunol.* **1987**, *138*, 3856–3862.

(12) Mollison, K. W.; Mandecki, W.; Zuiderweg, E. R. P.; Fayer, L.; Fey, T. A.; Krause, R. A.; Conway, R. G.; Miller, L.; Edalji, R. P.; Shallcross, M. A.; Lane, B.; Fox, J. L.; Greer, J.; Carter, G. W. Identification of Receptor Binding Residues in the Inflammatory Complement Protein C5a by Site-Directed Mutagenesis. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 292–296.

(13) Chenoweth, D. E.; Hugli, T. E. Human C5a and C5a Analogs as Probes of the Neutrophil C5a Receptor. *Molec. Immunol.* **1980**, *17*, 151–161.

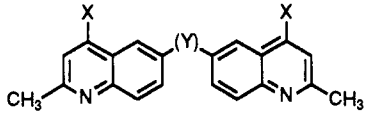
(14) Gerard, C.; Showell, H. J.; Hoeprich, P. D.; Hugli, T. E.; Stimler, N. P. Evidence for the Role of the Amino-terminal Region in the Biological Activity of the Classical Anaphylatoxin, Porcine C5a des-Arg-74. *J. Biol. Chem.* **1985**, *260*, 2613–2616.

(15) Preliminary results: Kawai, M.; Quincy, D. A.; Lane, B.; Mollison, K. W.; Luly, J. A.; Carter, G. W. Identification and Synthesis of a Receptor Binding Site of Human Anaphylatoxin C5a. *J. Med. Chem.* **1991**, *34*, 2068–2071.

(16) Olsen, U. B.; Selmer, J.; Kahl, J.-U. Complement C5a Receptor Antagonism by Protamine and Poly-L-Arg on Human Leukocytes. *Complement* **1988**, *5*, 153–162.

(17) Gerard, N. P.; Gerard, C. The Chemotactic Receptor for Human C5a Anaphylatoxin. *Nature* **1991**, *349*, 614–617.

Table I. Inhibition of C5a Binding by *N,N'*-Bis(4-amino-2-methyl-6-quinolyl)urea and Carboxamides



compd no.	X	Y	mp, °C (lit.)	inhibition of C5a binding: IC_{50} (μg/mL)[a]
1	NH_2	NHCONH	255–6 (255)[18]	3.3 (1)
2	NH_2	NHCOCONH	>300	2.0 (0.5)
3	$NH_2(HCl)_2$	$NHCOCH_2CONH$	255 dec (253)[20]	18 (5)
4	$NH_2(HCl)_2$	$NHCO(CH_2)_4CONH$	>300 (>300)[20]	>50
5	$NH_2(HCl)_2$	$NHCO(CH_2)_8CONH$	298 dec (301)[20]	>50
6	$NHCH_2Ph$	NHCOCONH	292–4 dec	>50
28[b]	$H(HCl)_2$	NHCONH	260–1 (262)[27]	>50

[a] Inhibition of binding of radiolabeled C5a to intact membrane-bound receptor prepared from human neutrophils (±SE). [b] Des(2,2'-dimethyl) analogue.

receptor antagonists on positively charged molecules, particularly structures containing amine functionalities. We report our discovery of the substituted 4,6-diaminoquinolines 1 and 7 (Figure 1) as inhibitors of C5a receptor binding and our efforts to enhance their potencies by chemical modification.

Chemistry

Compounds were prepared by procedures described for the preparation of *N,N'*-bis(4-amino-2-methyl-6-quinolyl)urea (1) and 6-*N*-(2-chlorocinnamoyl)-4,6-diamino-2-methylquinoline (7)[18,19] (Scheme I). 4,6-Diamino-2-methylquinoline (8) was condensed with the appropriate acyl chlorides to give compounds **2–5**, **7**, and **9–20**.[20] Displacement of the methyl ether in 6-(acetylamino)-4-methoxy-2-methylquinoline with substituted amines followed by amide hydrolysis and reaction with acyl chlorides afforded compounds **6** and **22–25**.

The 6-inverso amides were prepared by analogous methods shown in Scheme II. *p*-Aminobenzoic acid was condensed with ethyl acetoacetate to give 4-hydroxy-2-methylquinoline-6-carboxylic acid.[21] The benzyl- and *n*-octylamides were formed by diimide condensation. Methylation with dimethyl sulfate gave the methyl ethers which were displaced with ammonium acetate to give **26** and **27**.

