washed with 10 mL of H_2O, dried over $MgSO_4$, filtered, and evaporated. The resulting solid was chromatographed on Baker "flash" SiO_2 eluting with 5% MeOH–$CHCl_3$. Fractions with TLC R_f 0.5 were combined to yield 170 mg of light-yellow solid (11%): mass spectrum, m/e 524. The diester **10** (0.17 g, 0.000 32 mol) was dissolved in 2.5 mL of methoxyethanol and treated with 2.5 mL of 1.0 N NaOH solution (0.0025 mol). After 3 h at 22 °C the solution was taken to pH 7 by HOAc addition. The solvents were then removed at 2 mmHg. The residue was stirred with 10 mL of H_2O and filtered to yield the product, 50 mg (33%), as a white solid: UV max (pH 13) 246 nm (ϵ 21 360), 297 (10 030); mass spectrum, m/e 468 (bis trimethyl derivative). Anal. Calcd for $C_{22}H_{24}N_6O_6$: C, H, N.

5,10-Methylene-5,6,7,8-tetrahydro-8,10-dideazaminopterin (1). This reaction was conducted on 1.5 g of **7** in the same manner as described for the preparation of compound **10** to yield 1.7 g of crude product. Chromatography was conducted in the same manner as previously described with fractions that showed a TLC R_f = 0.4 being combined to yield the product as a tan solid (20%): mass spectrum, m/e 510. Anal. Calcd for $C_{22}H_{26}N_6O_5$: C; H; N, 16.9. Found N, 16.4.

The diester (**8**) was saponified in the manner described for the preparation of compound **11** to yield the product as a white solid (68%): UV max (pH 13) 305 nm (ϵ 5100), 242 (18 800), 225 sh (16 700), (pH 1) 310 nm (ϵ 4000), 232 (29 200); HPLC 97% pure; mass spectrum, m/e 454; NMR (Me_2SO-d_6, 400 MHz) δ 1.70 (1 H, m, C-7H), 1.80 (1 H, m, C-7H), 2.00 (1 H, m, CH), 2.05 (1 H, m, CH both of glutamate CH_2), 2.15 (2 H, m, C-9H), 2.35 (2 H, m, CH_2COOH), 2.54 (2 H, m, C-8H), 2.95 (1 H, t, C-10H), 3.47 (1 H, m, C-6H), 3.65 (2 H, m, 5,10-CH_2), 4.35 (1 H, m, CHNH), 6.25 (2 H, br s, NH_2), 6.40 (2 H, br s, NH_2), 7.50 (2 H, d, 3′,5′-H), 7.85 (2 H, d, 2′,6′-H), 8.42 (1 H, d, NH). Anal. Calcd for $C_{22}H_{26}N_6O_5$: C, H, N.

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Registry No. 1, 103003-96-9; 2, 88392-93-2; 3, 103003-97-0; 3 (TFA salt), 103024-65-3; 4, 103003-98-1; 5, 103003-99-2; 6, 103004-00-8; 7, 103004-01-9; 8, 103004-02-0; 9, 103004-03-1; 10, 103004-04-2; 11, 103004-05-3; diethyl L-glutamate hydrochloride, 1118-89-4; diethyl L-glutamate, 16450-41-2; dihydrofolate reductase, 9002-03-3; thymidylate synthase, 9031-61-2; folic acid, 59-30-3.

