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Design and Synthesis of 1,2-Bis(hydroxymethyl)pyrrolo[2,1-*a*]phthalazine Hybrids as Potent Anticancer Agents that Inhibit Angiogenesis and Induce DNA Interstrand Cross-links

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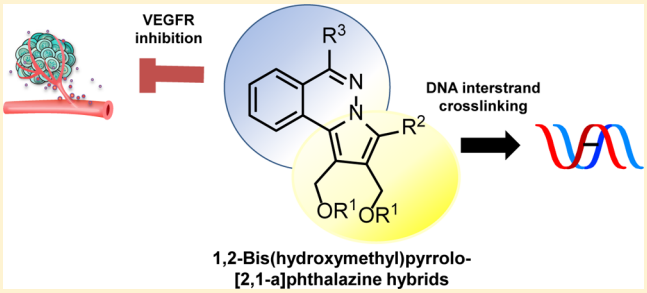
Supporting Information

ABSTRACT: Hybrid molecules are composed of two pharmacophores with different biological activities. Here, we conjugated phthalazine moieties (antiangiogenetic pharmacophore) and bis(hydroxymethyl)pyrrole moieties (DNA cross-linking agent) to form a series of bis(hydroxymethyl)pyrrolo-[2,1-*a*]phthalazine hybrids. These conjugates were cytotoxic to a variety of cancer cell lines by inducing DNA damage, arresting cell cycle progression at the G2/M phase, triggering apoptosis, and inhibiting vascular endothelial growth factor receptor 2 (VEGFR-2) in endothelial cells. Among them, compound **29d** encapsulated in a liposomal formulation (e.g., **29dL**) significantly suppressed the growth of small-cell lung cancer cell (H526) xenografts in mice. Based on immunohistochemical staining, the tumor xenografts in mice treated with **29dL** showed time-dependent decreases in the intensity of CD31, a marker of blood vessels, whereas the intensity of γ-H2AX, a marker of DNA damage, increased. The present data revealed that the conjugation of antiangiogenic and DNA-damaging agents can generate potential hybrid agents for cancer treatment.



INTRODUCTION

Antiangiogenic therapy (AAT) is a strategy for treating cancer involving starving cancer cells to death.[1] Bevacizumab, a monoclonal antibody that neutralizes vascular endothelial growth factor A (VEGF-A),[2] is an FDA-approved drug used for the treatment of metastatic solid tumors.[1] Several receptor tyrosine kinase inhibitors, which inhibit vascular endothelial growth factor receptors (VEGFRs), are also approved for cancer treatment as AAT agents.[1] Unfortunately, the rapid appearance of resistance to AAT agents causes treatment failure and even promotes aggressiveness.[3,4] However, AAT is still a relevant and promising strategy for fighting highly aggressive cancers.[5] Therefore, the development of novel AAT agents is desirable. Recently, a large number of small-molecule VEGFR inhibitors with diverse chemical scaffolds have been synthesized and evaluated for their therapeutic potential in oncology.[6] Some of these compounds, such as sunitinib and sorafenib, were approved for the treatment of selected cancers.[7,8]

Several phthalazine derivatives exhibited potent angiogenesis inhibition and anticancer activities.[9−13] Vatalanib (**1**) (Figure 1), an anilinophthalazine derivative,[9,14,15] can inhibit all VEGFRs, but it shows especially high selectivity for VEGFR-2, which is the principle endothelial VEGF signaling receptor and primary mediator for tumor angiogenesis.[16] This agent has been included in several clinical trials on the treatment of metastatic colorectal cancer, pancreatic adenocarcinoma, and relapsed lymphoma.[17−19] Substitution of the bioactive pharmacophores at positions 1 and 4 of the phthalazine core, such as with compounds **2** and **3** (Figure 1), provided derivatives with an improved inhibitory activity toward VEGFR-2 and cancer cells.[10−12,20,21] The conjugation of phthalazine and a triazole ring was an alternative approach for generating phthalazine-based VEGFR-2 inhibitors (**4**) with enhanced anticancer activity (Figure 1).[13] Moreover, phthalazine-based compound exerted diverse pharmacological activities, such as AMG900 (**5**), which acts as a potent inhibitor of aurora kinase, and phthalazino[1,2-*b*]quinazolinones (**6**), which is a p53 activator (Figure 1).[22−24] These studies revealed important information for phthalazine-based drug development.

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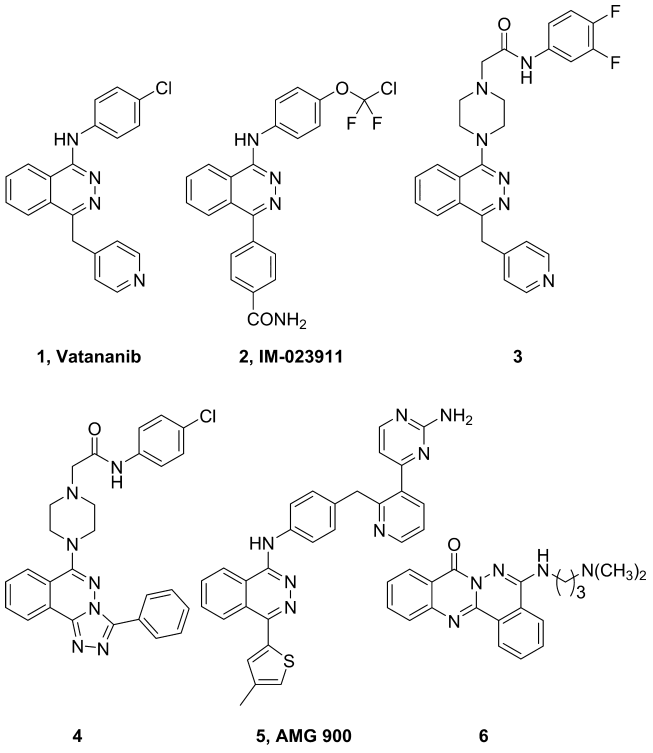

Figure 1. Phthalazine derivatives are potent antiangiogenesis inhibitors and anticancer agents.

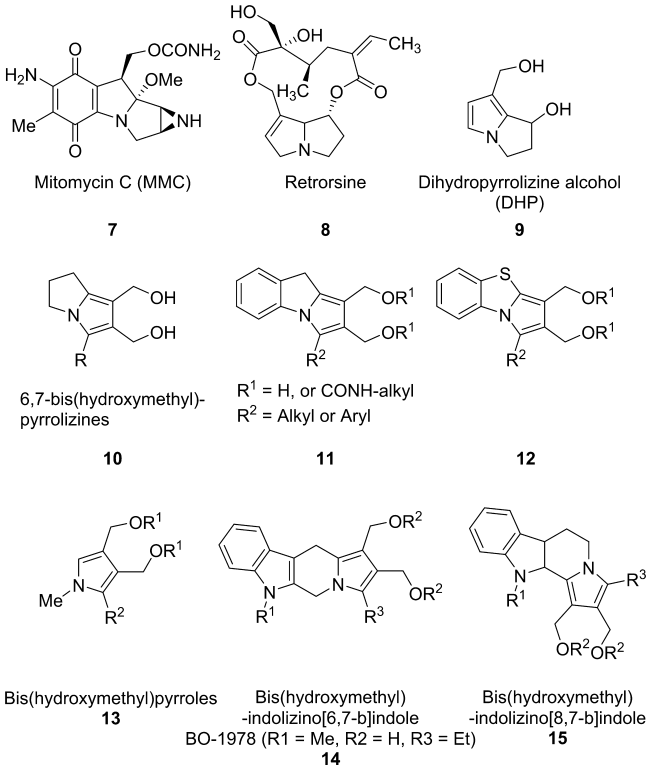

Figure 2. DNA cross-linking agents and their active metabolites.

On the other hand, DNA cross-linking agents are clinically used for cancer chemotherapy, particularly for late-stage and drug-resistant tumors.[25] In general, designed DNA cross-linking agents comprising two reactive electrophilic centers that attack DNA to form intrastrand or interstrand cross-links (ICLs) have potent cytotoxic activity, as they can interfere with replication and transcription, leading to cell death.[26] Mitomycin C (**7**) (Figure 2), a DNA cross-linking therapeutic frequently used in the clinic, possesses two reactive nucleophilic centers that are converted into DNA interactive groups via bioreductive activation,[27−30] and several naturally occurring pyrrolizidine alkaloids, such as retrorsine and its metabolite, dihydropyrrolizine alcohol (**8** and **9**) (Figure 2), are able to induce DNA cross-linking without enzymatic activation.[31−33] In addition, the pyrrolizine core, as in 1,2-bis(hydroxymethyl)pyrrolizines (**10**) (Figure 2), is a privileged motif in designing bifunctional DNA cross-linking agents with broad structural diversity.[34] We designed and synthesized several series of compounds containing pyrrolizine units that can form DNA ICLs (**11**−**12**) (Figure 2).[35,36]

The design of hybrid molecules is a potential strategy for developing new drugs. Hybrid molecules comprise two distinct biologically active domains that attack different targets, that is, one molecule that displays two modes of action.[37] Thus, hybrid molecules may mimic the combination therapies but with enhanced efficacy and reduced adverse side effects. We recently conjugated *β*-carbolines and bis(hydroxymethyl)-pyrroles (**13**) to form indolizino[6,7-*b*]indole (**14**) and indolizino[8,7-*b*]indole hybrids (**15**) and their bis-(alkylcarbamates) for antitumor studies (Figure 2).[38,39] The *β*-carboline component intercalates into DNA and inhibits topoisomerases,[40] while the bis(hydroxymethyl)pyrrole component induces DNA cross-link formation.[41] Of these hybrid molecules, BO-1978 (**14**, wherein R^1 = Me, R^2 = H, and R^3 = Et) potently inhibits topoisomerase I and II and induces DNA interstrand cross-linking (Figure 2),[38] but it also exhibits potent therapeutic efficacy against the growth of human non-small-cell lung cancer (NSCLC) xenografts.[42] These studies support that conjugation of bis(hydroxymethyl)pyrrole with another active pharmacophore is an effective strategy for identifying potent anticancer agents with dual mechanisms.[41]

While angiogenesis is a hallmark of tumor development and metastasis is now a validated target for cancer treatment,[43] the outcomes of AAT are still far from the desired overall benefits.[44,45] In the clinic, antiangiogenic drugs are most effective when they are used in combination with additional therapies, usually chemotherapy or immunotherapy.[46−49] On the other hand, the adverse effects of DNA cross-linking agents also limit their clinical application.[50] The development of novel cytotoxic drugs with increased efficacy but lowered incidence of adverse events is of great interest. Therefore, we propose to generate novel hybrid anticancer agents, which may display both antiangiogenesis and DNA cross-linking properties and provide increased anticancer efficacy and reduced toxicity.