Receptor Binding Assays. Compounds were tested for their ability to prevent the binding of radiolabeled C5a[22] to intact membrane-bound receptors[23] prepared from human neutrophils.[24] These data are reported as IC_{50}'s in Tables I and II. Specificity for binding was assessed by measuring the ability of the compounds to inhibit the binding of two other chemotactic agents, *N*-formylmethionylleucylphenylalanine (fMLF) or leukotriene B_4

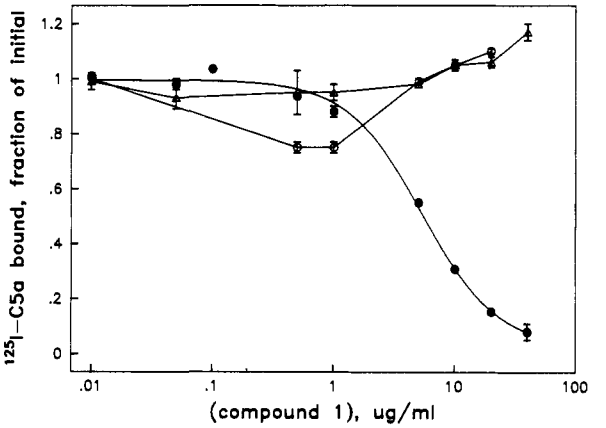


Figure 2. Inhibition of C5a binding by compound 1. These assays were performed by adding increasing concentrations of compound 1 to compete against a constant amount of either ^{125}I-C5a (●), ^{3}H-fMLF (○), or ^{3}H-LTB4 (△). All points are the averages of duplicate determinations.

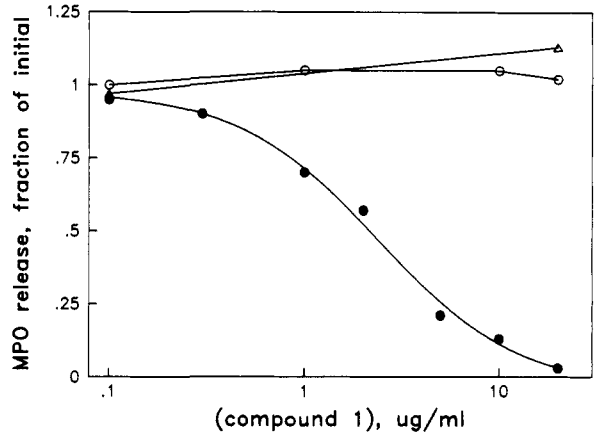
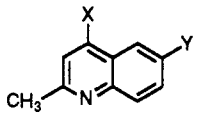


Figure 3. Inhibition of C5a-stimulated degranulation by compound 1. Freshly isolated human PMN were incubated with Cytochalasin B for 5 min and then distributed to the wells of microtiter plate. The cells were then stimulated with constant concentrations of a secretagogue containing various concentrations of compound 1. The rest of the assay was performed as described in the Experimental Section. The secretagogues used were C5a at 0.5 nM (●), fMLF at 10 nM (○), and PAF at 10 nM (△). At these concentrations all of the attractants released approximately equal amounts of MPO.

(18) Jensch, H. Neue Chemotherapeutika der 4-Amino-chinolin-Reihe. *Angew. Chem.* **1937**, *50*, 891–902.
(19) Peng, C.-T.; Daniels, T. C. The Synthesis of Some 6-N-Substituted Amide Derivatives of 4,6-Diaminoquinaldine and a Study of their in vitro Antibacterial Activity. *J. Am. Chem. Soc.* **1956**, *78*, 3703–3708.
(20) Pratt, M. G.; Archer, S. The Preparation of Some Amides of 4,6-Diaminoquinaldine. *J. Am. Chem. Soc.* **1948**, *70*, 4065–4069.
(21) Schock, R. U. The Preparation of Some N,N'-Bis-(4-quinaldyl)-a,o-diaminoalkanes as Potential Trypanocides. *J. Am. Chem. Soc.* **1957**, *79*, 1672–1675.
(22) Rollins, T. E.; Springer, M. S. Identification of the Polymorphonuclear Leukocyte C5a Receptor. *J. Biol. Chem.* **1985**, *260*, 7157–7160.
(23) Rollins, T. E.; Siciliano, S.; Springer, M. S. Solubilization of the Functional C5a Receptor from Human Polymorphonuclear Leukocytes. *J. Biol. Chem.* **1988**, *264*, 520–526.
(24) English, D.; Andersen, B. R. Single-step Separation of Red Blood Cells, Granulocytes, and Mononuclear Phagocytes on Discontinuous Density Gradients of Ficoll-Hypaque. *J. Immunolog. Methods* **1974**, *5*, 249–255.