Studies on Ca^{2+} Channel Antagonists. 5-[(3,4-Dimethoxyphenethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylpentyl Isothiocyanate, a Chemoaffinity Ligand Derived from Verapamil

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Reduction of **1** (verapamil) afforded amine **2**, which was converted with thiophosgene to isothiocyanate **3**, a chemoaffinity ligand for Ca^{2+} channels. Compound **3** showed concentration-dependent negative inotropic effects in rat right myocardial ventricular strips, EC_{50} = (4.56 ± 3.40) × 10^{-6} M (mean ± SD), being slightly less potent than **4** (gallopamil), EC_{50} = (1.95 ± 1.22) × 10^{-6} M. It displaced [^{3}H]gallopamil in rat myocardial membranes, IC_{50} = (3.42 ± 2.51) × 10^{-7} M, approximately equipotent with **1**. It showed irreversible antagonism of [^{3}H]gallopamil binding when preincubated at 10^{-5} M; only 25% of [^{3}H]gallopamil binding vs. control was observed. This agent may be a useful chemoaffinity ligand to aid in characterization of Ca^{2+} channels.

Influx of extracellular Ca^{2+} through ion channels is thought to be an important process in the excitation coupling of contraction of cardiac and smooth muscle.[1-4] Ca^{2+} channel antagonists like verapamil (**1**), nifedipine, diltiazem, prenylamine, and their congeners have provided important therapeutic advances in the treatment of a variety of cardiovascular diseases.[5-8] Study of a large number of these chemically diverse agents has provided significant advances in our understanding of the processes involved in Ca^{2+} mediated contraction of excitable tissues. Ligand binding studies have provided evidence indicating allosteric regulation of multiple sites by different classes of ligands.[9-13]

The use of chemoaffinity and photoaffinity ligands has aided our understanding of the biochemistry and pharmacology of these agents, especially the dihydropyridine Ca^{2+} channel antagonists.[14-16] In this paper, we report preparation of an isothiocyanate chemoaffinity ligand, **3**, derived from verapamil, **1**, and the characterization of its interaction with verapamil binding sites in rat myocardium membrane homogenates.

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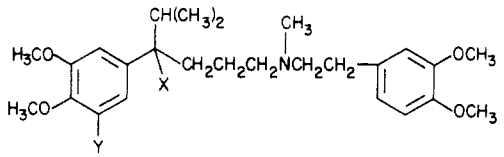
Structure–activity relationship studies on analogues of verapamil (1) have focused primarily on substituent changes in the aromatic ring adjacent to the quaternary chiral center.[17,18] However, the activity of related analogues suggests that neither the isopropyl group nor the carbonitrile group is required for Ca^{2+} channel antagonism.[19,20]

Isothiocyanate 3 was chosen as a target molecule based on its close structural similarity to 1, the ease of modification of the carbonitrile, and the chemical reactivity of this functionality. The isothiocyanate functional group has been successfully incorporated into various drug-related target molecules, e.g., various opioids,[21-23] phencyclidine,[24] clonidine,[25,26] and the aromatic ring of dihydropyridine Ca^{2+} channel antagonists.[27]

Chemistry. Reduction of verapamil (1) by lithium aluminum hydride (LAH) afforded the corresponding primary amine (2). The amine was readily characterized showing magnetically nonequivalent protons for the new methylene protons at δ 3.03 and 3.09, J_{gem} = 14.0 Hz (500-MHz ^1H NMR spectrum). Amine 2 was converted to the desired isothiocyanate 3 by using thiophosgene.



1, X = CN; Y = H (verapamil)
2, X = CH_2NH_2; Y = H
3, X = $CH_2N{=}C{=}S$; Y = H
4, X = CN; Y = OCH_3 (gallopamil, D600)

Pharmacological Results. Isothiocyanate 3 showed concentration-dependent negative inotropic effects in rat right myocardial ventricular strips (Figure 1). Its potency was slightly less than that of 4. The EC_{50} of 3 was (4.56 ± 3.40) × 10^{-6} M (mean ± SD, n = 4) and (1.95 ± 1.22) × 10^{-6} M (n = 6) and (2.51 ± 2.7) × 10^{-6} M (n = 4) for 4 and 1 (verapamil), respectively. Its effects on [^{3}H]gallopamil (0.1 μM) binding in rat myocardial membranes are shown in Figure 2. The IC_{50} of 3 was (3.42 ± 2.51) × 10^{-7} M (n = 3), virtually identical with 1 (3.62 ± 0.66) × 10^{-7} M (n = 3, not shown) and less potent than 4, IC_{50} = (1.60 ± 0.98) × 10^{-8} M (n = 5). Against [^{3}H]nitrendipine (0.05 nM) it had an IC_{50} of (3.58 ± 1.03) × 10^{-7} M (n = 3). With higher concentrations of [^{3}H]nitrendipine (0.55 nM), 3 displaced negligible amounts of [^{3}H]nitrendipine, as expected.[10] These observations indicate 3 had an affinity similar to 1 for the phenylalkylamine binding site.

