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Articles

Novel Non-Cross Resistant Diaminoanthraquinones as Potential Chemotherapeutic Agents

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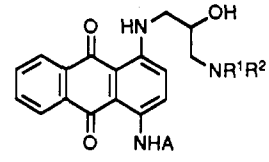
A novel series of diaminoanthraquinones was discovered initially as protein kinase C inhibitors with IC_{50}s in the 50–100 μM range. They exhibited potent tumor cell growth inhibitory activity in vitro without cross resistance to adriamycin. Further evaluation of two of the most active compounds NSC 639365 (3) and NSC 639366 (4) in human tumor cloning assay showed potent cytocidal activity. The results suggest therapeutical potentials against human tumors.

Introduction

The prerequisites for a successful surgical excision or radiation therapy in cancer patients are tumor accessibility and undisseminated disease. Unlike surgery and radiotherapy, chemotherapy provides treatments in a systemic manner, which seems more desirable particularly for disseminated or inoperable cancers. However, chemotherapy has not been able to successfully address tumor-sensitivity issues, which significantly reduce cancer curability in the clinic. Hence, new chemotherapeutic agents with novel biochemical mechanisms for treating cancer in general and tumor resistance in particular are urgently needed.

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine-specific protein kinases which play an important role in the control of cell growth and differentiation.[1] Recent reports suggest PKC may be involved in many aspects of human cancer treatment: multidrug resistance,[2] metastasis,[3] and chemopotentiation.[4] As part of our efforts to discover new chemotherapeutic agents based on PKC inhibition, we have designed and synthesized a new series of diaminoanthraquinones (DAAQ).



DAAQ

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(2) Staats, J.; Marquardt, D.; Center, M. S. Characterization of a Membrane-associated Protein Kinase of Multidrug-resistant HL60 cells which Phosphorylates P-glycoprotein. *J. Biol. Chem.* 1990, *265*, 4084–4090.
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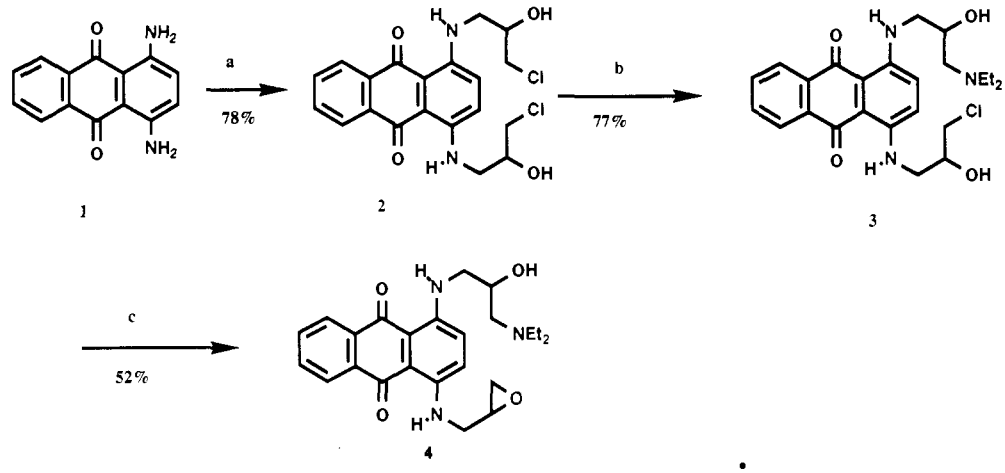
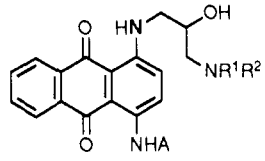
Scheme I



a) Epichlorohydrin, AcOH, 75°C, 1 hr; b) Diethylamine, EtOH, 25°C, 48 hr; c) NaOH, MeOH, 25°C, 48 hr.

Table I. Novel Diaminoanthraquinones (DAAQ)



compd	R^1	R^2	A	mp (°C)	anal.
3·HCl	Et	Et	$CH_2(OH)CH_2Cl$	105–108.5	C,H,N, Cl
4·fumarate	Et	Et	CH_2–epoxide	148–149	C,H,N
5·HCl	H	Pr	$CH_2(OH)CH_2Cl$	waxy	*a*
6·HCl	H	CH_2Ph	$CH_2(OH)CH_2Cl$	117 dec	*a*
7	Et	Et	H	75–77	C,H,N
8	NR^1R^2 = Cl		H	195–197	C,H,N,Cl

[a] Due to the extreme hygroscopic nature of these compounds, their combustion analysis results did not agree with the theoretical values.