Table II. Inhibition of C5a Binding by Substituted 4,6-Diaminoquinolines



compd no.	X	Y	mp, °C (lit.)	inhibition of C5a binding: IC_{50} (μg/mL)[a]
7	NH_2	NHCO-t-CHCH-(2-ClPh)	261–3 (286)[19]	12 (6)
8	NH_2	NH_2	193–4 (194)[18]	>100
9	NH_2	NHCO-t-CHCHPh	255–6 (252–4)[19]	30 (10)
10	NH_2	$NHCOCH_2CH_2Ph$	222–3 (227–8)[19]	>100
11	NH_2	NHCO-t-CHCH-(2,6-Cl_2Ph)	>300	8 (3)
12	NH_2	NHCOPh	246–7 (251–2)[19]	>100
13	NH_2	NHCO-2-naphthyl	270–1	30 (10)
14	NH_2	NHCO-*n*-C_3H_7	242–3 (244–5)[19]	>100
15	NH_2	NHCO-*n*-C_7H_{15}	227–9 (229–30)[19]	30 (10)
16	NH_2	NHCO-*n*-$C_{17}H_{35}$	134–5 (143–4)[19]	insol
17	NH_2	NHCO-t-CHCH-(2-CF_3Ph)	272–3	9 (2)
18	NH_2	NHCO-t-CHCH-(3-CF_3Ph)	235–6	4 (2)
19	NH_2	NHCO-t-CHCH-(4-CF_3Ph)	267–9	7 (4)
20	NH_2	NHCO-t-CHCH-(F_5Ph)	284–6	insol
21	OCH_3	NHCO-t-CHCH-(2-ClPh)	228–9	>50
22	$NHCH_2Ph$	NH_2	145–7	>100
23	$NHCH_2Ph$	NHCO-t-CHCH-(2-ClPh)	253–4	>100
24	$NHCH_2Ph$	NHCO-t-CHCH-(2-CF_3Ph)	265–7	>100
25	NH-n-C_8H_{17}	NHCO-t-CHCH-(2-ClPh)	227–8	>100
26	NH_2	CONH-*n*-C_8H_{17}	*b*	>100
27	NH_2	$CONHCH_2Ph$	*b*	>100

[a] Inhibition of binding of radiolabeled C5a to intact membrane-bound receptor prepared from human neutrophils (±SE). [b] Amorphous solid.

Table III. Inhibition of C5a- and fMLF-Induced Neutrophil Activation and Induction of Nonspecific MPO Release by Substituted Diaminoquinolines

compd no.	inhibition of C5a-induced neutrophil activation[a] (EC_{50}, μg/mL)	inhibition of fMLF-induced neutrophil activation[b] (EC_{50}, μg/mL)	induction of nonspecific MPO release[c] (EC_{50}, μg/mL)
1	15	0 at 25	0 at 25
2	4	0 at 20	0 at 20
7	4	5	0 at 20
11	4	4	0 at 20
17	4	4	0 at 20
23	<3	<3	5

[a] Inhibition of C5a-induced degranulation of human neutrophils as measured by the release of myeloperoxidase (MPO) activity. [b] Inhibition of fMLF-induced degranulation of human neutrophils as measured by the release of MPO activity. [c] Induction of nonspecific degranulation of human neutrophils in the absence of any stimulus as measured by the release of MPO activity.

(LTB_4). The selectivity results are shown in Figure 2.

Degranulation Assays. To determine whether the compounds were functional antagonists we measured their ability to inhibit the C5a-induced release of the azurophilic granule constituent myeloperoxidase (MPO) from intact neutrophils (shown in Table III). Specificity was assessed by measuring the effects of the compounds of *N*-formylmethionylleucylphenylalanine (fMLF) or platelet activating factor-induced degranulation. These data are shown in Figure 3 and in Table III. LTB4 was not used in the functional assay because it is a poor agent of degranulation in neutrophils. Several compounds were observed to cause the release of MPO in the absence of added C5a or fMLF. This nonspecific induction of MPO release from neutrophils for several compounds is also shown in Table III.

Results and Discussion

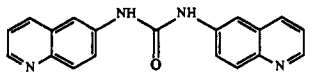
A search focusing on positively charged molecules containing amine functionalities led to the discovery that substituted 4,6-diaminoquinolines, as typified by *N,N'*-bis(4-amino-2-methyl-6-quinolyl)urea (compound 1 in Figure 1) are C5a antagonists. The ability of 1 to inhibit C5a binding is documented in Figure 2. It is highly selective for C5a as it has little effect on the binding of two other chemotactic agents, fMLF or leukotriene B_4 (LTB_4). In this experiment it inhibits C5a binding with an IC_{50} of 5 μg/mL. Averaged over experiments the IC_{50} was 3.3 μg/mL (Table I). The compound is an antagonist since as shown in Figure 3 it blocks C5a-induced degranulation. Again, the inhibition is highly selective as the compound has little effect on either fMLF or PAF induced degranulation.

The data in Table I suggest the relative relationship (whether spatial and/or electronic) of the two aminoquinoline moieties may be crucial for efficient binding to the C5a receptor. The urea 1 and oxamide 2 are approximately equipotent, while the potency of the malonamide 3, with an additional intervening atom and degree of rotational freedom, drops considerably. Increasing the number of intervening methylene units between the two heterocycles gave compounds 4 and 5 with decreased binding activity at the highest concentrations tested (50 μg/mL). A benzylamino substituent (secondary amine) at the C-4 position of the oxamide 6 also eliminates receptor binding activity.