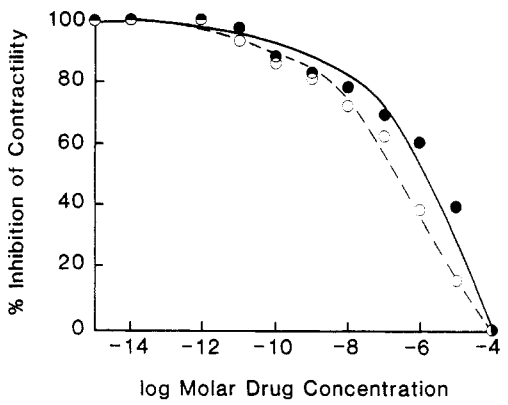


Figure 1. Inhibition of electrically stimulated contraction of rat right ventricle by 3 (●) and 4 (gallopamil, ○). Data are from representative experiments. Each point represents the mean of triplicate determinations.

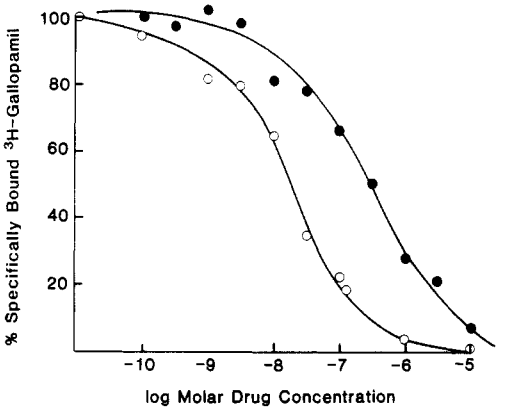


Figure 2. Displacement of [^{3}H]gallopamil from specific binding sites on rat myocardial membrane particulates by unlabeled 4 (gallopamil, ○) and by 3 (●). Data are from representative experiments. Each point represents the mean of triplicate determinations.

Isothiocyanate 3 produced irreversible antagonism of [^{3}H]gallopamil binding in myocardial membrane homogenates (Figure 3). After extensive washing of homogenates that had been preincubated with 3 (10^{-5} M), only 25 ± 5% (mean ± SE) of [^{3}H]gallopamil binding vs. controls was observed. When 4 was used in similar experiments 68.5 ± 4.5% (mean ± SE) of control binding capacity was observed, suggesting that the unlabeled 4 may not have been completely removed during the washing procedures. These results clearly indicate an irreversible effect of 3, which may result from covalent interaction of 3 with a portion of the Ca^{2+} channel. The utility of 3 in characterizing Ca^{2+} channels is under further study.

Derivatives of amine 2, with various functional groups[28-31] previously used for the design of chemoaffinity

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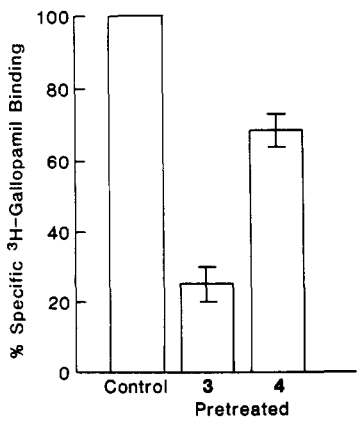