In the present work, we coupled a phthalazine base (**16**) bis(hydroxymethyl)pyrrol (**13**) to form 1,2-bis-(hydroxymethyl)pyrrolo[2,1-*a*]phthalazines (**17**) (Figure 3). To better understand the structure−activity relationship (SAR), a series of new hybrids were synthesized with various substituents, such as Me, Et, or a substituted aryl group at C3 and secondary amine side chains at C6 (Figure 3). The systematic chemical modifications of the phthalazine derivatives allowed us to determine the SAR through cytotoxicity analysis. In addition, these diols were converted to the corresponding alkylcarbamates to understand whether the alkylcarbamate group could serve as a better leaving group and favor the interaction with DNA over what was achieved with the OH group. Experiments were also conducted to

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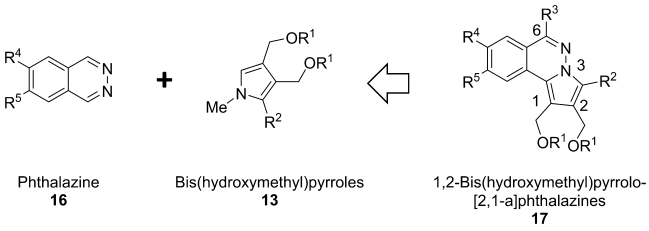


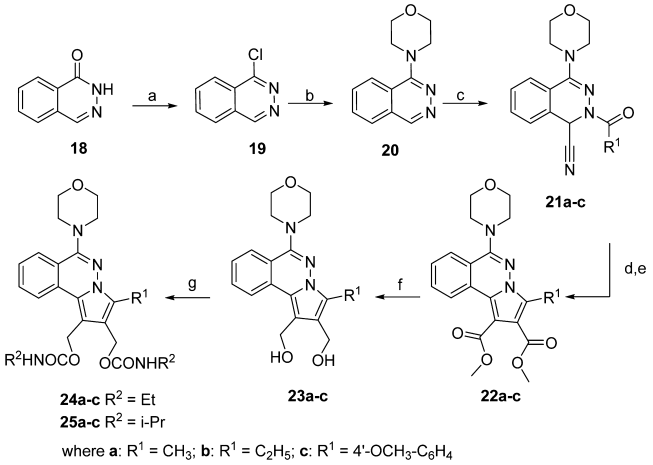
Figure 3. Retrosynthetic analysis for the synthesis of pyrrolo[2,1-*a*]phthalazine hybrids.

demonstrate that these novel hybrids not only possessed significant antiangiogenic activity in endothelial cells but also displayed potent DNA cross-linking activity in cancer cells. Furthermore, we confirmed the in vivo anti-small-cell lung cancer (SCLC) activity of one of the derivatives using liposomes as a delivery system.

RESULTS AND DISCUSSION

Chemistry. As shown in Scheme 1, commercially available 1-phthalazinone (**18**) was treated with phosphorus oxychloride

Scheme 1. Chemical Synthesis of 6-Morpholinopyrrolo[2,1-*a*]phthalazine Derivatives[a]

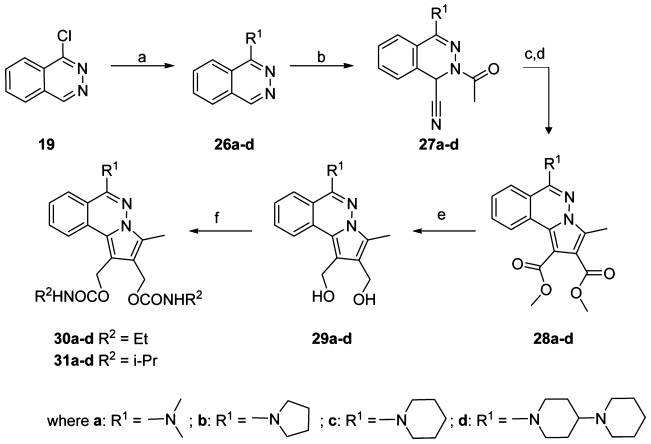


[a]Reagents and conditions: (a) phosphorus oxychloride, reflux; (b) morpholine, TEA, ethanol, reflux; (c)TMSCN, $AlCl_3$, R^1COCl, DCM, rt; (d) HBF_4, AcOH, 60 °C; (e) DMAD, DMF, 100 °C; (f) LAH, ether, DCM, 0−25 °C; (g) R^2NCO, TEA, DMF.

to give 1-chlorophthalazine (**19**) by the reported procedure.[51] The reaction of **19** with morpholine in ethanol in the presence of TEA gave compound **20**, which was then reacted with trimethylsilyl cyanide (Me_3SiCN) and various alkyl or aryl acid chlorides in dichloromethane (DCM) to afford compounds **21a−c**. Treatment of **21a−c** with tetrafluoroboric acid (HBF_4) in ether yielded the intermediate hydrofluoroborate salts, which were immediately reacted with dimethyl acetylenedicarboxylate (DMAD) to afford diester derivatives **22a−c**.[52] Diesters **22a−c** were reduced to generate the corresponding 6-morpholinopyrrolo[2,1-*a*]phthalazine dimethanol derivatives **23a−c** by treatment with lithium aluminum hydride (LAH) in a mixture of ether/DCM in an ice bath. Compounds **23a−c** were further converted into their corresponding bis(alkylcarbamate) congeners **24a−c** (R^2 = Et) or **25a−c** (R^2 = *i*-Pr) in good yields by treatment with ethyl isocyanate or isopropyl isocyanate, respectively, in the presence of trimethylamine (TEA). These derivatives (**23a−c**, **24a−c**, and **25a−c**) with alkyl (such as Me or Et) and aryl (4′-MeO-Ph) substituents at C3 would allow us to study the effect of the substituent at C3 on the cytotoxicity.

Our preliminary antitumor screening showed that C3-Me derivatives (e.g., **23a**, **24a**, and **25a**) were the more cytotoxic than the corresponding C3-Et or C3-phenyl-substituted compounds (e.g., **23b−c**, **24b−c**, and **25b−c**). We therefore focused on preparing C3-Me-pyrrolo[2,1-*a*]phthalazines bearing various secondary amines at C6 for antitumor evaluation. As shown in Scheme 2, compound **19** was first reacted with

Scheme 2. Chemical Synthesis of 6-Substituted Pyrrolo[2,1-*a*]phthalazine Derivatives[a]



[a]Reagents and conditions: (a) secondary amines R_1NH, TEA, ethanol, reflux; (b) TMSCN, $AlCl_3$, CH_3COCl, DCM, rt; (c) HBF_4, AcOH, 60 °C; (d) DMAD, DMF, 100 °C; (e) LAH, ether, DCM, 0−25 °C; (f) R^2NCO, TEA, DMF.

various secondary amines in ethanol in the presence of TEA to obtain compounds **26a−d** by the previously described method.[53] As described above, the reactions of compounds **26a−d** with Me_3SiCN and acetyl chloride in the presence of a catalytic amount of $AlCl_3$ yielded compounds **27a−d**, which were converted into the corresponding hydrofluoroborate salts (tetrafluoroboric acid/ether) and then reacted with DMAD to give diesters **28a−d**. The reductions of diesters **28a−d** by treatment with LAH/ether/DCM afforded the corresponding bis(hydroxymethyl) derivatives **29a−d**. Compounds **29a−d** were further converted into their corresponding bis(alkylcarbamate) congeners (**30a−d** or **31a−d**) in good yields by treatment with ethyl isocyanate or isopropyl isocyanate, respectively, in the presence of TEA. These derivatives (**29a−d**, **30a−d**, and **31a−d**) with Me at the C3 position and different secondary amine side chains at C6 would allow us to study the effect of the substituent at C6 on the cytotoxicity.

Biological Data. For the SAR analysis, the in vitro cytotoxicity of the 1,2-bis(hydroxymethyl)pyrrolo[2,1-*a*]phthalazines (**23a−c**, **24a−c**, and **25a−c**) with morpholine substituents at C6 (R^1 = morpholine) was first examined in human lymphoblastic leukemia CCRF-CEM (Table 1). In these derivatives, different sized functional groups at C3 (e.g., R^2 = Me, Et, or 4′-MeO-Ph) were introduced. The SAR analysis clearly showed that increasing the size of the substituent at the C3 position generally attenuated the cytotoxicity of the bis(hydroxymethyl)pyrrolo[2,1-*a*]phthalazine derivative toward CCRF-CEM cells (i.e., 4′-

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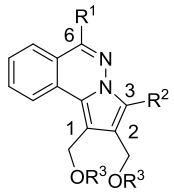
Table 1. Cytotoxicity of 1,2-Bis(hydroxymethyl)pyrrolo[2,1-*a*]phthalazines and Their Biscarbamate Derivatives



Compound	R^1	R^2	R^3	IC_{50} (μM) CCRF-CEM	IC_{50} (μM) CEM/VBL
23a	morpholine	Me	H	0.76 ± 0.03	0.56 ± 0.06 [0.74×][a]
23b	morpholine	Et	H	3.04 ± 0.38	2.55 ± 0.12 [0.84×]
23c	morpholine	4′-MeO-C_6H_4	H	6.63 ± 1.74	5.75 ± 0.62 [0.86 ×]
24a	morpholine	Me	CONHEt	0.69 ± 0.22	0.45 ± 0.03 [0.65×]
24b	morpholine	Et	CONHEt	3.44 ± 0.82	5.11 ± 0.91 [1.48 ×]
24c	morpholine	4′-MeO-C_6H_4	CONHEt	6.12 ± 0.88	7.21 ± 0.25 [1.18 ×]
25a	morpholine	Me	CONH-*i*-Pr	1.17 ± 0.18	0.96 ± 0.16 [0.82×]
25b	morpholine	Et	CONH-*i*-Pr	3.68 ± 0.25	1.58 ± 0.20 [0.43×]
25c	morpholine	4′-MeO-C_6H_4	CONH-*i*-Pr	2.85 ± 1.52	3.07 ± 0.43 [1.07×]
29a	dimethylamine	Me	H	3.33 ± 0.39	2.94 ± 0.26 [0.88×]
30a	dimethylamine	Me	CONHEt	0.73 ± 0.05	0.55 ± 0.06 [0.75×]
31a	dimethylamine	Me	CONH-*i*-Pr	1.54 ± 0.22	0.97 ± 0.17 [0.63×]
29b	pyrrolidine	Me	H	0.92 ± 0.07	0.47 ± 0.05 [0.51×]
30b	pyrrolidine	Me	CONHEt	1.42 ± 0.09	2.10 ± 0.09 [1.47×]
31b	pyrrolidine	Me	CONH-*i*-Pr	4.11 ± 0.15	3.16 ± 0.18 [0.77×]
29c	piperidine	Me	H	1.04 ± 0.12	1.33 ± 0.13 [1.28×]
30c	piperidine	Me	CONHEt	1.28 ± 0.09	1.23 ± 0.20 [0.96×]
31c	piperidine	Me	CONH-*i*-Pr	1.92 ± 0.11	1.73 ± 0.09 [0.90×]
29d	1,4′-bipiperidine	Me	H	0.23 ± 0.03	0.25 ± 0.04 [1.09×]
30d	1,4′-bipiperidine	Me	CONHEt	4.51 ± 0.29	4.49 ± 0.57 [1.00×]
31d	1,4′-bipiperidine	Me	CONH-*i*-Pr	3.61 ± 0.1	3.61 ± 0.71 [1.00×]
cisplatin				16.53 ± 0.90	8.88 ± 1.86 [0.54×]
vinblastine				1.41 ± 0.10	392.48 ± 44.75 [278.3×]

[a]Resistance factor.

Table 2. In Vitro Cytotoxicity of 1,2-Bis(hydroxymethyl)pyrrolo[2,1-*a*]phthalazine Derivatives to Human Solid Tumor Cell Lines

Compound	IC_{50} (μM) HCT-116	H460	H526	PaCa S1
23a	1.67 ± 0.18	2.64 ± 0.52	0.10 ± 0.01	2.16 ± 0.09
23b	11.53 ± 1.66	18.57 ± 0.18	0.75 ± 0.12	13.02 ± 0.93
23c	5.20 ± 0.43	18.72 ± 1.81	1.95 ± 0.26	36.63 ± 1.04
24a	1.70 ± 0.21	2.48 ± 0.73	0.21 ± 0.03	17.23 ± 3.48
29b	3.96 ± 0.06	3.87 ± 0.73	0.30 ± 0.04	11.73 ± 1.16
29d	0.40 ± 0.09	0.60 ± 0.11	0.014 ± 0.004	0.30 ± 0.08
30a	2.45 ± 0.65	3.47 ± 0.47	0.57 ± 0.09	8.01 ± 1.40
cisplatin	9.17 ± 0.67	5.11 ± 0.22	0.73 ± 0.07	27.04 ± 0.98

MeO-Ph < Et < Me), except that the C3-4′-MeO-Ph-substituted compound (**25c**) was slightly more cytotoxic than the C3-Et-substituted derivative (**25b**). Although good leaving groups were introduced to C1 and C2 (i.e., R^3 = ethyl or iso-pro carbamate) (**24a−c** or **25a−c**), their cytotoxicity toward leukemia cells was similar to those of the corresponding compounds with a bis-diol moiety at C1 and C2 (**23a−c**).