The acylated compounds in Table II containing one aminoquinoline group and an aliphatic or aromatic acyl side chain also gave a narrow profile of activity for inhibiting binding of C5a to its receptor. The size, substitution, and orientation of the side chain proved crucial. The cinnamoyl side chain without the *o*-chloro group in 9 gave an approximately 3-fold decrease in binding potency with respect to the parent compound 7. Reduction of the double bond in 9 eliminated any activity in 10. The addition of another chloro group in 11 gave a negligible increase in potency. The 2-naphthoyl (13) and *n*-octanoyl (15) side chains gave compounds with modest activities,

while the smaller benzoyl (12) and butyryl (14) analogues were inactive. The three trifluoromethyl analogues (17–19) had comparable activities. Substitution of the 4-aminoquinoline group with methoxyl group eliminated inhibition of 21. The 4-N-substituted aminoquinolines 23–25 were all inactive as were the inverso amide analogues 26–27.

The results in Tables I and II indicate that the structural requirements for C5a receptor binding by these compounds fit a very narrow profile. The spatial or electronic relationship of the two 4-aminoquinoline rings may be important as seen in compounds 1 → 2 → 3 → 4. The 4-amino group appears essential as seen with loss of inhibition by 21. This amino group must also remain unencumbered in order to retain activity as noted by the lack of binding inhibition by 23–25. The bis(desamino-desmethyl) compound 28 was also found to be inactive. Only the larger hydrophobic side chains or small changes in aromatic substitution appear tolerated on the 6-amino group.



28

The specificity for receptor binding was also found to be somewhat selective for inhibitor structure. As seen in Figures 2 and 3 and Table III, the symmetrical compound 1 had little effect of fMLF or LTB4 binding and also failed to inhibit fMLF- or PAF-induced degranulation. Similarly compound 2 had no effect on fMLF-induced degranulation. Neither 1 or 2 by themselves caused nonspecific degranulation of PMN's as measured by levels of released myeloperoxidase (MPO) in the absence of active peptides. Thus the symmetrical compounds as typified by 1 appear to be highly selective C5a receptor antagonists. In contrast compounds 7, 11, and 17 were generally nonspecific, inhibiting both C5a- and fMLF-induced degranulation equally well. The *N*-benzyl analogue 23 was a potent inhibitor of both C5a- and fMLF-induced degranulation and did induce modest nonspecific degranulation at slightly higher concentrations.

The cationic nature of the anaphylatoxin C5a has suggested that searching for receptor antagonists among cationic compounds may be fruitful. Indeed, we have discovered that a class of substituted diaminoquinoline derivatives will inhibit the binding of C5a to its receptor and that very specific structural elements are necessary for binding. The recent report of the deduced human C5a receptor sequence confirms the anionic nature of the receptor.[17] The acidic N-terminal region of the receptor surface would be expected to have an affinity for basic compounds. Exactly how compounds such as 1 align with or mimic the important binding elements of C5a with its receptor cannot convincingly be surmised from the data presented here. It is suggested that the "soft" or diffuse nature of protonated aminoquinoline nucleus ($pK_a \approx 9$) could be recognized by the same features of the receptor that bind the guanidinium side chain of crucial arginine residues of C5a. Additional screening of aromatic amines or quinoline/pyridine compounds did not yield promising results. The narrow structure–activity relationship observed with these diaminoquinolines suggests that even simple cationic heterocycles can bind to a small and selective region of the C5a receptor, thereby effectively blocking the activity of this very potent mediator of inflammation.

Experimental Section

Chemistry. General Procedures. Melting points were determined on a Thomas-Hoover "Unimelt" capillary apparatus and are uncorrected. Mass spectra were recorded with a MAT 731 spectrometer. Proton NMR spectra were recorded on a Varian XL-200 NMR spectrometer. Chemical shifts are given on the δ scale. Spectra were measured at ambient temperature for solutions in chloroform-*d*, methanol-d_4, or dimethyl-d_6 sulfoxide, with tetramethylsilane (δ 0.00) as the internal standard. Thin-layer chromatography was performed on plates (250 μm) of silica gel-GHLF$_{254}$ (Analtech), and indication was effected with UV light, iodine, ninhydrin, or ceric sulfate (1%)–sulfuric acid (10%) spray.

Cautionary Note. Compound 1 has been reported to cause lymphosarcoma in mice.[25] Caution is warranted in the handling of these compounds.

Bis(4-amino-2-methyl-6-quinolyl)oxalamide (2). 4,6-Diamino-2-methylquinoline (8) (1.0 g, 5.8 mmol) was placed in an oven-dried 100-mL three-necked flask under nitrogen followed by 6 mL of glacial acetic acid. Oxalyl chloride (0.25 mL, 2.89 mmol) was added dropwise to this solution over a 3-min period. A heavy precipitate formed, and the mixture was stirred for 1 h at room temperature. After addition of diethyl ether (25 mL), the solid was filtered, washed with ether, and dried under vacuum. The hydrochloride salt was dissolved in 75 mL of warm water, and the solution was cooled to room temperature and made basic with 2.5 N sodium hydroxide. The precipitated solid was filtered, washed with water, and dried at 50 °C at high vacuum. Recrystallization was effected by dissolving the solid in warm DMF (30 mL), filtering, and adding diethyl ether to the point of turbidity. The crystals were collected by filtration, washed with ether, and dried under high vacuum: yield 0.52 g (45%); mp 243 °C dec.