Figure 3. Specifically bound [^{3}H]gallopamil in rat myocardial membrane particulates that had been preincubated with buffer (control), **3**, and **4** as means ± SE (n = 3).

ligands for other targets, may also be useful in characterizing Ca^{2+} channels.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer 283 spectrometer. Absorptions are expressed in units of frequency (cm^{-1}). High-resolution NMR spectra were recorded on a Bruker WM-500-MHz spectrometer. Chemical shifts are expressed in parts per million (δ) relative to Me_4Si used as the internal standard. High-resolution chemical ionization mass spectra (methane) were obtained on a VG-7070 mass spectrometer by direct insertion probe.

(±)-5-[(3,4-Dimethoxyphenethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylpentylamine (2). To 1.50 g (3.05 mmol) of verapamil hydrochloride (**1**·HCl) in 50 mL of dry THF was added 500 mg (13.2 mmol) of lithium aluminum hydride. The mixture was stirred at reflux for 12 h, cooled, and quenched by addition of 5% aqueous NaOH (3 mL). The mixture was stirred for 30 min at room temperature and filtered. The residue was washed with 50 mL of Et_2O. The organic phases were combined, dried ($MgSO_4$), filtered, and concentrated affording **2** as a colorless oil (1.36 g, 97%): ^{1}H NMR δ 6.7–6.9 (6 H, m, aromatic), 3.8–3.9 (12 H, 4 s, OCH_3's), 3.03 and 3.09 (2 H, 2d, J_{gem} = 14.0 Hz, CH_2NH_2), 2.71 (2 H, dd, CH_2), 2.58 (2 H, dd, CH_2), 2.28 (3 H, s, NCH_3), 1.91 [1 H, septet, $CH(CH_3)_2$], 1.79 (2 H, t, CH_2), 0.79 (3 H, d, CH_3), 0.74 ppm (3 H, d, CH_3).

(±)-5-[(3,4-Dimethoxyphenethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylpentyl Isothiocyanate (3). To 1.49 g (3.25 mmol) of primary amine **2** and 1.32 g (13.0 mmol, 4.0 equiv) of triethylamine in 50 mL of dry $CHCl_3$ was added 0.50 mL (6.56 mmol, 2.0 equiv) of thiophosgene via syringe. The mixture was stirred at room temperature for 1 h and at reflux for 10 h. The mixture was then cooled and washed with H_2O (3 × 75 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated. The residue was chromatographed on silica gel, eluting with MeOH–EtOAc (10:90), affording **3** as a brown oil (1.03 g, 63%): ^{1}H NMR δ 6.65–6.85 (6 H, m, aromatic), 3.8–3.9 (12 H, 4 s, OCH_3's), 3.98 and 3.87 (2 H, 2 d, J_{gem} = 14.1 Hz, CH_2N=C=S), 2.75 (2 H, m, CH_2), 2.63 (2 H, m, CH_2), 2.31 (3 H, s, NCH_3), 1.97 (2 H, m, CH_2), 1.78 (1 H, m, $CH(CH_3)_2$), 1.42 and 1.32 (2 H, m, CH_2), 0.82 ppm (6 H, d, J = 6.5 Hz, $CH(CH_3)_2$); IR (neat) 2090, 2170 (C=N=S stretch) cm^{-1}; CIMS (methane), m/e 529 (M + 29, 25), 501 (MH^+, base peak), 349 ($MH^+ - C_9H_{12}O_2$, 95); high-resolution CIMS (methane), calcd for $C_{28}H_{41}N_2O_4S$, (MH^+) 501.2787; found, 501.2745. Anal. Calcd for $C_{28}H_{40}N_2S_4$, C, H, N.