Because C3-Me-substituted bis(hydroxymethyl)pyrrolo[2,1-*a*]phthalazine derivatives (**23a**, **24a**, and **25a**) were generally more cytotoxic than those with ethyl or 4′-MeO-Ph substituents at the C3 position, C3-Me-substituted bis-(hydroxymethyl)pyrrolo[2,1-*a*]phthalazine derivatives with various secondary aliphatic and heterocyclic amines at C6 (e.g., R^1 = dimethylamine, pyrrolidine, piperidine, and 1,4′-bipiperidine) (**29a−d**, **30a−d**, and **31a−d**) were synthesized. Together with morpholine substituents at C6, compounds with different C6 substituents, that is, those in different series of derivatives, such as bis-diol (**23a** and **29a−d**), bis-ethylcarbamate (**24a** and **30a−d**), and bis-iso-pro carbamate (**25a** and **31a−d**), did not exhibit consistent cell killing potency against CCRF-CEM cells (Table 1). However, among those with various secondary aliphatic and heterocyclic amines at C6, the derivatives with morpholine substituents at C6 were generally the most cytotoxic toward leukemia cells. Intriguingly, a bipiperidine substituent at C6 of a bis-diol derivative (compound **29d**) afforded the most potent compound in this

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J. Med. Chem. 2019, 62, 2404−2418

study; its IC_{50} against CCRF-CEM cells was 0.23 ± 0.03 μM. Compound **29d** was thus selected for mechanistic and animal studies.

Furthermore, as shown in Table 1, these newly synthesized compounds were 2.5- to 72-fold more cytotoxic to CCRF-CEM cells than cisplatin, and more than 90% of them had IC_{50} values less than 5 μM. In addition, the CCRF-CEM/VBL cell line, an acquired multidrug resistant (MDR) cell line featuring P-glycoprotein overexpression, was used to determine whether these newly synthesized compounds displayed cross-resistance to MDR reagents. Compared with the parental CCRF-CEM cells, CCRF-CEM/VBL cells were first confirmed as remarkably resistant cells to vinblastine (Table 1). However, CCRF-CEM/VBL cells showed no obvious cross-resistance to any of the newly synthesized molecules. Some of the new compounds were even more cytotoxic to CCRF-CEM/VBL than they were to parental CCRF-CEM. The range of the resistant factor (RF) values was 0.43−1.48. Among all compounds examined, compound **29d**, with RF = 1.09, was the most cytotoxic toward drug-resistant CCRF-CEM/VBL cells.

In addition to leukemia cells, several compounds were selected for further evaluation of their antiproliferative activity in a number of human solid tumor cell lines, including colorectal cancer HCT-116 cells, pancreatic cancer PaCa-S1 cells, NSCLC H460 cells, and SCLC H526 cells (Table 2). Among **23a−c**, compound **23a** displayed the most activity toward these four solid tumor cell lines, consistent with the previous observation that smaller substituents at C3 result in stronger cytotoxicity. Intriguingly, SCLC H526 cells were the most susceptible to the tested compounds; the IC_{50} values ranged from nanomolar to submicromolar levels. Of the compounds examined, compound **29d** exhibited highly potent antitumor activity against a broad spectrum of tumor cell lines.

The pyrrolo[2,1-*a*]phthalazine hybrid molecules were designed by assembling a DNA cross-linking moiety and an antiangiogenic moiety. This DNA cross-linking moiety, bis(hydroxymethyl)pyrrole, has been widely used as a scaffold for the development of a variety of therapeutic agents.[34,54] The two adjacent hydroxymethyl groups serve as leaving groups and lead to the formation of electrophilic centers that attack DNA to form cross-links. The alkaline gel electrophoresis was performed to demonstrate the DNA ICL-inducing activity of the pyrrolo[2,1-*a*]phthalazine derivatives. As shown in Figure 4A, compound **29d** dose-dependently induces a significant amount of DNA ICLs in the concentration range of 0.1−1 μM, while compound **23b** required higher concentrations (>10 μM) to achieve the same effect. These results showed the consistency between the cytotoxicity toward cancer cell lines and in vitro DNA cross-linking activity of the pyrrolo[2,1-*a*]phthalazine derivatives, confirming that the induction of DNA ICLs is one of the mechanisms causing cell death.

While cell cycle interference occurs in cells treated with DNA cross-linking agents, the effects of compound **29d** were further investigated on cell cycle progression in H460 cells. As shown in Figure 4B and Supporting Information Figure S1, cell cycle progression was obviously influenced by compound **29d**. By increasing the concentration of compound **29d**, the G2/M phase accumulation was observed at low concentrations (0.125 and 0.25 μM), the S phase accumulation was observed at a moderate concentration (0.5 μM), and the G1 phase accumulation was observed at high concentrations (1 and 2 μM). Accordingly, the pattern of cell cycle interference

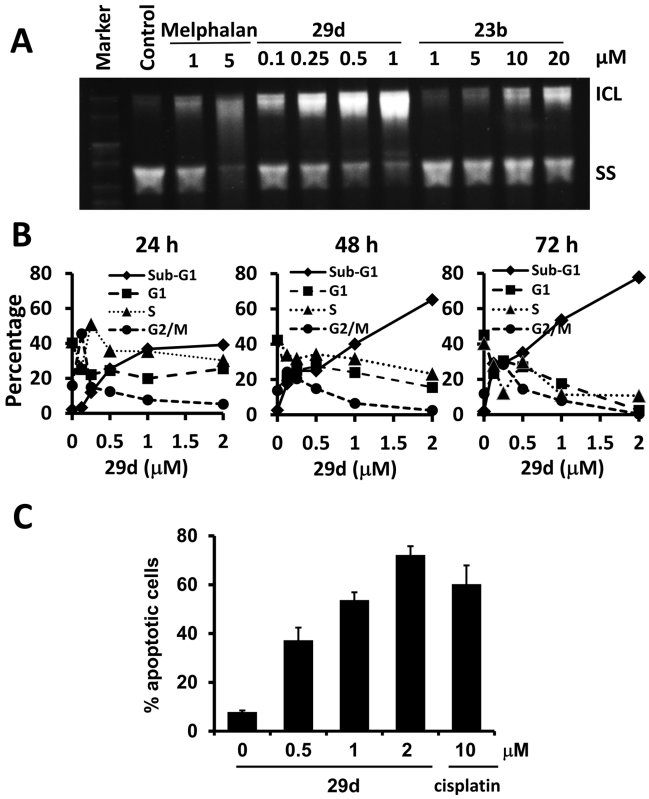

Figure 4. Induction of DNA ICL, cell cycle progression interference, and apoptotic cell death by phthalazine derivatives. (A) Plasmid pEGFP-N1 was incubated with various concentrations of compounds **29d** or **23b** at 37 °C for 2 h. After linearization by digestion with BamH1, the DNA was denatured under alkaline conditions. The interstrand cross-linked and SS DNA molecules were electrophoretically separated on alkaline gel. Melphalan was included as a positive control. (B) Growing H460 cells were treated with various concentrations of compound **29d** for 24, 48, and 72 h. At the end of the treatment, the cells were harvested, fixed, stained with propidium iodide, and subjected to flow cytometric analysis. The cell cycle phases at 24, 48, and 72 h were analyzed with ModFit LT 3.0 software. Data are the average of 2 experiments. (C) H460 cells were treated with various concentrations of compound **29d** for 48 h. At the end of the treatment, the cells were harvested, stained with Annexin V-FITC and propidium iodide, and subjected to flow cytometric analysis. The Annexin V^+ cells (the first and fourth quadrants) represented late and early apoptotic cells. Cisplatin (10 μM) was used as a positive control. The data are the average of 3 independent experiments. Bars, SE.

induced by compound **29d** was typically attributed to the formation of DNA cross-links.[38,39] In addition, we noticed time- and dose-dependent accumulation of the sub-G1 population, which is an indicator of apoptotic cells. Furthermore, it was confirmed that compound **29d** induced apoptotic cell death by using an Annexin V staining assay (the Supporting Information, Figure S2). As summarized in Figure 4C, Annexin V^+ cells (the first and fourth quadrants) were significantly and dose-dependently increased in H460 cells treated with various concentrations of compound **29d**. Together, these results indicate that compound **29d** induces DNA damage and hence interferes with cell cycle progression, triggering apoptotic cell death.

There are several critical steps involved in angiogenesis and blood capillary formation, such as endothelial cell survival, proliferation, migration, organization, and remodeling into a

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J. Med. Chem. 2019, 62, 2404−2418

capillary-like structure.[55] Tumor-induced angiogenesis is facilitated by the production of a number of angiogenic factors.[56] Approximately 30 endogenous pro-angiogenic factors are known to participate in angiogenesis. These hybrids were designed to challenge traditionally combinational therapies using antiangiogenic and DNA interstrand cross-linking agents. To demonstrate the antiangiogenic properties of these hybrids, the cytotoxicity of compounds **29d** and **23b** and vatalanib were first evaluated toward human endothelial EA.hy926 cells. As shown in Supporting Information Figure S3, compound **29d** was more cytotoxic to EA.hy926 cells (IC_{50} = 1.41 ± 0.31 μM) than were compound **23b** (IC_{50} = 8.84 ± 3.89 μM) and vatalanib (IC_{50} = 19.98 ± 7.54 μM). However, compared with the IC_{50} values of these compounds against the four human solid tumor cell lines analyzed, compounds **29d** and **23b** were approximately 5- to 100-fold less toxic to EA.hy926 cells.

VEGF, the most potent mediator of angiogenesis,[56] stimulates endothelial cell survival, proliferation, migration, and invasion via activation of specific tyrosine kinase receptors, VEGFRs, through autophosphorylation.[57] The in vivo angiogenic response to VEGF in tumors is mainly mediated via the activation of VEGFR-2.[58] Phthalazine is the core structure of vatalanib, and it is the moiety that binds VEGFR-2 and inhibits its activation.[9] We therefore performed western blotting analysis to examine the levels of VEGFR-2 and phosphorylated VEGFR-2 (p-VEGFR-2) in endothelial EA.hy926 cells treated with **29d**, **23b**, and vatalanib for 12 h.[59] As shown in Figure 5, these compounds at noncytotoxic

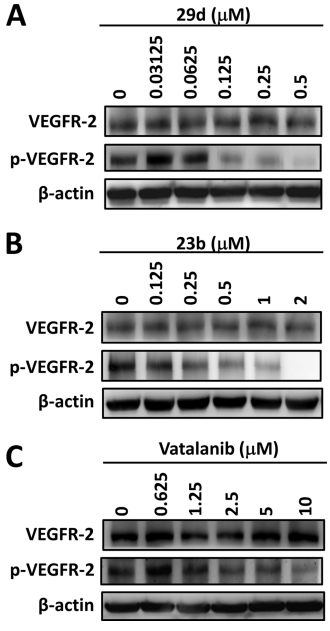

Figure 5. Suppression of VEGFR-2 phosphorylation by the phthalazine derivatives and vatalanib. EA.hy926 cells were treated with various concentrations of **29d** (A), **23b** (B), or vatalanib for 12 h. At the end of the treatment period, the levels of VEGFR-2 and p-VEGFR were determined by western blot analysis.

doses did not significantly change the protein levels of VEGFR-2 but dose-dependently suppressed p-VEGFR-2 protein levels, indicating that **29d** and **23b** could inhibit VEGFR-2 activation to a similar extent as achieved with vatalanib. However, compound **29d** was more potent than **23b** and vatalanib in decreasing p-VEGFR-2. Because one of the main effects of the VEGFR pathway is to enhance the migration of endothelial cells, it was observed that compound **29d** inhibited the migration of EA.hy926 cells at the same dose range in which VEGFR-2 activation was inhibited using the transwell migration assay (the Supporting Information, Figure S4). These results support that our hybrid designs retain the ability to inhibit VEGFR-2.