Malonamide 3, adipamide 4, and sebacamide 5 were prepared in an analogous fashion as oxalamide 2, using malonyl chloride, adipoyl chloride, or sebacoyl chloride in place of oxalyl chloride, respectively. Physical data for these compounds are given in Table I.

Bis(4-(benzylamino)-2-methyl-6-quinolyl)oxalamide (6). A warm solution of benzylamine hydrochloride (1.5 g, 10.4 mmol) in water (2 mL) was added to a mixture of 6-acetamido-4-methoxy-2-methylquinoline (2.0 g, 8.7 mmol) in 1-butanol (20 mL). The mixture was stirred for 4 days at 100 °C and cooled in an ice bath, and the resulting precipitated solid was filtered, washed with ether, and dried by suction to afford 2.1 g (71%) of 6-acetamido-4-(benzylamino)-2-methylquinoline hydrochloride. This material was heated in 25% hydrochloric acid (33 mL) for 5 h at 90 °C. After cooling, the separated solid was filtered, washed with ether, and dried by suction. The solid was dissolved in water and made basic (pH >10) with 5 N sodium hydroxide to afford a yellow solid that was filtered, washed with water, and dried. 6-Amino-4-(benzylamino)-2-methylquinoline was obtained in 43% yield (0.98 g). A 0.5-g (1.9-mmol) portion of this material was dissolved in glacial acetic acid, and the solution was treated with oxalyl chloride (0.17 mL) for 1 h at room temperature with stirring. Ether was then added (25 mL), and the mixture was stirred for 30 min. The solid was filtered, washed with ether, and dried by suction. It was dissolved in water (25 mL) and warm ethanol (200 mL) with additional water added to achieve complete solution. After the solution was cooled to room temperature, 5 N sodium hydroxide was added to give a yellow precipitate that was filtered and dried under high vacuum: yield 0.46 g (41%); mp 292–294 °C dec; MS (FAB) m/z 581 (M + 1).

Preparation of the 6-Acylated Derivatives of 4,6-Diamino-2-methylquinoline. The procedure described by Pratt and Archer[20] was used to prepare these derivatives of 4,6-diamino-2-methylquinoline. Physical data for new compounds are recorded in Table II. The method is illustrated with the preparation of 4-amino-6-((2′-(trifluoromethyl)cinnamoyl)amino)-2-methylquinoline (17). 4,6-Diamino-2-methylquinoline (8) (0.5 g, 2.9 mmol) was placed in an oven-dried, 100-mL, three-necked flask under nitrogen followed by 3 mL of glacial acetic acid. A solution of 2-(trifluoromethyl)cinnamoyl chloride (0.68 g, 2.89 mmol) in 1 mL of glacial acetic acid was added dropwise over a 2-min period to this mixture. A heavy precipitate resulted. The mixture was

(25) Hunter, D. T.; Hill, J. M. Surfen: A Quinoline with Oncogenic and Heparin-Neutralizing Properties. *Nature* **1961**, *191*, 1378–1379.

stirred at room temperature for 1 h, diethyl ether (25 mL) was added, and the hydrochloride salt was filtered, washed with ether, and dried at room temperature in vacuo. The hydrochloride salt was dissolved in a warm mixture of 50% aqueous methanol, and the solution was filtered. The solution was made basic with sodium bicarbonate solution, and the resulting solid was filtered, washed with water, and dried at 50 °C under high vacuum. Recrystallization from hot methanol gave 0.68 g (63%) of the desired product, mp 272–273 °C.

6-((2′-Chlorocinnamoyl)amino)-4-methoxy-2-methylquinoline (21). A solution of 2-chlorocinnamoyl chloride (prepared by treatment of 2-chlorocinnamic acid with oxalyl chloride and catalytic DMF in dichloromethane) (0.27 g) in 1 mL of acetic acid was added to a solution of 6-amino-4-methoxy-2-methylquinoline (0.25 g, 1.33 mmol) in glacial acetic acid (3 mL). A heavy precipitate formed, and the mixture was stirred for 1 h at room temperature. Ether (25 mL) was added, and the solid was filtered, washed with ether, and dried by suction. The hydrochloride salt was suspended in warm water (25 mL) and ethanol (25 mL) and heated until dissolved. After cooling to room temperature, 5 N sodium hydroxide was added to give a white precipitate that was filtered and washed with water. Recrystallization from ethanol afforded **21**: yield 0.27 g (58%); mp 228–229 °C; MS (FAB) m/z 353 (M + 1).