Pharmacological Experiments. Isolated myocardial strips were dissected from the right ventricle of male Sprague-Dawley rats (300–400 g) and cut into longitudinal segments approximately 2 mm wide and 5 mm in length. Preparations were suspended in a 100-mL multichambered muscle chamber containing aerated (O_2–CO_2, 95:5) Krebs–Henseleit buffer at 30 °C. The salt solution had the following composition (mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; glucose, 10.0. The pH was adjusted to 7.4 and continuously monitored. Muscles were isometrically contracted against a working tension of 1 g. Contractions were induced by short square stimuli (2 ms) at a rate of 1 pulse/s and a voltage just sufficient to induce contractions.

Cumulative dose–response curves were generated after an equilibration period of 1–1.5 h. Drug was then added and effects measured at conditions of equilibrium, which was considered attained after a 40-min exposure time. Steady-state contraction amplitudes in the absence of drug was set as 100%. The depression of this steady-state contraction amplitude occurring under the influence of increasing drug concentrations was compared with the control contraction amplitudes and expressed in percentage.

Binding Experiments. [^{3}H]Gallopamil (85 Ci/mmol) and [^{3}H]nitrendipine (81 Ci/mmol) were obtained from NEN-Dupont (Boston, MA). A solution of (1 mM) of **3**·HCl and **4** was prepared daily in distilled deionized water. A 1 mM solution of nifedipine HCl was prepared daily in ethanol. Solutions of **3** (1 mM) were prepared daily by first dissolving it in Me_2SO/0.1 N HCl. Subsequent dilutions were made in distilled, deionized water to the appropriate concentrations.

Myocardial membrane particulates were prepared from male Sprague-Dawley rats (300–400 g), which were sacrificed by cervical dislocation. The heart was immediately removed and perfused through the aorta with ice-cold 10 mM Tris·HCl buffer, pH 7.4. The ventricles were dissected away from the atria, finely minced with scissors, and homogenized at a tissue concentration of 100 mg original wet tissue weight/mL of buffer. The resulting homogenate was stirred for 15 min on ice in the presence of an equal volume of 1 M KCl to remove contractile proteins and then filtered through four layers of cheesecloth. Tissue homogenates were washed 3 times by centrifugation at 50000g for 10 min after which the final pellet was resuspended in fresh buffer at a concentration of 20 mg original ventricular weight/mL of buffer.

For gallopamil binding assays, 200-μL aliquots of tissue homogenate were incubated with 0.1 nM [^{3}H]gallopamil and either **4** or **3** in a total incubation volume of 2 mL for 30 min at 25 °C. Separate experiments showed this time was sufficient for binding equilibrium to be established. Incubations were terminated by rapid vacuum filtration over Whatman GF/B glass fiber filters presoaked for at least 30 min in 1% polyethyleneimine. The filters were rinsed with 3-mL (4×) aliquots of ice-cold buffer, and the amount of trapped radioactivity was determined by liquid scintillation spectrometry at 38–42% efficiency. Binding in the presence of 10 μM **4** gallopamil was defined as nonspecific. Protein was determined by the method of Lowry et al.[32] using bovine serum albumin as standard.

In order to determine the tightness of binding of **3** to the putative phenylalkylamine binding site, myocardial membrane particulates were prepared as above with the final tissue pellet being resuspended at a concentration of 50 mg wet ventricular weight/mL of Tris buffer. A 4-mL aliquot of this final tissue homogenate was incubated in the presence of either **4**, **3**, or buffer in a total volume of 5 mL for 30 min at 0–4 °C. At the end of the incubation period, 20 mL of fresh buffer was added and the resulting diluted incubate centrifuged for 10 min at 50000g. The resulting pellet was resuspended in 25 mL of fresh ice-cold buffer and the homogenate allowed to stand for 20 min on ice to allow the dissociation of membrane-bound drug. The dissociation half-life for **4** under similar conditions was previously determined to be approximately 10 min (unpublished data). At the end of the 20-min dissociation period, the homogenate was again centrifuged as above and the whole procedure repeated again. The total number of wash and dissociation cycles was 6. The final pellet was resuspended in 8 mL of buffer and used in standard competition assays. A 0.6-mL aliquot (0.7 mg of protein) of the final homogenate was incubated in the presence of 0.1 nM

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[^{3}H]gallopmil as above in a 1-mL total assay volume. The remaining procedures were as described above. Each data point shown represents the mean of triplicate determinations. Each experiment was repeated at least 3 times.