Synthesis

An example of the synthesis of DAAQ is depicted in Scheme I. Reaction of the commercially available 1,4-diamino-9,10-anthracinedione (1) with epichlorohydrin gave rise to the bis-chlorohydrin 2,[5] which, upon treatment with *N,N*-diethylamine, furnished the quinone chlorohydrin 3 in 60% overall yield. Ring closure of 3 in the presence of base resulted in the potent antiproliferative compound 4. Analogs that can be synthesized in a similar fashion are listed in Table I.

Results and Discussion

The current new series of DAAQ (Table I) was initially designed as PKC inhibitors. When evaluated in the rat brain PKC mixed isozymes, analogs 3, 4, and 6 exhibited inhibitory activity in μM concentrations (data not shown), whereas compounds 2, 5, 6, and 8 were inactive. They did not inhibit c-AMP-dependent protein kinase (PKA) at the highest concentration tested (>218 μM, data not shown). Preliminary structure–activity relationship (SAR) studies on protein kinase C activity suggest that the terminal amino group on the side chain is important to PKC inhibition. When the best two compounds 3 (NSC 639365) and 4 (NSC 639366) were tested in the human mammary tumor MCF-7 in vitro system, they inhibited the tumor cell growth at nM level shown in Table II. In addition, at the same concentration level, the compounds demonstrated similar potent tumor cell growth inhibition against two adriamycin-resistant lines, human mammary carcinoma MCF-7/Adr and human myeloma 8226/Adr, with the latter cell line slightly more sensitive than its parent line to the two best compounds 3 and 4. There appears to be a lack of direct correlation between the DAAQ's antitumor and PKC inhibitory activities. One cannot, however, completely rule out the possibility that

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Table II. Summary of Inhibitory Activities In Tumor Cell Growth Systems (IC_{50}, μM)

entry	MCF-7	MCF-7/ADR	8226	8226/ADR
3	0.43 ± 0.20 (n = 6)	0.72 ± 0.30 (n = 5)	0.21	0.07 ± 0.01 (n = 2)
4	0.04 ± 0.02 (n = 4)	0.02 ± 0.01 (n = 3)	0.01	0.009
5	3.45 ± 1.75 (n = 2)	8.70 ± 6.0 (n = 2)	9.60	12.50
6	8.20 ± 1.7 (n = 2)	12.45 ± 0.05 (n = 2)	11.10	14.30
7	6.00 ± 1.7 (n = 2)	NT[b]	NT	NT
ADR[a]	0.063 ± 0.02 (n = 9)	3.1 ± 0.50 (n = 11)	0.033 ± 0.02 (n = 2)	0.560 ± 0.15 (n = 2)
MTXN[c]	0.005	4.0	0.03	0.18

[a] Adriamycin (ADR) when complexed with iron was reported to inhibit PKC. Hannun, Y. A.; Foglesong, R. J.; Bell, R. M. The Adriamycin-Iron(III) Complex is a Potent Inhibitor of Protein Kinase C. *J. Biol. Chem.* **1989**, *264*, 9960–9966. [b] NT: not tested. [c] MTXN: mitoxantron.

Table III. Summary of Activity in a Human Tumor Cloning System

compd	concn (μg/mL)	exposure time	no. of responses[a]/ no. of evaluable (%)
3	0.5	1 h	2/5 (40)
	5.0	1 h	4/5 (80)
	50.0	1 h	5/5 (100)
	0.5	continuous	2/4 (50)
	5.0	continuous	4/4 (100)
	50.0	continuous	4/4 (100)
4	0.5	1 h	4/10 (40)
	5.0	1 h	8/10 (80)
	50.0	1 h	8/10 (80)
	0.5	continuous	8/9 (89)
	5.0	continuous	8/9 (89)
	50.0	continuous	9/9 (100)

[a] Response is defined as ≤50% survival of tumor colony forming units.