Angiogenesis is the process of generating new blood vessels as extensions of the existing vasculature. Evaluating the vascular behavior and monitoring tube formation are generally used to experimentally determine whether a compound is capable of interrupting angiogenesis.[60] A tube formation assay using human endothelial EA.hy926 cells was conducted to compare the antiangiogenic activity of newly synthesized hybrids **29d** with vatalanib. The results (Figure 6A) show that

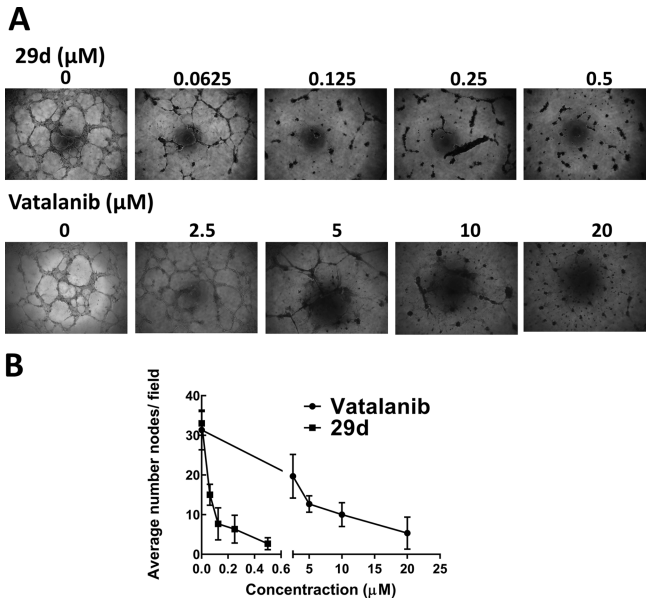

Figure 6. Inhibition of tube formation by a phthalazine derivative **29d** and vatalanib. EA.hy926 cells were cultured on Matrigel-coated plates and treated with various concentrations of **29d** and vatalanib as described in the Experimental Section. (A) Representative images; (B) average number of nodes per field.

robust tubular structures were formed in the control culture, whereas preincubation with **29d** or vatalanib dose-dependently decreased tube formation. However, compound **29d** was approximately 50-fold more potent than vatalanib in suppressing tube formation (Figure 6B), indicating that hybrid compound **29d** obviously retained antiangiogenic activity by the inhibition of VEGFR-2 activation.

On the basis of in vitro data, the therapeutic efficacy of compound **29d** was subsequently evaluated in nude mice bearing human SCLC H526 xenografts. To overcome the poor solubility of **29d**, a liposomal formulation was successfully developed to encapsulate compound **29d** (**29dL**). Liposomal **29dL** was prepared by mixing **29d** with 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), soybean phosphatidylcholine (SPC), cholesterol (CHO), and PEG-2000. For the antitumor experiments, mice bearing H526 tumors were intravenously (iv) administered various doses of liposomal **29dL** via the tail vein every other day six times (Q2D × 6). As shown in Figure 7A, the average volume of the H526 tumors was dose-dependently decreased by treatment with **29dL**. At

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J. Med. Chem. 2019, 62, 2404−2418

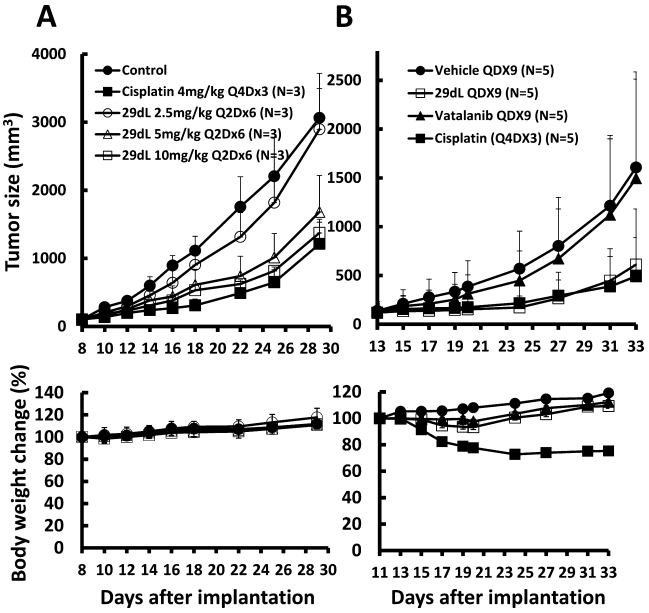

Figure 7. Suppression of tumor growth by liposomal **29d** (**29dL**) using the H526 xenograft model. (A) H526 cells were subcutaneously injected into nude mice. When the tumor size reached approximately 100 mm^3, the mice (n = 5 for each group) were iv administered vehicle or various doses of **29dL** via the tail vein every other day for a total of 6 injections (Q2D × 6). (B) As described in (A), the mice (n = 5 for each group) were treated with vehicle, **29dL** (10 mg/kg), vatalanib (100 mg/kg), or cisplatin (4 mg/kg). **29dL** was given daily for 9 days (QD × 9) and cisplatin every 4 days three times (Q4D × 3) via the tail vein, while vatalanib was orally given for 9 consecutive days. Upper panel, averaged tumor size ± SE; lower panel, averaged body weight ± S.E.

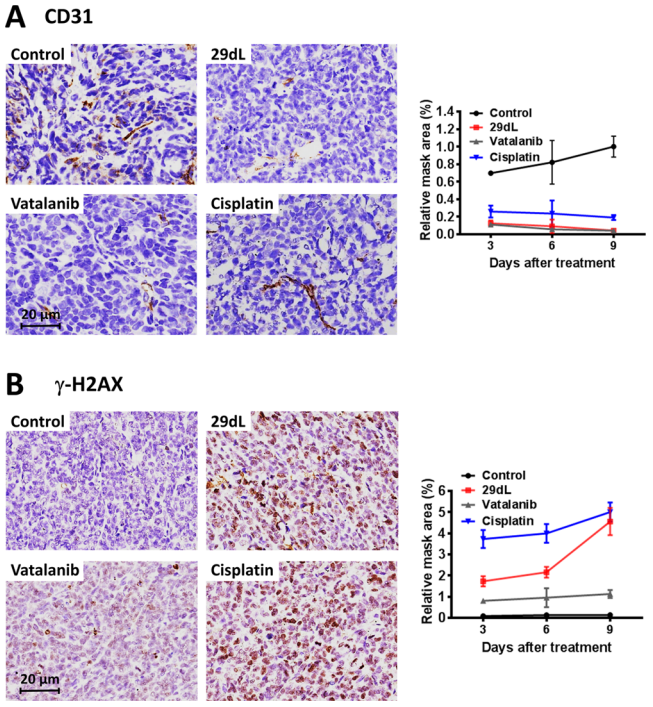

Figure 8. Differential effects on CD31 (blood vessel marker) and γ-H2AX (DNA damage marker) in tumors treated with **29dL**, cisplatin, or vatalanib. (A) Tumor xenografts of H526 cells were treated with **29dL**, cisplatin, or vatalanib as described in Figure 7. Tumors at days 3, 6, and 9 after the initial treatment were harvested, embedded in paraffin, sectioned, and IHC stained with antibodies against CD31 (A) and γH2AX (B). Representative images of IHC stained cells at day 6 after treatment are shown in the left panel. The relative intensities of CD31 and γH2AX in the tumor sections are shown in the right panel. Data are the mean ± SE of 15 randomly selected fields from 3 tumor sections.

the maximal achievable dose (10 mg/kg), liposomal **29dL** suppressed tumor growth by approximately 55% on D30. In addition, no body weight change was observed in mice treated with **29dL**, indicating that mice could tolerate **29dL** at the doses used. Then, the efficacy of **29dL** (10 mg/kg, QD × 9 via iv injection) was compared with that of vatalanib (100 mg/kg, QD × 9 given orally) and cisplatin (4 mg/kg, Q4D × 3 via iv injection). The results of Figure 7B revealed that **29dL** was more potent than vatalanib and almost as efficacious as cisplatin on D33, that is, it suppressed H526 tumors by approximately 60%. Notably, liposomal **29dL** at the tested dosages did not cause body weight loss, which further supports its low toxicity, whereas approximately 25% body weight loss was observed in cisplatin-treated mice (Figure 7B). These results suggest that liposomal **29dL** potently suppresses H526 xenografts but is less systematically toxic.

In a separate experiment, tumors at days 3, 6, and 9 were excised after drug treatment for histopathological examination. Tumor xenograft tissue sections were immunohistochemically (IHC) stained with primary antibodies (anti-rabbit CD31 antibody and anti-mouse γ-H2AX antibody) followed by HRP-conjugated secondary antibody. The intensities of the anti-CD31 and anti-γ-H2AX staining indicated the blood vessel density and the DNA damage, respectively. As shown in Figure 8A, significantly reduced blood vessel formation was observed in tumors treated with **29dL**, vatalanib, or cisplatin compared with that in the vehicle control. The CD31 intensity increased in a time-dependent manner in untreated tumors, whereas on day 9, the intensity was reduced to 4% in tumors treated with **29dL** or vatalanib (Figure 8A). Because cisplatin at the tested dose also significantly suppressed the tumor growth, decreased CD31 expression in cisplatin-treated tumors was also observed but to a lesser extent than what was seen in those treated with **29dL** or vatalanib. These results indicated that **29dL** was almost as potent as vatalanib in inhibiting angiogenesis in an in vivo system.

Because compound **29d** is also a DNA-damaging agent, remarkably increased signals of γ-H2AX were observed in tumors treated with cisplatin or **29dL**, but the signals increase by much less in tumors treated with vatalanib (Figure 8B). The quantitative analysis data are shown in Figure 8B. Signals for γ-H2AX were almost absent in the control tumor. However, time-dependent increases in the γ-H2AX signals in tumors treated with cisplatin and **29dL** were observed. In tumors treated with vatalanib, a moderate signal from γ-H2AX was observed, which may be indirectly caused by the inhibition of blood vessel formation by vatalanib. Taken together, compound **29dL** displays unique activity, for example, this hybrid compound may kill cancer cells via DNA damage and suppress blood vessel formation via the inhibition of VEGFR-2.

CONCLUSIONS

AAT for patients with various types of cancers initially shows clinical benefits, but those benefits rapidly disappear because most patients became refractory or acquire resistance to

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J. Med. Chem. 2019, 62, 2404−2418

angiogenic inhibitors.[61] In animal studies, insufficient doses of VEGF blockers were apt to result in vascular regrowth and limit the tumor-suppression effects.[62] Furthermore, several studies have shown that VEGF blockades suppressed not only tumor vessel formation but also healthy vessel formation, resulting in life-threatening problems such as hemorrhagic and thrombotic events.[63,64] The identification of novel AATs specific for tumors that do not impact the signaling pathways essential for the maintenance of healthy vessels is of utmost importance.

Combination therapy has been frequently used to treat patients with cancer. Because each drug has its own ADME characteristics, it remains to be a great challenge to overcome the pharmacology and toxicology of various drugs given separately. It may also limit the designs of hybrid molecules. However, we have successfully synthesized a series of new compounds with potential anticancer effects. They may simultaneously target cancer cells and tumor microenvironment. Of these novel 1,2-bis(hydroxymethyl)pyrrolo[2,1-*a*]phthalazine hybrids, we found that compound **29d** was able to inhibit the phosphorylation of VEGFR, leading to the suppression of vascular formation, and induce DNA cross-linking, resulting in cell cycle arrest followed by apoptosis. Furthermore, compound **29d** exhibited significant antitumor and antivascular properties in tumor xenograft models. Compound **29d** is composed of two different biological pharmacophores, allowing it to target cancer cells by inducing DNA damage and endothelial cells by suppressing VEGFR activation. Thus, compound **29d** is a promising new compound with potential as an antitumor and antivascular agent that could be superior to common combinational anticancer therapies.