4-(Benzylamino)-6-((2′-chlorocinnamoyl)amino)-2-methylquinoline (23). A warm solution of benzylamine hydrochloride in water (1 mL) was added to a mixture of 6-acetamido-4-methoxy-2-methylquinoline (1.0 g, 4.3 mmol) in 1-butanol (10 mL), after which complete solution occurred. The solution was stirred for 3 days at 100 °C under a nitrogen atmosphere and cooled, and the precipitated solid was filtered, washed with ether, and dried to afford 1.0 g (75%) of 6-acetamido-4-(benzylamino)-2-methylquinoline hydrochloride. A solution of this material (0.76 g, 2.49 mmol) in 25% hydrochloric acid (12 mL) was heated for 5 h at 90 °C and subsequently cooled. The precipitated solid was filtered, washed with water, and dried at 50 °C under high vacuum for several hours to give 0.38 g (58%) of 6-amino-4-(benzylamino)-2-methylquinoline. This material was dissolved in glacial acetic acid (3 mL) to which was added dropwise a solution of 2-chlorocinnamoyl chloride in glacial acetic acid over a 5-min period. The reaction mixture was stirred for 1 h at room temperature and diluted with diethyl ether (25 mL). The solid was filtered, washed with ether, and dried by suction. It was then dissolved in a hot mixture of methanol (50 mL) and water (25 mL) and cooled, and 5 N sodium hydroxide was added to give a yellow precipitate that was filtered, washed with water, and dried under high vacuum. Recrystallization from 2-propanol afforded pale yellow crystals: yield 0.32 g (52%); mp 253–254 °C; MS (FAB) m/z 428 (M + 1).

6-(2′-Chlorocinnamoyl)-4-(*n*-octylamino)-2-methylquinoline (25). This compound was prepared in an analogous fashion as **23**, using *n*-octylamine hydrochloride in place of benzylamine hydrochloride in the first step of the sequence.

4-Hydroxy-2-methylquinoline-6-carboxylic Acid *n*-Octylamide. 4-(Dimethylamino)pyridine (60 mg, 0.49 mmol), *n*-octylamine (1 mL, 6.1 mmol), and 1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride (1.1 g, 5.7 mmol) were added to a mixture of 4-hydroxy-2-methylquinoline-6-carboxylic acid[21] (1.0 g, 4.9 mmol) in *N,N*-dimethylformamide (20 mL). The reaction mixture was stirred overnight at room temperature and the solid was filtered, washed with diethyl ether, and dried in vacuo: yield 1.4 g (90%); MS (FAB) m/z 315 (M + 1).

4-Methoxy-2-methylquinoline-6-carboxylic Acid *n*-Octylamide. A mixture 4-hydroxy-2-methylquinoline-6-carboxylic acid *n*-octylamide (1.3 g, 4.1 mmol) and dimethyl sulfate (0.7 mL, 7.4 mmol) in toluene (10 mL) was heated for 3 h at 100 °C with vigorous stirring. The toluene was decanted off, and the remaining oil was dissolved in water (15 mL) and treated with 1 mL of 35% aqueous sodium hydroxide. The solid was filtered and washed with water. Purification was achieved by means of flash chromatography on silica gel using 4% methanol in dichloromethane as eluant. The product **23** was obtained as a crystalline solid: yield 0.91 g (67%); mp 143–145 °C; MS (FAB) m/z 329 (M + 1).

4-Amino-2-methylquinoline-6-carboxylic Acid *n*-Octylamide (26). A mixture of 4-methoxy-2-methylquinoline-6-carboxylic acid *n*-octylamide (0.75 g, 2.3 mmol) and ammonium acetate (2.5 g, 32.4 mmol) was heated for 3 h at 135 °C, cooled, and then poured into water. The solid was filtered, washed with water, and dried by suction. It was then dissolved in 50% aqueous methanol, the solution made basic with 10% aqueous sodium hydrogen carbonate. The precipitated solid was filtered, washed with water, and dried in vacuo at 50 °C: yield 495 mg (69%); MS (FAB) m/z 314 (M + 1).

4-Amino-2-methylquinoline-6-carboxylic Acid Benzylamide (27). This compound was prepared in an analogous fashion as **26** by a three-step sequence from 4-hydroxy-2-methylquinoline-6-carboxylic acid using benzylamine in place of *n*-octylamine: MS (FAB) m/z 292 (M + 1).