Table IV. Tumor-Specific Activity of Compounds (1-h Exposure)

	3			4		
tumor type	0.5[a]	5.0	50.0	0.5	5.0	50.0
breast	0/1[b]	0/1	1/1	0/2	1/2	1/2
lung, non-small cell	0/1	1/1	1/1			
ovary	1/2	2/2	2/2	2/2	2/2	2/2
gastric	1/1	1/1	1/1	1/1	1/1	1/1
colon				1/5	4/5	4/5
total	2/5	4/5	5/5	4/10	8/10	8/10

[a] Concentration in μg/mL. [b] Number of responses (defined as ≤50% survival of tumor colony forming units) per number tested.

the compounds' PKC inhibitory activity contributes at least in part to their non-cross resistant property. For example, PKC isozyme inhibitory attributes of these compounds in relationship to their antitumor properties need to be exploited. The two most active compounds, 3 and 4, were further evaluated in the human tumor cloning assay (HTCA), which is an in vitro soft agar technique originally developed to predict for response or lack of response of an individual patient's tumor to a particular antineoplastic agent.[6] Prospective studies of the HTCA have shown the assay can correctly predict for response to single agent chemotherapy 60% of the time and can predict for lack of response 85% of the time.[7,8] Nevertheless, the assay has not been widely utilized to predict human tumor response. The HTCA has also been actively utilized to screen for tumor specific activity of potential new antitumor agents.[9,10] In this study we have utilized the HTCA to explore (in a pilot manner) the activity of 3 and 4 against primary human tumor colony forming units.

Overall a total of 31 patient specimen tumors (11 for compound 3 and 20 for compound 4) were plated. Five of the compound 3 specimens and 10 of the compound 4 were evaluable (acceptable colony formation and positive control).

The in vitro human tumor cloning results are summarized in Table III. Based on limited data shown, there were no major differences between a 1-h and a continuous exposure to either agent. There did appear to be a concentration–response effect for both agents. Table IV details the tumor-specific activity of both compounds. As can be seen in that table, both compounds appear to have rather broad spectrums of cytotoxicity against breast, non-small cell lung, ovarian, and gastric human tumor colony forming units.

Based on a limited number of human tumors tested, it appears that both compounds 3 and 4 possess considerable cytotoxicity against primary human tumor colony forming units. Additional in vitro testing against these colony forming units is needed to try to judge just how active these agents could be in a clinical trial situation. Moreover, animal toxicology/pharmacokinetic work will help determine whether or not the concentrations we have tested in vitro can be achieved in an in vivo system.

Structure modification of 4 to fine tune efficacy vs toxicity continues. In addition, the four optical isomers resulting from the two chiral centers on the molecule have been synthesized. Additional biological results on 3, 4, and their optical isomers and a complete SAR study will be reported in due course.

Experimental Section

General Procedures. Melting points (uncorrected) were determined in an open capillary with a Mel-Temp II melting point apparatus. IR spectra were determined with a Mattson Galaxy 2020 spectrophotometer. NMR spectra were determined with a Bruker AM 300 MHz or a Varian Gemini 300 MHz spectrometer containing tetramethylsilane as internal standard. Microanalyses were performed by Atlantic Microlabs, Inc., Norcross, GA, and were within 0.4% of the calculated values, unless stated otherwise. Chromatography was done using the flash column technique on silica gel 60 supplied by EM Science.

1,4-Bis[(3-chloro-2-hydroxypropyl)amino]-9,10-anthracenedione (2). To a solution of 1,4-diaminoanthraquinone (10 g, 42.0 mmol) in glacial acetic acid (200 mL) at 70 °C was added epichlorohydrin (32.8 mL, 420 mmol) all at once. The reaction mixture was stirred at 75 °C for 1 h, and the volatiles were removed in vacuo. The resulting residue was chromatographed (CH_2Cl_2 with a CH_3OH gradient of up to 1%) to provide a blue solid of the title compound (13.8 g, 78%): mp 167–169 °C; ^{1}H NMR (DMSO-d_6) δ 3.43–3.63 (m, 6H), 3.76–3.78 (m, 4H), 4.22 (br s, 2H), 6.92–6.94 (m, 2H), 7.69–7.72 (m, 2H), 8.24–8.27 (m, 2H), 10.81 (br s, 2H); IR (KBr) cm^{-1} 3350, 1640, 1570, 1250, 1170, 1010, 720. Anal. ($C_{20}H_{20}Cl_2N_2O_4 \cdot 0.25H_2O$) C, H, N.