EXPERIMENTAL SECTION

General. All commercial chemicals and solvents were of reagent grade and were used without further purification unless otherwise specified. Melting points were determined in open capillaries on a Fargo MP-2D melting point apparatus. Thin-layer chromatography analyses were performed on silica gel 60 F254 (Merck KGaA, Darmstadt, Germany) with shortwavelength ultraviolet (UV) light for visualization. High-performance liquid chromatography (HPLC) was performed on an Elite LaChrom instrument (HITACHI) with a Mightysil RP-18 (250 × 4.6 mm) column. Compounds were detected by UV light at 260 nm. The mobile phase was acetonitrile/tetrahydrofuran (THF; 80:20 v/v) at a flow rate of 1 mL/min. The purities of all tested compounds were ≥95% based on analytical HPLC. ^{1}H NMR spectra and ^{13}C NMR spectra were taken on Bruker AVANCE Top-Spin spectrometers (400 or 500 MHz) in the solvents indicated. Proton chemical shifts (δ) are reported in parts per million (ppm) relative to $(CH_3)_4Si$ (TMS), and coupling constants (J) are reported in Hertz (Hz). NMR peak splittings are given by the following abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet; and br, broad. Most of the 1,2-bis(hydroxymethyl)pyrrolo[2,1-*a*]phthalazine analogues were characterized by high-resolution mass spectrometry (HRMS) on a Waters HDMS G1 instrument with ESI$^+$ in centroid mode, and the samples were dissolved in MeOH. Notably, some of the carbamate analogues were decomposed by the ESI$^+$ ion source. However, these carbamate phthalazine analogues were characterized using a Thermo Scientific Orbitrap Elite Mass Spectrometer with a low-power ESI$^+$ source. The HPLC chromatograms and ^{1}H NMR, ^{13}C NMR, and HRMS spectra of the new compounds are presented in Supporting Information Figure S5.

*1-Chlorophthalazine (**19**).*[65] A mixture of phthalazin-1(2*H*)-one (**18**, 5.0 g, 34.0 mmol) and phosphorus oxychloride ($POCl_3$) (25 mL) was heated under stirring at 100 °C for 2 h. After cooling to rt, the excess $POCl_3$ was completely removed by distillation under reduced pressure. The residue was triturated with toluene (2 × 25 mL) followed by THF (100 mL). The solid product was collected by filtration and washed with THF. The solid product was then dissolved in DCM, washed with saturated aqueous $NaHCO_3$ solution, dried over sodium sulfate, and concentrated under reduced pressure to give **19**. Yield 4.6 g (82%); mp 119−121 °C (lit.[65] mp 132−134 °C). ^{1}H NMR (DMSO-d_6): δ 8.18−8.22 (2H, m, ArH), 8.31−8.35 (2H, m, ArH), 9.73 (1H, s, ArH). ^{13}C NMR (DMSO-d_6): δ 124.8, 126.1, 128.4, 128.7, 135.0, 151.5, 155.3. HRMS [ESI$^+$]: calcd for $C_8H_5ClN_2$, 165.0220 [M + H]$^+$; found, 165.0212.

*4-(Phthalazin-1-yl)morpholine (**20**).*[66] Morpholine (1.89 mL, 22.0 mmol) was added dropwise to a solution of **19** (3.64 g, 20.0 mmol) in ethanol (120 mL) containing triethylamine (6.96 mL, 50.0 mmol). The reaction mixture was heated at reflux for 18 h, and the solvent was removed under reduced pressure to give a dry residue. The residue was cooled to rt. The obtained crude material was diluted with water and extracted twice with DCM. The separated organic layer was dried over sodium sulfate and concentrated in vacuo to give (**20**). Brown solid; yield 3.8 g (80%); mp 125−127 °C ^{1}H NMR (DMSO-d_6): δ 3.40 (4H, t, J = 4.8 Hz, 2 × CH_2), 3.88 (4H, t, J = 4.8 Hz, 2 × CH_2), 7.94−7.97 (2H, m, ArH), 8.09−8.14 (2H, m, ArH), 9.31 (1H, s, ArH). ^{13}C NMR (DMSO-d_6): δ 51.4, 51.7, 66.5, 120.7, 124.4, 124.5, 127.5, 128.5, 132.4, 132.5, 148.4, 159.7. HRMS [ESI$^+$]: calcd for $C_{12}H_{13}N_3O$, 216.1137 [M + H]$^+$; found, 216.1094.

*2-Acetyl-4-morpholino-1,2-dihydrophthalazine-1-carbonitrile (**21a**).* To a solution of **20** (2.0 g, 9.3 mmol) in DCM (30 mL) containing a catalytic amount of $AlCl_3$ was added dropwise Me_3SiCN (2.32 mL, 18.6 mmol). Acetyl chloride (1.0 mL, 14.0 mmol) was then added dropwise to the above mixture, and the reaction was stirred for 4 h at rt. The reaction mixture was poured into ice water, and the organic layer was washed sequentially with water, 5% NaOH solution, and water. The solution was dried over sodium sulfate and concentrated under vacuum to give **21a**. Yield 2.36 g (89%); mp 140−142 °C. ^{1}H NMR ($CDCl_3$): δ 2.32 (3H, s, $COCH_3$), 3.13−3.18 (2H, m, CH_2), 3.44−3.49 (2H, m, CH_2), 3.81−3.86 (2H, m, CH_2), 3.94−3.98 (2H, m, CH_2), 6.70 (1H, s, CH), 7.42−7.44 (1H, m, ArH), 7.53−7.59 (3H, m, ArH). ^{13}C NMR ($CDCl_3$): δ 16.9, 49.8, 55.8, 66.7, 116.4, 121.5, 124.0, 123.7, 129.2, 130.3, 132.8, 155.5, 169.6. HRMS [ESI$^+$]: calcd for $C_{15}H_{16}N_4O_2$, 285.1352 [M + H]$^+$; found, 285.1341.

Compounds **21b** and **21c** were prepared following the synthetic procedure used to prepare **21a**.

*4-Morpholino-2-propionyl-1,2-dihydrophthalazine-1-carbonitrile (**21b**).* Compound **21b** was prepared from **20** (2.0 g, 9.3 mmol), Me_3SiCN (2.3 mL, 18.6 mmol), and propionyl chloride (1.2 mL, 14.0 mmol). Yield 2.46 g (89%); mp 146−148 °C. ^{1}H NMR (DMSO-d_6): δ 1.05 (3H, t, J = 5.6 Hz, CH_3), 2.53−2.58 (1H, m, CH), 2.71−2.75 (1H, m, CH), 3.03 (2H, m, CH_2), 3.35 (2H, m, CH_2), 3.70 (2H, m, CH_2), 3.87 (2H, m, CH_2), 7.04 (1H, s, CH), 7.64 (3H, m, ArH), 7.80 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 49.8, 51.8, 65.7, 116.4, 121.5, 124.0, 123.7, 129.2, 130.3, 132.8, 155.5, 169.6. HRMS [ESI$^+$]: calcd for $C_{16}H_{18}N_4O_2$, 299.1508 [M + H]$^+$; found, 299.1519.

*2-(4-Methoxybenzoyl)-4-morpholino-1,2-dihydrophthalazine-1-carbonitrile (**21c**).* Compound **21c** was prepared from **20** (3.0 g, 14.0 mmol), Me_3SiCN (3.5 mL, 28.0 mmol), and *p*-anisoyl chloride (2.26 mL, 16.0 mmol). Yield 4.2 g (87%); mp 162−164 °C; ^{1}H NMR (DMSO-d_6): δ 2.92−2.96 (2H, m, CH_2), 3.23−3.26 (2H, m, CH_2), 3.69−3.71 (2H, m, CH_2), 3.80−3.83 (2H, m, CH_2), 3.83 (3H, s, OCH_3), 6.99−7.01 (2H, m, ArH), 7.05 (1H, s, CH), 7.70−7.73 (5H, m, ArH), 7.87−7.89 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 42.1, 49.8, 55.8, 66.0, 113.6, 116.8, 121.2, 124.8, 126.7, 127.7, 129.6, 130.8, 132.8, 132.9, 155.0, 162.4, 169.0; HRMS [ESI$^+$]: calcd for $C_{21}H_{20}N_4O_3$, 377.1614 [M + H]$^+$; found, 377.1623.

*Dimethyl-3-methyl-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-dicarboxylate (**22a**).* To a solution of **21a** (2.0 g, 7.0 mmol) in warm acetic acid (50 mL) was added dropwise HBF_4 (1.55 mL). The mixture was allowed to stir at 50−60 °C for 30 min. After cooling to rt, the yellow solid salt was collected by filtration, and the filter cake was washed with dry ether. The solid salt was dissolved in DMF (20 mL), and DMAD (1.6 mL, 13 mmol) was slowly added to this

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solution. The reaction mixture was heated at 90−100 °C for 16 h. The solvent was removed by evaporation in vacuo. The residue was crystallized from MeOH to give **22a**. Yield 1.5 g (56%); mp 182−183 °C. ^{1}H NMR (DMSO-d_6): δ 2.64 (3H, s, CH_3), 3.30−3.31 (4H, m, 2 × CH_2), 3.80 (3H, s, $COOCH_3$), 3.88 (3H, s, $COOCH_3$), 3.87−3.88 (4H, m, 2 × CH_2), 7.62−7.65 (1H, m, ArH), 7.79−7.82 (1H, m, ArH), 8.05−8.07 (1H, m, ArH), 8.24−8.26 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 13.9, 52.4, 52.7, 55.6, 66.2, 106.7, 115.8, 117.7, 121.0, 124.6, 126.7, 128.9, 130.3, 133.0, 156.2, 159.7, 165.6, 165.9; HRMS [ESI$^+$]: calcd for $C_{20}H_{21}N_3O_5$, 406.1379 [M + Na]$^+$; found, 406.1385.

Compounds **22b** and **22c** were prepared following the synthetic procedure used to prepare **22a**.

*Dimethyl 3-Ethyl-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-dicarboxylate (**22b**).* Compound **22b** was prepared from **21b** (2.0 g, 6.7 mmol), HBF_4 (1.30 mL), and DMAD (1.5 mL, 12.0 mmol). Yield 1.6 g (60%); mp 182−183 °C. ^{1}H NMR (DMSO-d_6): δ 1.21 (3H, t, CH_3), 3.14−3.15 (2H, m, CH_2), 3.30 (4H, m, 2 × CH_2), 3.80 (3H, s, $COOCH_3$), 3.87 (7H, m, 2 × CH_2 and $COOCH_3$), 7.63 (1H, m, ArH), 7.80 (1H, m, ArH), 8.05−8.06 (1H, m, ArH), 8.26−8.27 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 13.9, 23.0, 52.4, 52.7, 55.6, 66.2, 106.7, 115.8, 117.7, 121.0, 124.6, 126.7, 128.9, 130.3, 133.0, 156.2, 159.7, 165.6, 165.9. HRMS [ESI$^+$]: calcd for $C_{21}H_{23}N_3O_5$, 420.1535 [M + Na]$^+$; found, 420.1552.

*Dimethyl 3-(4-Methoxyphenyl)-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-dicarboxylate (**22c**).* Compound **22c** was prepared from **21c** (2.0 g, 5.3 mmol), HBF_4 (1.02 mL), and DMAD (1.7 mL, 13.0 mmol). Yield 1.9 g (77%); mp 192−194 °C. ^{1}H NMR (DMSO-d_6): δ 3.17 (4H, t, J = 4.5 Hz, 2 × CH_2), 3.72 (3H, s, OCH_3), 3.81 (4H, t, J = 4.5 Hz, 2 × CH_2), 3.83 (3H, s, $COOCH_3$), 3.88 (3H, s, $COOCH_3$), 7.04 (2H, d, J = 9.0 Hz), 7.57 (2H, d, J = 9.0 Hz, ArH), 7.66−7.69 (1H, m, ArH), 7.82−7.85 (1H, m, ArH), 8.06−8.07 (1H, m, ArH), 8.73−8.75 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 51.5, 52.4, 52.7, 55.6, 66.2, 106.7, 113.7, 115.8, 117.7, 121.0, 124.6, 126.7, 128.3, 128.9, 130.2, 133.0, 156.2, 159.7, 165.6, 165.9. HRMS [ESI$^+$]: calcd for $C_{26}H_{25}N_3O_6$, 498.1641 [M + Na]$^+$; found, 498.1637.