Binding Assays. Isolation of PMN. Fresh PMN's were isolated by the procedure of English and Anderson[24] with an additional step to lyse erythrocytes. After separation of Ficoll-Hypaque, cells were resuspended in lysing solution (0.15 M ammonium chloride, 0.1 mM EDTA, and 10 mM potassium bicarbonate, pH 7.3) and the cells were washed with the same solution. The cells were then resuspended to 4×10^5/mL in Hank's balanced salt solution (HBSS). PMN membranes were prepared as described.[23]

C5a Receptor Binding Assays. Test compounds were dissolved at appropriate concentrations in 1% aqueous dimethyl sulfoxide (DMSO) containing 1 mM sodium chloride. The C5a binding inhibitor assay was performed as follows: 200 μL of assay buffer (HBSS containing 10 mM *p*-toluenesulfonyl fluoride [pMSF] and 0.5% bovine serum albumin [BSA] to pH 7.2 with 25 mM HEPES) and 20 μL of ^{125}I-iodinated-C5a[22] (typically 20 pM final concentration) were added sequentially into 12 × 75-mm polypropylene tubes at 22 °C. Either 10 μL of the DMSO solution containing test compound or 10 μL of 1% aqueous DMSO were combined in the tube. Binding was initiated by the addition of 20 μL of a solution containing intact PMN membranes (typically 0.5 μg of protein) to each tube. After incubating each tube for 90 min at room temperature, 2 mL of assay buffer was added to the tubes and the contents vortexed and filtered through a Whatman GF/C or GF/B filter which had been soaked in 0.33% polyethyleneimine. An additional 2 mL of assay buffer was added to the tube and used to wash the filter. The filters were punched out and counted in a Packard Gamma Counter. Nonspecific background was determined by inclusion of at least 20 ng of unlabeled C5a. The percent inhibition was calculated as follows:

$$\% \text{ inhibition} = 100 \times \frac{\text{CPM}_{\text{solvent}} - \text{CPM}_{\text{sample}}}{\text{CPM}_{\text{solvent}} - \text{CPM}_{\text{nonspecific background}}}$$

The IC_{50}'s were determined by fitting the concentration dependence of inhibition with the four-parameter logistic equation of DeLean.[26]

Degranulation of PMN. Freshly isolated PMN's (4×10^5/mL) were preincubated with test compound or solvent alone (1% aqueous DMSO) for 20 min at 37 °C followed by the addition of Cytochalasin B (final concentration 5 μg/mL). After an additional 5-min incubation, 115 μL of the treated cells were distributed to 96-well flat-bottom plates containing 10 μL of C5a or fMLF (or PAF) at final concentrations of 0.3 or 10 nM, respectively, and incubated for 5 min at 37 °C. The plates were then removed from the incubator, and all further manipulations were performed at room temperature. To each well of the plate were added 50 μL of 0.1 M sodium dihydrogen phosphate, pH 6.0, and 25 μL of a freshly prepared solution containing 9 volumes of 3,3′-dimethoxybenzidine dihydrochloride (3.2 mg/mL) and 1 volume of 0.05% hydrogen peroxide. After 2 min, the reaction was quenched by the addition of 25 μL of a sodium azide solution to each well and the absorbance at 450 nm was measured. All values are the averages of duplicate or triplicate determinations and have the background absorbance, obtained in the absence of stimulus, substracted. The EC_{50}'s were calculated from the

(26) DeLean, A.; Munson, P. J.; Rodbard, D. Simultaneous Analysis of Families of Sigmoidal Curves: Application to Bioassay, Radioligand Assay, and Physiological Dose-Response Curves. *Am. J. Physiol.* 1978, *235*, E97–E102.

(27) Haskelberg, L. Derivatives of 6-Nitro and 6-Aminoquinoline. *J. Org. Chem.* 1947, *12*, 434–436.

four-parameter logistic equation as described above. In some experiments, for example those in Figure 3, there was no preincubation with the test compounds, rather they were added to the cells with the degranulatory stimuli. Results were the same with or without the preincubation.

Registry No. 1, 3811-56-1; 2, 137872-78-7; 3, 137872-79-8; 4, 137895-36-4; 5, 137872-80-1; 6, 137872-81-2; 7, 137872-82-3; 8, 5443-31-2; 9, 137872-83-4; 10, 101890-98-6; 11, 137872-84-5; 12, 6269-68-7; 13, 137872-85-6; 14, 6954-99-0; 15, 109094-06-6; 16, 103270-77-5; 17, 137872-86-7; 18, 137872-87-8; 19, 137872-88-9; 20, 137872-89-0; 21, 137872-90-3; 22, 137872-91-4; 23, 137872-92-5; 24, 137872-93-6; 25, 137872-94-7; 26, 137872-95-8; 27, 137872-96-9; 28, 137872-97-0; oxalyl chloride, 79-37-8; malonyl chloride, 1663-67-8; adipoyl chloride, 111-50-2; sebacoyl chloride, 111-19-3; benzylamine hydrochloride, 3287-99-8; *n*-octylamine hydrochloride, 142-95-0; *n*-octylamine, 111-86-4; 6-acetamido-4-methoxy-2-methylquinoline, 100795-23-1; 6-acetamido-4-(benzylamino)-2-methylquinoline hydrochloride, 137872-98-1; 6-amino-4-(benzylamino)-2-methylquinoline, 137872-91-4; 2-chlorocinnamoyl chloride, 35086-82-9; 6-amino-4-methoxy-2-methylquinoline, 84264-27-7; 4-hydroxy-2-methylquinoline-6-carboxylic acid *n*-octylamide, 137872-99-2; 4-hydroxy-2-methylquinoline-6-carboxylic acid, 103853-88-9; 4-methoxy-2-methylquinoline-6-carboxylic acid *n*-octylamine, 137873-00-8; anaphylatoxin C5a, 80295-54-1.