1-[[3-(Diethylamino)-2-hydroxypropyl]amino]-4-[(3-chloro-2-hydroxypropyl)amino]-9,10-anthracenedione Hydrochloride (3). To a solution of 2 (1 g, 2.36 mmol) in ethanol (150 mL) was added dropwise, under nitrogen, diethylamine (1 mL, 9.66 mmol) over 1.5 h. The reaction was stirred for 48 h at room temperature and the solvent removed in vacuo. The residue was chromatographed (CH_2Cl_2/MeOH, 25/1 followed by CH_2Cl_2/MeOH/NH_4OH, 9/1/2) and the product recrystallized (CH_2Cl_2/ether/hexanes) to give a blue solid (769 mg, 77%): mp 165 °C; ^{1}H NMR ($CDCl_3$) δ 1.10 (t, J = 7 Hz, 6H, CH_3), 2.63 (m, 4H, CH_2), 2.74 (m, 2H, CH_2), 3.44 (m, 2H, CH_2), 3.56 (m, 1H, CH), 3.63 (m, 1H, CH), 3.73 (m, 2H, CH_2), 4.17 (b, 1H, NH), 4.19 (b, 1H, NH), 7.22 (s, 2H, ArH-2,3), 7.68 (m, 2H, ArH-6,7), 8.3 (m, 2H, ArH-5,8). Anal. ($C_{24}H_{30}O_4N_3Cl$), C, H, N.

To a cold solution of free base 3 (0.531 g, 2.36 mmol) in acetone/dichloromethane (50%, 15 mL) was slowly added concentrated HCl (8 drops); a color change from purple to red was observed. The solvent was removed in vacuo and the extremely hygroscopic purple solid removed from the flask by scraping (617 mg, 100%): mp 105–108.5 °C; ^{1}H NMR (D_2O) δ 1.40 (t, J = 7.3 Hz, 6H, CH_3), 2.90 (m, 4H, CH_2), 3.29 (m, 4H, CH_2), 3.38 (m, 3H, CH_2, CH), 3.73 (m, 3H, CH_2, CH), 3.92 (b, 1H, NH), 4.15 (b, 1H, NH), 6.23 (s, 2H, ArH-2,3), 7.26 (m, 2H, ArH-6,7), 7.32 (m, 2H, ArH-5,8); IR KBr (disc) cm^{-1}, 3284, 2987, 1633, 1587, 1527, 1450, 1400, 1364, 1270, 1176, 1100, 1049, 1020, 734. Anal. ($C_{24}H_{30}ClN_3O_4 \cdot 2HCl$) C, H, N, Cl.

1-[[3-(Diethylamino)-2-hydroxypropyl]amino]-4-[(2,3-epoxypropyl)amino]-9,10-anthracenedione Fumaric Acid

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Salt (4). To a solution of **3** (1 g, 1.78 mmol) in methanol (50 mL) was added KOH (0.3 g, 5.36 mmol) and the reaction was stirred at room temperature for 48 h. Solvent was removed in vacuo and the residue was chromatographed ($CHCl_3$ with a CH_3OH gradient up to a ratio of 10:1) to give **4**, free base (391 mg, 52%): mp 98 °C; ^1H NMR ($CDCl_3$) δ 1.06 (t, J = 7.1 Hz, 6H, CH_3), 2.62 (m, 7H, CH_2), 2.87 (m, 1H, CH_2), 3.27 (m, 1H, CH_2), 3.49 (m, 2H, CH_2), 3.59 (m, 1H, CH_2), 3.77 (m, 1H, CH), 3.96 (m, 1H, CH), 7.35 (s, 2H, ArH-2,3), 7.71 (m, 2H, ArH-6,7), 8.35 (m, 2H, ArH-5,8), 10.80 (b, 1H, NH), 10.92 (b, 1H, NH). Anal. ($C_{24}H_{29}N_3O_4$) C, H, N.

To a solution of free base **4** (100 mg, 0.236 mmol) in anhydrous acetone (8 mL) under nitrogen was added fumaric acid (13.7 mg, 0.118 mmol). The reaction mixture was stirred for 1 h and diluted by Et_2O (4 mL) to yield a hygroscopic purple solid of title compound (100 mg, 88%): mp 148–149 °C; ^1H NMR δ 1.02 (t, 6H, J = 7 Hz), 2.55–2.75 (m, 7H), 2.81 (t, 1H, J = 4 Hz), 3.85–3.98 (m, 2H), 6.53 (s, 1H), 8.25 (m, 2H), 10.85 (m, 1H), 10.99 (m, 1H); IR (KBr) cm^{-1} 3397, 3217, 3070, 2992, 2922, 2864, 1580, 1521, 1366, 1260, 1235, 1173, 1019, 734, 663. Anal. ($C_{24}H_{29}N_3O_4 \cdot 0.5C_4H_4O_4 \cdot H_2O$) C, H, N.