*(3-Methyl-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-diyl)-dimethanol (**23a**).* A solution of **22a** (1.5 g, 3.9 mmol) in DCM (50 mL) was added dropwise to a stirred suspension of LAH (0.37 g, 9.7 mmol) in diethyl ether (20 mL) at 0−5 °C. After completion of the reaction (2 h), the excess LAH was decomposed by the addition of water (2 mL) and NH_4OH (2 mL). The reaction mixture was filtered through a pad of Celite and washed well with DCM. The combined filtrate and washings were concentrated to dryness in vacuo. The residue was crystallized from ethanol to give **23a**. Yield 1.0 g, (80%), mp 184−186 °C. ^{1}H NMR (DMSO-d_6): δ 2.46 (3H, s, CH_3), 3.23 (4H, m, 2 × CH_2), 3.88 (4H, m, 2 × CH_2), 4.58 (3H, br s, OCH_2 and OH, exchangeable), 4.78 (1H, d, J = 4.5 Hz, OH, exchangeable), 4.82−4.83 (2H, m, OCH_2), 7.44−7.47 (1H, m, ArH), 7.72−7.75 (1H, m, ArH), 7.96−7.98 (1H, m, ArH), 8.28−8.30 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.3, 51.9, 53.8, 54.4, 66.5, 114.4, 115.7, 118.3, 121.6, 123.2, 123.6, 125.3, 126.0, 130.2, 132.3, 154.1. HRMS [ESI$^+$]: calcd for $C_{18}H_{21}N_3O_3$, 310.1556 [M + H−H_2O]$^+$; found, 310.1569.

Compounds **23b** and **23c** were prepared following the synthetic procedure used to prepare **20a**.

*(3-Ethyl-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-diyl)-dimethanol (**23b**).* Compound **23b** was prepared from **22b** (1.4 g, 3.5 mmol) and LAH (0.43 g, 10.5 mmol). Yield 1.2 g (90%); mp 180−182 °C. ^{1}H NMR (DMSO-d_6): δ 1.21 (3H, t, J = 7.5 Hz, CH_3), 2.96 (2H, q, J = 7.5 Hz, CH_2), 3.21 (4H, t, J = 4.5 Hz, 2 × CH_2), 3.86 (4H, t, J = 4.5 Hz, 2 × CH_2), 4.57 (3H, br s, OCH_2 and OH, exchangeable), 4.76 (1H, t, J = 5.1 Hz, OH, exchangeable), 4.82 (2H, d, J = 5.1 Hz, OCH_2), 7.42−7.45 (1H, m, ArH), 7.70−7.74 (1H, m, ArH), 7.95−7.96 (1H, m, ArH), 8.28−8.30 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 13.5, 16.5, 51.3, 53.1, 53.8, 65.8, 113.7, 115.2, 117.5, 120.4, 123.0, 124.7, 125.4, 128.3, 129.7, 131.7, 153.4. HRMS [ESI$^+$]: calcd for $C_{19}H_{23}N_3O_3$, 342.1818 [M + H−H_2O]$^+$; found, 324.1712.

*(3-(4-Methoxyphenyl)-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-diyl)dimethanol (**23c**).* Compound **23c** was prepared from **22c** (1.2 g, 2.86 mmol) and LAH (0.30 g, 8.86 mmol). Yield 0.9 g (83%); mp 190−192 °C. ^{1}H NMR (DMSO-d_6): δ 3.14 (4H, t, J = 4.0 Hz, 2 × CH_2), 3.83 (7H, m, 2 × CH_2 and OCH_3), 4.52 (2H, d, J = 5.0 Hz, OCH_2), 4.85 (1H, t, J = 5.0 Hz, OH, exchangeable), 4.86−4.90 (3H, m, OCH_2 and OH, exchangeable), 7.05 (2H, d, J = 8.8 Hz, ArH), 7.47−7.50 (1H, m, ArH), 7.73 (2H, d, J = 8.8 Hz, ArH), 7.75−7.78 (1H, m, ArH), 7.97−7.98 (1H, m, ArH), 8.37−8.39 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 51.8, 54.0, 54.4, 55.5, 66.4, 113.8, 115.7, 116.5, 119.1, 122.3, 123.1, 124.2, 125.9, 126.0, 127.1, 130.2, 131.5, 132.5, 154.2, 158.8. HRMS [ESI$^+$]: calcd for $C_{24}H_{25}N_3O_4$, 419.1923, [M + H−H_2O]$^+$; found, 402.1818, [M + Na]$^+$; found, 442.1743.

General Procedure for the Preparation of bis-(alkylcarbamate) Derivatives (24a−c and 26a−c). The alkyl isocyanate (4.0 equiv) was added to a solution of bis(hydroxymethyl) derivative (**23a−c**, 1.0 equiv) and TEA (4.0 equiv) in dry DMF or THF. The reaction mixture was stirred for 24−48 h at rt under argon. After completion of the reaction, the reaction mixture was concentrated to dryness in vacuo. The residue was triturated with ether, and the desired product was collected by filtration.

*(3-Methyl-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-diyl)bis-(methylene) bis(ethylcarbamate) (**24a**).* Compound **24a** was prepared from **23a** (0.15 g, 0.5 mmol), TEA (0.25 mL, 2.0 mmol), and ethyl isocyanate (0.15 mL, 2.0 mmol). Yield 0.16 g (75%); mp 171−173 °C. ^{1}H NMR (DMSO-d_6): δ 0.97 (6H, t, J = 6.5 Hz, 2 × CH_3), 2.46 (3H, s, CH_3), 2.97−2.98 (4H, m, 2 × CH_2), 3.23 (4H, m 2 × CH_2), 3.86 (4H, m, 2 × CH_2), 5.17 (2H, s, OCH_2), 5.38 (2H, s, OCH_2), 7.01 (2H, br s, 2 × NH, exchangeable), 7.48−7.51 (1H, m, ArH), 7.75−7.77 (1H, m, ArH), 7.99−8.01 (1H, m, ArH), 8.10−8.11 (1H, m, ArH), ^{13}C NMR (DMSO-d_6): δ 9.35, 15.5, 35.4, 51.9, 56.5, 57.3, 66.4, 109.5, 116.1, 117.6, 119.2, 122.9, 125.2, 126.2, 129.6, 132.7, 154.6, 156.5, 156.6. HRMS [ESI$^+$]: calcd for $C_{24}H_{31}N_5O_5$, 470.2403 [M + Na]$^+$; found, 492.2222.

*(3-Ethyl-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-diyl)bis-(methylene) bis(ethylcarbamate) (**24b**).* Compound **24b** was prepared from **23b** (0.14 g, 0.44 mmol), TEA (0.2 mL, 1.7 mmol), and ethyl isocyanate (0.14 mL, 1.7 mmol). Yellow solid; Yield 0.16 g (75%); mp 165−167 °C. ^{1}H NMR (DMSO-d_6): δ 0.97 (6H, t, J = 7.0 Hz, 2 × CH_3), 1.19 (3H, t, J = 6.5 Hz, CH_3), 2.98−3.00 (6H, m, 3 × CH_2), 3.20 (4H, m, 2 × CH_2), 3.85−3.87 (4H, m, CH_2), 5.17 (2H, s, OCH_2), 5.38 (2H, s, OCH_2), 7.00−7.05 (2H, br s, 2 × NH, exchangeable), 7.49−7.52 (1H, m, ArH), 7.75−7.78 (1H, m, ArH), 7.99−8.01 (1H, m, ArH), 8.09−8.10 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 13.9, 15.5, 15.6, 17.2, 35.5, 51.9, 66.4, 109.4, 116.1, 116.9, 119.1, 122.9, 126.2, 126.4, 129.7, 130.7, 132.7, 154.5, 156.4, 156.6. HRMS [ESI$^+$]: calcd for $C_{25}H_{33}N_5O_5$, 484.2560 [M + Na]$^+$; found, 506.2379.

*(4-Methoxyphenyl-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-diyl) bis(methylene)-bis(ethylcarbamate) (**24c**).* Compound **24c** was prepared from **23c** (0.2 g, 0.47 mmol), TEA (0.25 mL, 1.7 mmol), and ethyl isocyanate (0.15 mL, 1.7 mmol). Yield 0.16 g (64%); mp 168−170 °C. ^{1}H NMR (DMSO-d): δ 0.98 (3H, t, J = 7.0 Hz, CH_3), 1.00 (3H, t, J = 7.0 Hz, CH_3), 2.99−3.02 (4H, m, 2 × CH_2), 3.13 (4H, m, 2 × CH_2), 3.81 (4H, m, 2 × CH_2), 3.82 (3H, s, OCH_3), 5.10 (2H, s, OCH_2), 5.45 (2H, s, OCH_2), 7.05−7.06 (2H, br s, 2 × NH, exchangeable), 7.05 (2H, d, J = 8.5 Hz, ArH), 7.53−7.56 (1H, m, ArH), 7.60 (2H, d, J = 8.5 Hz, ArH), 7.78−7.81 (1H, m, ArH), 7.97−8.02 (1H, m, ArH), 8.16−8.18 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 14.9, 34.9, 51.1, 54.9, 56.6, 56.7, 65.8, 110.0, 113.2, 113.4, 116.2, 117.3, 119.3, 121.7, 122.8, 125.8, 126.1, 127.6, 129.0, 131.0, 131.1, 132.3, 154.1, 155.7, 156.0, 158.6. HRMS [ESI$^+$]: calcd for $C_{30}H_{35}N_5O_6$, 562.2665 [M + Na]$^+$; found, 584.2484.

*(3-Methyl-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-diyl)bis-(methylene) bis(isopropylcarbamate) (**25a**).* Compound **25a** was prepared from **23a** (0.15 g, 0.5 mmol), TEA (0.25 mL, 2.0 mmol), and isopropyl isocyanate (0.18 mL, 2.0 mmol). Yield 0.18 g (79%); mp 159−161 °C. ^{1}H NMR (DMSO-d_6): δ 1.01 (12H, d, J = 6.0 Hz, 4 × CH_3), 2.48 (3H, s, CH_3), 3.24 (4H, m, 2 × CH_2), 3.59 (2H, d, J = 6.0 Hz, 2 × CH), 3.87 (4H, m, 2 × CH_2), 5.19 (2H, s, OCH_2), 5.40 (2H, s, OCH_2), 6.95 (2H, br s, 2 × NH, exchangeable), 7.49−7.52 (1H, m, ArH), 7.75−7.78 (1H, m, ArH), 8.00−8.02 (1H, m, ArH), 8.10−8.12 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.36, 23.0, 23.7,

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J. Med. Chem. 2019, 62, 2404−2418

42.7, 51.9, 56.3, 57.1, 66.4, 109.5, 116.1, 117.6, 119.2, 122.9, 125.2, 126.1, 126.4, 129.6, 132.7, 154.6, 155.8, 156.0. HRMS [ESI$^+$]: calcd for $C_{26}H_{35}N_5O_5$, 498.2716 [M + Na]$^+$; found, 520.2535.

*(3-Ethyl-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-diyl)bis-(methylene) bis(isopropylcarbamate) (**25b**).* Compound **25b** was prepared from **23b** (0.15 g, 0.44 mmol), TEA (0.22 mL, 1.7 mmol), and isopropyl isocyanate (0.17 mL, 1.7 mmol). Yield 0.18 g (81%); mp 147−149 °C; ^{1}H NMR (DMSO-d_6): δ 1.00 (12H, d, J = 6.5 Hz, 4 × CH_3), 1.19 (3H, t, J = 7.5 Hz, CH_3), 2.96 (2H, q, J = 7.5 Hz, CH_2), 3.23 (4H, m, 2 × CH_2), 3.56−3.62 (2H, m, CH), 3.86 (4H, m, 2 × CH_2), 5.17 (2H, s, OCH_2), 5.38 (2H, s, OCH_2), 6.91−6.97 (2H, br s, 2 × NH, exchangeable), 7.49−7.52 (1H, m, ArH), 7.74−7.77 (1H, m, ArH), 7.99−8.01 (1H, m, ArH), 8.08−8.10 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 13.9, 17.2, 23.0, 51.9, 66.4, 109.5, 116.1, 122.9, 126.2, 126.4, 129.7, 130.7, 132.7, 154.5, 155.7, 156.0. HRMS [ESI$^+$]: calcd for $C_{27}H_{37}N_5O_5$, 512.2873 [M + Na]$^+$; found, 534.2692.