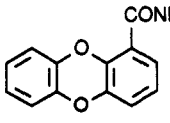
Potential Antitumor Agents. 64. Synthesis and Antitumor Evaluation of Dibenzo[1,4]dioxin-1-carboxamides: A New Class of Weakly Binding DNA-Intercalating Agents

Ho H. Lee, Brian D. Palmer, Maruta Boyd, Bruce C. Baguley, and William A. Denny*

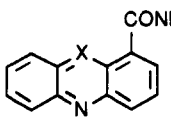
Cancer Research Laboratory, University of Auckland School of Medicine, Private Bag, Auckland, New Zealand.
Received May 7, 1991

A series of substituted dibenzo[1,4]dioxin-1-carboxamides has been synthesized and evaluated for in vitro and in vivo antitumor activity. The required substituted dibenzo[1,4]dioxin-1-carboxylic acids were prepared by a variety of methods. No regiospecific syntheses were available for many of these, and separation of the mixtures of regioisomers obtained was sometimes difficult. The dibenzo[1,4]dioxin-1-carboxamides are active against wild-type P388 leukemia in vitro and in vivo, with structure–activity relationships resembling those for both the acridine-4-carboxamide and phenazine-1-carboxamide series of DNA-intercalating antitumor agents. In all three series, substituents placed peri to the carboxamide sidechain (the 5-position in the acridines, and the 9-position in the phenazines and dibenzo-[1,4]dioxins) enhance activity and potency. The 9-chlorodibenzodioxin-1-carboxamide was also curative against the remotely sited Lewis lung carcinoma. Several of the compounds showed much lower levels of cross-resistance to the P388/AMSA line than classical DNA-intercalating agents, which suggests that their primary mechanism of action may not be via interference with topoisomerase IIα. This is of interest with regard to the development of drugs to combat resistance mechanisms which arise by the expression of the topo IIβ isozyme.

In a general study of the antitumor properties of linear tricyclic carboxamides, we recently[1] noted the in vivo antileukemic activity of the DNA-intercalating dibenzo-[1,4]dioxin-1-carboxamide (1). While DNA-intercalating



1



2 : X = CH
3 : X = N

agents form an important class of anticancer drugs, a common limitation of such compounds is their poor extravascular distributive properties.[2-4] This is particularly true for compounds where a cationic charge is located on the DNA-binding chromophore, for example acridine-based compounds such as 2. A previous study[5] of analogues of 2 showed that activity against remotely sited Lewis lung tumors was exhibited only by those analogues where the acridine chromophores were uncharged at physiological pH.

Structures such as the parent dioxin 1, with small neutral chromophores, are therefore of particular interest, since they are likely to have better ability to diffuse efficiently into solid tumor tissue. While this compound has only modest antitumor activity,[1] recent work[6] with similar phenazinecarboxamides (3) has demonstrated that dramatic improvements in activity can be achieved by suitable substitution of the chromophore. A recent survey[7] of tricyclic carboxamides showed that 1 was virtually inactive as a frameshift mutagen, unlike many acridine-based derivatives. In the present work we therefore outline the

(1) Palmer, B. D.; Rewcastle, G. W.; Atwell, G. J.; Baguley, B. C.; Denny, W. A. Potential Antitumor Agents. 54. Chromophore requirements for in vivo antitumor activity among the general class of tricyclic carboxamides. *J. Med. Chem.* **1988**, *31*, 707-712.
(2) Jain, R. K. Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. *J. Natl. Cancer Inst.* **1989**, *81*, 570-576.
(3) Durand, R. E. Distribution and activity of antineoplastic drugs in a tumor model. *J. Natl. Cancer Inst.* **1989**, *81*, 146-152.
(4) Denny, W. A. DNA-Intercalating agents as antitumor drugs: prospects for future design. *Anti-Cancer Drug Des.* **1989**, *4*, 241-263.
(5) Denny, W. A.; Atwell, G. J.; Rewcastle, G. W.; Baguley, B. C. Potential Antitumor Agents. 49. 5-Substituted derivatives of N-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide with in vivo solid tumor activity. *J. Med. Chem.* **1987**, *30*, 658-663.
(6) Rewcastle, G. W.; Denny, W. A.; Baguley, B. C. Potential Antitumor Agents. 51. Synthesis and antitumor activity of phenazine-1-carboxamides. *J. Med. Chem.* **1987**, *30*, 843-851.
(7) Denny, W. A.; Turner, P. M.; Atwell, G. J.; Rewcastle, G. W.; Ferguson, L. R. Structure–activity relationships for the mutagenic activity of tricyclic intercalating agents in *Salmonella typhimurium*. *Mutat. Res.* **1990**, *232*, 233-241.

0022-2623/92/1835-0258$03.00/0