1-[[3-(Propylamino)-2-hydroxypropyl]amino]-4-[(3-chloro-2-hydroxypropyl)amino]-9,10-anthracenedione Hydrochloride Salt (5). A mixture of **2** (1 g, 2.36 mmol), propylamine (1.39 g, 23.6 mmol), and KI (0.26 g, 160 mmol) in CH_3OH (20 mL) was stirred at room temperature for 4 h. After the addition of KCO_3 (0.5 g, 3.62 mmol), the reaction was stirred at room temperature for an additional 16 h. The reaction mixture was evaporated in vacuo and the blue residue was dissolved in CH_2Cl_2, which was washed three times with water. The organic layer was dried ($MgSO_4$), filtered, and evaporated. The blue free base residue was chromatographed (CH_2Cl_2 with an increasing CH_3OH gradient) to give a blue solid which was dissolved in acetone and treated with HCl gas until a color change from blue to purple was noted. The product thus obtained (105 mg, 10%) was extremely hygroscopic: ^1H NMR ($CDCl_3$) 0.94 (m, 3H, CH_3), 1.55 (m, 2H, CH_2), 2.64 (m, 2H, CH_2), 2.77 (m, 3H, CH_2 and CH), 3.52–3.27 (m, 7H, CH and CH_2), 4.06 (b, 2H, OH), 6.86 (m, 1H, ArH-3), 7.14 (m, 1H, ArH-2), 7.58 (m, 2H, ArH-6,7), 8.14 (m, 2H, ArH-5,8), 10.70 (b, 2H, NH); MS on the free base (FAB) $m/_z$ 446 (M + 1, 20), 235 (100). Anal. ($C_{23}H_{28}N_3O_4Cl$) H; C, N, Cl: calcd, 61.95, 9.42, 7.95; found, 56.98, 8.65, 7.30.

1-[[3-(Benzylamino)-2-hydroxypropyl]amino]-4-[(3-chloro-2-hydroxypropyl)amino]-9,10-anthracenedione Hydrochloride Salt (6). To a solution of **2** (1 g, 2.36 mmol), benzylamine (2.02 g, 18.88 mmol), and potassium iodide (0.25 g, 153 mmol) in a solution of methanol (40 mL) and dichloromethane (5 mL) stirred at reflux for 5 h was added potassium carbonate (0.7 g, 5.06 mmol). The reaction was stirred at reflux for an additional 16 h. Solvent was removed and the blue product was dissolved in dichloromethane and washed three times with water. The organic layer was dried over magnesium sulfate and the solvent was removed. The blue free base product was purified by silica gel chromatography (eluant: dichloromethane with an increasing methanol gradient). The title product was formed (210 mg, 17%) by bubbling hydrochloride gas through a stirring solution of the free base in acetone until a color change was noted: extremely hygroscopic, mp 117 °C dec; NMR ($CDCl_3$) 2.82 (m, 2H, CH_2), 2.96 (m, 2H, CH_2), 3.53 (m, 4H, CH_2), 3.93 (d, J = 4.8 Hz, 2H, CH_2), 4.07 (m, 2H, CH), 7.08 (d, J = 4.92 Hz, 2H, ArH-2,3), 7.34 (m, 5H, ArH (benzyl)), 7.63 (m, 2H, ArH-6,7), 8.15 (m, 2H, ArH-5,8), 10.80 (b, 2H, NH); MS (FAB) $m/_z$ 496 (M + 2, 50), 494 (M + 1, (100)). Anal. ($C_{27}H_{28}N_3O_4Cl \cdot HCl$) H; C, N, Cl: calcd, 61.14, 7.92, 13.375; found, 54.73, 7.13, 11.89.

Protein Kinase C Inhibition. Rat brain protein kinase C was purified as previously described.[11] The enzyme was purified to apparent homogeneity as determined by a single band on silver stained SDS-polyacrylamide. The PKC assay was performed employing mixed micelles as previously described by Bell et al.[12] The assay contained in a total volume of 250 μL, 0.3% Triton X-100 containing 10 mol % dioleoylphosphatidylserine, 2 mol % dioleoylglycerol, 20 mM Hepes buffer pH 7.5, 100 μM $CaCl_2$, 10 mM $MgCl_2$, 200 μg/mL histone, 20 μM [δ^{32}P]ATP, and 10 μL of DMSO or compound in DMSO. The reaction was started with 10 μL of purified PKC (1 μg/mL). The specific activity of control reactions were 1.28 μmol min^{-1} mg^{-1}. Control reactions in the absence of phosphotidylserine and diacylglycerol were subtracted from the reactions containing lipids. Potential inhibitors were added to the assay mixture in DMSO at a final concentration of 0.1, 1, and 5 mol %. These amounts are equivalent to 4.3, 43, and 218 μM, respectively. The IC_{50} value is the concentration of inhibitor which gives 50% inhibition of the protein kinase C maximal activity.