*(3-(4-Methoxyphenyl)-6-morpholinopyrrolo[2,1-a]phthalazine-1,2-diyl) bis(methylene) bis(isopropylcarbamate) (**25c**).* Compound **25c** was prepared from **23c** (0.2 g, 0.47 mmol), TEA (0.25 mL, 1.8 mmol), and isopropyl isocyanate (0.2 mL, 1.8 mmol). Yield 0.18 g (64%); mp 152−154 °C. ^{1}H NMR (DMSO-d_6): δ 0.99−1.06 (12H, m, 4 × CH_3), 3.14 (4H, m, 2 × CH_2), 3.61−3.63 (2H, m, 2 × CH), 3.80 (4H, m, 2 × CH_2), 3.84 (3H, s, OCH_3), 5.11 (2H, s, OCH_2), 5.46 (2H, s, OCH_2), 6.97−7.02 (2H, br s, 2 × NH, exchangeable), 7.06 (2H, d, J = 8.5 Hz, ArH), 7.54−7.57 (1H, m, ArH), 7.62 (2H, d, J = 8.5 Hz, ArH), 7.79−7.82 (1H, m, ArH), 8.02−8.03 (1H, m, ArH), 8.17−8.18 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 22.4, 23.1, 46.2, 51.1, 55.0, 65.8, 110.0, 113.3, 114.8, 121.7, 122.8, 125.8, 128.8, 131.2, 133.5, 147.4, 154.1, 158.6. HRMS [ESI$^+$]: calcd for $C_{32}H_{39}N_5O_6$, 590.2979 [M + Na]$^+$; found, 612.2797.

*N,N-Dimethylphthalazin-1-amine (**26a**).* Dimethylamine (90.0 mL, 240.0 mmol) was added slowly to a solution of 1-chlorophthalazine (**19**) (10.0 g, 60.0 mmol) and TEA (28.0 mL, 200.0 mmol) in ethanol (200 mL). The reaction mixture was stirred at rt for 48 h. After completion of the reaction, the solvent was evaporated, and the residue was diluted with water and then extracted with DCM (2 × 200 mL). The organic layer was dried over sodium sulfate and concentrated in vacuo to give the desired product **26a**. Yield 8.0 g (80%); mp 69−71 °C (lit.[44] mp 67 °C). ^{1}H NMR (DMSO-d_6): δ 3.12 (6H, s, $N(CH_3)_2$), 7.90−7.93 (2H, m, ArH), 8.03−8.05 (1H, m, ArH), 8.14−8.15 (1H, m, ArH), 9.18 (1H, s, ArH). ^{13}C NMR (DMSO-d_6): δ 42.8, 120.3, 125.0, 127.1, 128.6, 131.9, 132.1, 146.8, 159.8.

Compounds **26b−d** were prepared following the synthetic procedure used to prepare **26a**.

*1-(Pyrrolidin-1-yl)phthalazine (**26b**).* Compound **26b** was prepared from **19** (10.0 g, 60.0 mmol), pyrrolidine (20.0 mL, 240.0 mmol), and TEA (30.0 mL, 200.0 mmol). Yield 10.0 g (83%); mp 90−91 °C (lit.[45] mp 88−89 °C). ^{1}H NMR (DMSO-d_6): δ ^{1}H NMR (DMSO-d_6): δ 1.95−1.98 (4H, m, 2 × CH_2), 3.80−3.83 (4H, m, 2 × CH_2), 7.80−7.88 (2H, m, ArH), 7.94−7.95 (1H, m, ArH), 8.27−8.29 (1H, m, ArH), 8.97 (1H, s, ArH). ^{13}C NMR (DMSO-d_6): δ 25.8, 51.1, 119.2, 125.2, 126.5, 128.7, 130.9, 131.6, 144.1, 155.7. HRMS [ESI$^+$]: calcd for $C_{12}H_{13}N_3$, 200.1188 [M + H]$^+$; found, 200.1140.

*1-(Piperidin-1-yl)phthalazine (**26c**).* Compound **26c** was prepared from **19** (10.0 g, 60.0 mmol), piperidine (24.0 mL, 240.0 mmol), and TEA (34.0 mL, 240.0 mmol). Yield 10.0 g, (78%), mp 130−132 °C. ^{1}H NMR (DMSO-d_6): δ 1.64−1.67 (2H, m, CH_2), 1.75−1.79 (4H, m, 2 × CH_2), 3.35−3.37 (4H, m, 2 × CH_2), 7.91−7.94 (2H, m, ArH), 8.01−8.07 (2H, m, ArH), 9.25 (1H, s, ArH). ^{13}C NMR (DMSO-d_6): δ 24.6, 26.0, 52.4, 121.0, 124.4, 127.3, 128.5, 132.2, 132.4, 147.8, 160.4. HRMS [ESI$^+$]: calcd for $C_{13}H_{15}N_3$, 214.1344 [M + H]$^+$; found, 214.1364.

*1-([1,4′-Bipiperidin]-1′-yl)phthalazine (**26d**).* Compound **26d** was prepared from **19** (10.0 g, 60.0 mmol), 1,4′-bipiperidine (20.0 g, 120.0 mmol), and TEA (34 mL, 240.0 mmol). Yield 10.2 g (57%); mp 140−142 °C. ^{1}H NMR (DMSO-d_6): δ 1.37−1.42 (2H, m, CH_2), 1.48−1.53 (4H, m, CH_2), 1.75−1.81 (2H, m, CH_2), 1.88−1.91 (2H, m, CH_2), 2.43 (1H, m, CH), 2.57 (4H, m, 2 × CH_2), 2.94−3.00 (2H, m, CH_2), 3.87−3.90 (2H, m, CH_2), 7.92−7.94 (2H, m, ArH), 8.04−8.06 (2H, m, ArH), 9.25 (1H, s, ArH). HRMS [ESI$^+$]: calcd for $C_{18}H_{24}N_4$, 297.2079 [M + H]$^+$; found, 297.2092.

Compounds **27a−d** were prepared following the synthetic procedure used to prepare **21a**.

*2-Acetyl-4-(dimethylamino)-1,2-dihydrophthalazine-1-carbonitrile (**27a**).* Compound **27a** was prepared from **26a** (2.5 g, 14.4 mmol), Me_3SiCN (3.6 mL, 28.8 mmol), and acetyl chloride (1.5 mL, 20.0 mmol). Yield 2.78 g (80%); mp 118−120 °C. ^{1}H NMR (DMSO-d_6): δ 2.25 (3H, s, $COCH_3$), 2.90 (6H, s, $(NCH_3)_2$), 7.08 (1H, s, CH), 7.61−7.68 (3H, m, ArH), 7.82−7.83 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 20.9, 116.7, 121.7, 127.0, 127.5, 129.6, 130.6, 132.6, 155.9, 171.2. HRMS [ESI$^+$]: calcd for $C_{13}H_{14}N_4O$, 243.1246 [M + H]$^+$; found, 243.1241.

*2-Acetyl-4-(pyrrolidin-1-yl)-1,2-dihydrophthalazine-1-carbonitrile (**27b**).* Compound **27b** was prepared from **26b** (5.0 g, 25.0 mmol), Me_3SiCN (6.3 mL, 50.0 mmol), and acetyl chloride (2.7 mL, 37.5 mmol). Yield 5.7 g (85%); mp 123−125 °C. ^{1}H NMR (DMSO-d_6): δ 1.81−1.85 (2H, m, CH_2) 2.00 (2H, m, CH_2), 2.21 (3H, s, $COCH_3$), 3.28−3.34 (2H, m, CH_2), 3.70−3.75 (2H, m, CH_2), 7.06 (1H, s, CH), 7.59−7.66 (2H, m, ArH), 7.81−7.82 (2H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 20.8, 25.3, 49.8, 116.9, 122.7, 126.7, 127.3, 129.7, 130.4, 132.3, 153.5, 170.8. HRMS [ESI$^+$]: calcd for $C_{15}H_{16}N_4O$, 269.1402 [M + H]$^+$; found, 269.1416.

*2-Acetyl-4-(piperidin-1-yl)-1,2-dihydrophthalazine-1-carbonitrile (**27c**).* Compound **27c** was prepared from **26c** (5.0 g, 23.5 mmol), Me_3SiCN (6.0 mL, 47.0 mmol), and acetyl chloride (2.5 mL, 35.0 mmol). Yield 5.7 g (87%); mp 135−137 °C. ^{1}H NMR (DMSO-d_6): δ 1.59−1.64 (4H, m, 2 × CH_2), 1.78−1.80 (2H, m, CH_2), 2.24 (3H, s, $COCH_3$), 3.08−3.12 (2H, m, CH_2), 3.30−3.34 (2H, m, CH_2), 7.07 (1H, s, CH), 7.58−7.67 (3H, m, ArH), 7.80−7.82 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 21.0, 24.5, 25.3, 50.3, 116.7, 121.8, 126.6, 127.5, 129.5, 130.7, 132.7, 155.6, 171.3. HRMS [ESI$^+$]: calcd for $C_{16}H_{18}N_4O$, 283.1559 [M + H]$^+$; found, 283.1536.

*4-([1,4′-Bipiperidin]-1′-yl)-2-acetyl-1,2-dihydrophthalazine-1-carbonitrile (**27d**).* Compound **27d** was prepared from **26d** (2.0 g, 6.7 mmol), Me_3SiCN (1.69 mL, 13.5 mmol), and acetyl chloride (0.88 mL, 10.0 mmol). Yield 1.45 g, (60%); mp 160−162 °C. ^{1}H NMR (DMSO-d_6): δ 1.42−1.45 (1H, m, CH_2), 1.70−1.82 (6H, m, 3 × CH_2), 2.10−2.26 (6H, m, 3 × CH_2), 2.65 (3H, s, $COCH_3$), 2.66−2.73 (1H, m, CH), 2.95−3.00 (3H, m, CH_2), 3.75−3.78 (1H, m, CH_2), 3.86−3.89 (1H, m, CH_2), 7.00 (1H, s, CH), 7.61−7.69 (3H, m, ArH), 7.79−7.81 (1H, m, ArH). HRMS [ESI$^+$]: calcd for $C_{21}H_{27}N_5O$, 366.2294 [M + H]$^+$; found, 366.2308.

Compounds **28a−d** were prepared following the synthetic procedure used to prepare **22a**.

*Dimethyl 6-(dimethylamino)-3-methylpyrrolo[2,1-a]-phthalazine-1,2-dicarboxylate (**28a**).* Compound **28a** was prepared from **27a** (2.7 g, 11.0 mmol), HBF_4 (2.15 mL), and DMAD (2.5 mL, 22.0 mmol) in DMF (30 mL). Yield 1.5 g (45%); mp 164−166 °C. ^{1}H NMR (DMSO-d_6): δ 2.63 (3H, s, CH_3), 2.98 (6H, s, $N(CH_3)_2$), 3.80 (3H, s, $COOCH_3$), 3.88 (3H, s, $COOCH_3$), 7.60−7.63 (1H, m, ArH), 7.76−7.79 (1H, m, ArH), 8.03−8.05 (1H, m, ArH), 8.23−8.24 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 10.5, 52.0, 52.8, 107.0, 112.2, 117.5, 120.5, 123.2, 127.3, 128.1, 128.2, 130.2, 132.8, 156.9, 164.9, 167.1. HRMS [ESI$^+$]: calcd for $C_{18}H_{19}N_3O_4$, 364.1273 [M + Na]$^+$; found, 364.1308.