Acknowledgment. We acknowledge support of this work by the National Heart, Lung, and Blood Institute through research Grant HL-34052 to W. L. Nelson and by the Veterans Administration through a merit review grant to J. C. Giacomini.

Registry No. (±)-**1**·HCl, 23313-68-0; (±)-**2**, 102852-52-8; (±)-**3**, 102852-53-9; (±)**3**·HCl, 102852-54-0; Ca, 7440-70-2.

Amino-Substituted *p*-Benzoquinones

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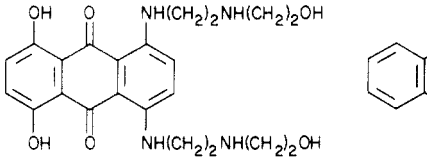
Based on the observation of outstanding antineoplastic activity of a number of amino-substituted anthraquinones, thioxanthones, and *N*-(aminoethyl)-substituted naphthalimides, four types of amino-substituted *p*-benzoquinones were designed, synthesized, and their biological activity evaluated. Although none of these compounds exhibited inhibitory activity against P388 leukemia, 2,5-bis[[4-[(dimethylamino)methyl]phenyl]amino]-3,6-dibromo-1,4-benzoquinone and the corresponding dichloro compound demonstrated good inhibitory activity against the proliferating human colon adenocarcinoma in vitro. The dichloro compound was also found to be active against the leukemia L1210 screening in vitro. 2,5-Bis[[2-(dimethylamino)ethyl]amino]-1,4-benzoquinone possessed inhibitory activity against *Neisseria catarrhali*.

A common *o*-aminoquinoid unit was reported[1] among several antitumor antibiotics including streptonigrin, actinomycin D, the mitomycins, and porfiromycin. On the basis of this concept, the AB ring units of streptonigrin[2,3] and amino-containing benzoquinones[4] and naphthoquinones[5] were synthesized and evaluated for their biological activity. Knowledge gained through these studies led to the proposition of a N–O–O working hypothesis.[6] Adamson[7] subsequently postulated a structural modification approach for the anthracyclines including adriamycin and daunomycin. Repeated structural designs and syntheses, together with a structural lead from the National Cancer Institute,[8] culminated in the synthesis of a dihydroxylated amino-containing anthraquinone DHAQ,[9] 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]-amino]anthraquinone (**1**), which displayed outstanding anticancer activity and is being evaluated in cancer patients.[10-12]



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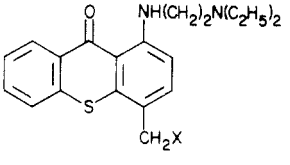

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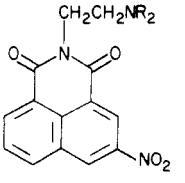

3

The DHAQ side chain contains an (aminoethyl)amino unit in common with two other types of antineoplastic agents **2** (e.g., hycanthone,[13,14] X = OH) and **3** (e.g., mitonafide,[15-17] R = CH_3). The presence of the distal nitrogen atom among the three structurally different ring systems,[8-14] together with the report that DNA binding or intercalation may not be the true mechanism of action for the anticancer activity of DHAQ,[18] suggested that incorporation of the substituted (aminoethyl)amino group and related side chains into other simpler ring systems should be studied. Consequently, synthesis of compounds containing the following amino-substituted side chains attached to the opposite sides of *p*-benzoquinone was conducted.

Type I (Compounds 4a–c). *p*-Benzoquinones containing the 2-[(dimethylamino)ethyl]amino side chains with or without substituents at other positions.

Type II (Compounds 5a,b). The ethylene linkage of

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