Human Tumor Cell Growth Inhibition. MCF-7, a human breast tumor cell line, and MCF-7/ADR, an adriamycin resistant line of MCF-7 cells, were obtained from the National Cancer Institute, Frederick, MD. Human myeloma cell line 8226 and its adriamycin-resistant line 8226/ADR were obtained from William Dalton at the University of Arizona, Tucson, AZ. Tumor cells were trypsinized with 0.05% trypsin obtained from GIBCO (Grand Island Biological Co. Laboratories, Grand Island, NY), counted with a hemacytometer, and seeded at a concentration of 7500 cells/well in a 96-well microtiter plate and allowed to attach to the surface overnight. To determine a concentration response, test agents were serially diluted and 100 μL/well added at 2× final concentration to quadruplicate cultures to bring the total volume of each well to 200 μL. The microtiter plate was then incubated at 37 °C, 5% CO_2 for 24 h with [^{3}H]thymidine added at a concentration of 0.5 μCi/well in 50 μL culture medium during the last 4 h of incubation. Supernatant was then aspirated and 50 μL of 0.05% trypsin (GIBCO) was added to each well. Cells were checked microscopically to determine detachment from surfaces, and plates were harvested with a cell harvester (PHD, Cambridge Technology, Inc.). Filter papers corresponding to wells were placed in scintillation vials and counted to determine the amount [^{3}H]thymidine incorporated by the cells. Counts per minute (CPM) of quadruplicate cultures were averaged and a percent of control calculated for each test agent concentration by the following formula:

$$\% \text{ control} = \frac{\text{mean CPM test agent}}{\text{mean CPM control (media only)}} \times 100$$

The IC_{50} (concentration necessary to inhibit the proliferation of cells by 50% compared to control) of each test agent was determined by plotting percent control vs log concentration for each dilution and then linear forecasting the 50% control value onto the x axis on a Quattro Pro computer program. Only regression values with a correlation coefficient greater then 0.9 were considered acceptable.

Human Tumor Cloning Assay. Collection of Tumor Cells. Malignant effusions and ascites were obtained by standard techniques. They were placed in sterile glass vacuum containers, with 10 units of preservative-free heparin per 1 mL of malignant fluid. Solid tumor samples were minced into 2–5-mm fragments in the operating room and were placed immediately in McCoy's 5A medium plus 10% newborn calf serum, 1% penicillin and streptomycin, 15 mM HEPES buffer, and 2 mM pyrurate (all from GIBCO). Single-cell suspensions were prepared from six solid tumors and effusions as described previously.[7] After preparation of single-cell suspensions, the cells were washed in McCoy's 5A medium plus 10% heat-inactivated fetal calf serum (GIBCO).

Exposure to Agents. Stock solutions of both **3** and **4** were prepared in 100% DMSO and stored at −70 °C. Cells were incubated with and without drug for 1 h at 37 °C in McCoy's 5A medium plus 10% heat-inactivated fetal calf serum.[13] They were then centrifuged at 150g for 10 min, washed twice in McCoy's solution, and placed in the assay system described below. For continuous exposure to the agents, the cells were placed in the media–agar mixture and plated with the cells in the petri dishes.

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Assay for Tumor Colony Forming Units. The culture system utilized has been extensively described elsewhere.[6,7,9,10] In brief, the cells were suspended in 0.3% agar in enriched Connaught Medical Research Laboratories medium 1066 (GIBCO) supplemented with horse serum, glutamine, insulin, asparagine, and penicillin.[7] One milliliter of the resultant mixture was pipeted onto a 1-mL feeder layer in 35-mm plastic petri dishes (Falcon Plastics).[7] The final concentrations of cells in each dish was 5×10^5 cells in each milliliter of agar medium.

To ensure the presence of an excellent single cell suspension, a positive control consisting of orthosodium vandate (vanadium) (Sigma) at a concentration of 200 μg/mL was utilized. For an experiment to be considered evaluable, at least 20 colonies had to be present on control plates and the vanadium had to produce less than a 30% survival of colony forming units. The use of a positive control has been shown to greatly increase the reproducibility of the human tumor cloning assay.[10]

Colonies (>50 cells) usually appeared on day 14 of culture. The number of colonies was determined by counting the colonies on an inverted stage microscope.