*Dimethyl-3-methyl-6-(pyrrolidin-1-yl)pyrrolo[2,1-a]phthalazine-1,2-dicarboxylate (**28b**).* Compound **28b** was prepared from **27b** (5.0 g, 18.6 mmol), HBF_4 (3.6 mL), and DMAD (4.5 mL, 37.0 mmol). Yield 2.5 g (48%); mp 176−178 °C. ^{1}H NMR (DMSO-d_6) 1.92 (4H, m, 2 × CH_2), 2.57 (3H, s, CH_3), 3.65 (4H, m, 2 × CH_2), 3.78 (3H, s, $COOCH_3$), 3.86 (3H, s, $COOCH_3$), 7.53−7.57 (1H, m, ArH), 7.72−7.75 (1H, m, ArH), 8.13−8.15 (1H, m, ArH), 8.20−8.21 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 10.5, 25.7, 51.3, 51.9, 52.7, 106.7, 111.7, 117.8, 120.1, 123.0, 127.4, 127.7, 128.1, 129.4, 132.4, 153.7, 165.0, 167.3. HRMS [ESI$^+$]: calcd for $C_{20}H_{21}N_3O_4$, 368.1610 [M + H]$^+$; found, 368.1581.

*Dimethyl-3-methyl-6-(piperidin-1-yl)pyrrolo[2,1-a]phthalazine-1,2-dicarboxylate (**28c**).* Compound (**28c**) was prepared from (**27c**) (5.0 g, 17.7 mmol), HBF_4 (3.4 mL), and DMAD (3.7 mL, 30.0

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J. Med. Chem. 2019, 62, 2404−2418

mmol). Yield 3.0 g (54%); mp 182−183 °C. ^{1}H NMR (DMSO-d_6): δ 1.67 (2H, m, CH_2), 1.79 (4H, m, 2 × CH_2), 2.65 (3H, s, CH_3), 3.28 (4H, m, 2 × CH_2), 3.82 (3H, s, $COOCH_3$), 3.90 (3H, s, $COOCH_3$), 7.64−7.67 (1H, m, ArH), 7.79−7.82 (1H, m, ArH), 7.99−8.00 (1H, m, ArH), 8.25−8.26 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 10.5, 24.5, 25.7, 52.1, 52.4, 52.8, 107.0, 112.4, 117.6, 120.6, 123.2, 126.9, 128.1, 128.3, 130.4, 133.0, 157.0, 164.9, 167.1. HRMS [ESI$^+$]: calcd for $C_{21}H_{23}N_3O_4$, 382.1767 [M + H]$^+$; found, 382.1796.

*Dimethyl-6-([1,4′-bipiperidin]-1′-yl)-3-methylpyrrolo[2,1-a]-phthalazine-1,2-dicarboxylate (**28d**).* Compound **28d** was prepared from **27d** (1.3 g, 3.5 mmol), HBF_4 (0.7 mL), and DMAD (0.8 mL, 6.5 mmol). Yield 1.0 g (70%); mp 189−191 °C. ^{1}H NMR (DMSO-d_6): δ 1.43−1.46 (3H, m, CH_2), 1.68−1.72 (2H, m, CH_2), 1.88−1.91 (2H, m, CH_2), 2.02−2.04 (2H, m, CH_2), 2.14−2.16 (2H, m, CH_2), 2.65 (3H, s, CH_3), 3.01 (4H, m, 2 × CH_2), 3.17 (1H, m, CH), 3.49−3.51 (3H, m, CH_2), 3.81 (3H, s, $COOCH_3$), 3.86−3.88 (2H, m, CH_2), 3.89 (3H, s, $COOCH_3$), 7.64−7.68 (1H, m, ArH), 7.81−7.85 (1H, m, ArH), 7.99−8.01 (1H, m, ArH), 8.26−8.28 (1H, m, ArH),. ^{13}C NMR (DMSO-d_6): δ 12.6, 24.5, 26.2, 28.1, 50.4, 51.5, 58.2, 58.9, 67.5, 70.4, 111.6, 118.1, 123.4, 127.6, 128.5, 129.8, 132.8, 133.5, 147.4, 165.9, 175.7. HRMS [ESI$^+$]: calcd for $C_{26}H_{32}N_4O_4$, 465.2502 [M + H]$^+$; found, 465.2501.

Compounds **29a−d** were prepared following the synthetic procedure used to prepare **23a**.

*(6-(Dimethylamino)-3-methylpyrrolo[2,1-a]phthalazine-1,2-diyl)dimethanol (**29a**).* Compound **29a** was prepared from **28a** (1.5 g, 4.3 mmol) and LAH (0.4 g, 10.9 mmol). Yield 1.0 g (80%), mp 164−166 °C. ^{1}H NMR (DMSO-d_6): δ 2.45 (3H, s, CH_3), 2.92 (6H, s, $N(CH_3)_2$), 4.56 (3H, s, OCH_2 and OH, exchangeable), 4.75 (1H, t, J = 5.3 Hz, OH, exchangeable), 4.80 (2H, d, J = 5.3 Hz, OCH_2), 7.41−7.44 (1H, m, ArH), 7.69−7.72 (1H, m, ArH), 7.95−7.96 (1H, m, ArH), 8.26−8.28 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.3, 43.1, 53.8, 54.4, 114.2, 116.1, 118.2, 121.3, 123.0, 123.5, 125.1, 126.4, 130.2, 132.1, 155.0. HRMS [ESI$^+$]: calcd for $C_{16}H_{19}N_3O_2$, 286.1556 [M + H−H_2O]$^+$; found, 268.1450.

*(3-Methyl-6-(pyrrolidin-1-yl)pyrrolo[2,1-a]phthalazine-1,2-diyl)-dimethanol (**29b**).* Compound **29b** was prepared from **28b** (2.2 g, 6.23 mmol) and LAH (0.6 g, 15.5 mmol). Yield 1.6 g (83%), mp 160−162 °C. ^{1}H NMR (DMSO-d_6): δ 1.93−1.95 (4H, m, 2 × CH_2), 2.43 (3H, s, CH_3), 3.58−3.60 (4H, m, 2 × CH_2), 4.52−4.56 (3H, m, OCH_2 and OH, exchangeable), 4.72 (1H, t, J = 5.2 Hz, OH, exchangeable), 4.81 (2H, d, J = 5.2 Hz, OCH_2), 7.39−7.42 (1H, m, ArH), 7.68−7.71 (1H, m, ArH), 8.05−8.06 (1H, m, ArH), 8.26−8.28 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.3, 25.3, 51.3, 53.8, 54.5, 113.8, 116.6, 117.9, 120.8, 122.4, 123.3, 124.7, 126.7, 130.3, 131.8, 152.5. HRMS [ESI$^+$]: calcd for $C_{18}H_{21}N_3O_2$ 312.1712 [M + H−H_2O]$^+$; found, 294.1606.

*(3-Methyl-6-(piperidin-1-yl)pyrrolo[2,1-a]phthalazine-1,2-iyl)-dimethanol (**29c**).* Compound **29c** was prepared from **28c** (2.5 g, 6.5 mmol) and LAH (0.6 g, 16.3 mmol). Yield 1.7 g, (80%), mp 159−161 °C. ^{1}H NMR (DMSO-d_6): δ 1.64 (2H, m, CH_2), 1.77 (4H, m, 2 × CH_2), 2.44 (3H, s, CH_3), 3.19 (4H, m, 2 × CH_2), 4.56 (3H, br s, OCH_2 and OH, exchangeable), 4.75 (1H, t, J = 4.7 Hz, OH, exchangeable), 4.81 (2H, d, J = 4.7 Hz, OCH_2), 7.42−7.45 (1H, m, ArH), 7.69−7.72 (1H, m, ArH), 7.88−7.90 (1H, m, ArH), 8.26−8.27 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.3, 24.6, 25.9, 52.6, 53.8, 54.4, 114.2, 116.2, 118.2, 121.4, 123.0, 123.5, 125.2, 126.0, 130.2, 132.1, 155.0. HRMS [ESI$^+$]: calcd for $C_{19}H_{23}N_3O_2$, 326.1869 [M + H]$^+$; found, 326.1917.

*(6-([1,4′-Bipiperidin]-1′-yl)-3-methylpyrrolo[2,1-a]phthalazine-1,2-diyl)dimethanol (**29d**).* Compound **29d** was prepared from **28d** (0.65 g, 1.31 mmol) and LAH (0.10 g, 4.5 mmol). Yield 0.42 g, (78%), mp 183−185 °C. ^{1}H NMR (DMSO-d_6): δ 1.41−1.42 (2H, m, CH_2), 1.50−1.53 (4H, m, 2 × CH_2), 1.75−1.81 (2H, m, CH_2), 1.86−1.88 (2H, m, CH_2), 2.44 (3H, s, CH_3), 2.45−2.47 (1H, m, CH), 2.52−2.54 (4H, m, 2 × CH_2), 2.83−2.88 (2H, m, CH_2), 3.59−3.65 (2H, m, CH_2), 4.45 (1H, t, J = 5.0 Hz, OH, exchangeable), 4.56 (2H, d, J = 5.0 Hz, OCH_2), 4.62 (1H, t, J = 5.0 Hz, OH, exchangeable), 4.82 (2H, d, J = 5.0 Hz, OCH_2), 7.41−7.44 (1H, m, ArH), 7.68−7.71 (1H, m, ArH), 7.89−7.90 (1H, m, ArH), 8.26−8.27 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 8.7, 24.6, 26.1, 27.6, 49.8, 50.9, 53.5, 54.0, 61.8, 113.8, 115.8, 117.8, 121.1, 122.7, 123.2, 124.8, 125.6, 129.7, 131.6, 154.0. HRMS [ESI$^+$]: calcd for $C_{24}H_{32}N_4O_2$, 409.2604 [M + H]$^+$; found, 409.2638. In addition, DEPT-135, COSY, HMQC and HMBC spectra to further confirm the structure of compound **29d** (Supporting Information Figure S6).

Compounds **30a−d** and **31a−d** were prepared following the general procedure for the preparation of bis(alkylcarbamate) derivatives.

*(6-(Dimethylamino)-3-methylpyrrolo[2,1-a]phthalazine-1,2-diyl)bis(methylene) bis(ethylcarbamate) (**30a**).* Compound **30a** was prepared from **29a** (0.15 g, 0.5 mmol), ethyl isocyanate (0.2 mL, 2.0 mmol), and TEA (0.3 mL, 2.0 mmol). Yield 0.12 g (53%); mp 149−151 °C. ^{1}H NMR (DMSO-d_6): δ 0.98 (6H, t, J = 7.0 Hz, 2 × CH_3), 2.47 (3H, s, CH_3), 2.93 (6H, s, $N(CH_3)_2$), 2.97−3.00 (4H, m, 2 × CH_2), 5.18 (2H, s, OCH_2), 5.38 (2H, s, OCH_2), 7.02−7.04 (2H, br s, 2 × NH, exchangeable), 7.48−7.51 (1H, m, ArH), 7.73−7.76 (1H, m, ArH), 7.99−8.01 (1H, m, ArH), 8.09−8.10 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.3, 15.5, 35.5, 43.0, 56.6, 57.3, 109.3, 116.5, 117.3, 119.1, 122.8, 125.0, 126.0, 126.9, 129.6, 132.5, 155.5, 156.5, 156.6. HRMS [ESI$^+$]: calcd for $C_{22}H_{29}N_5O_4$, 428.2298 [M + Na]$^+$; found, 450.2118.

*(3-Methyl-6-(pyrrolidin-1-yl)pyrrolo[2,1-a]phthalazine-1,2-diyl)-bis(methylene) bis(ethylcarbamate) (**30b**).* Compound **30b** was prepared from **29b** (0.16 g, 0.5 mmol), ethyl isocyanate (0.2 mL, 2.0 mmol), and TEA (0.3 mL, 2.0 mmol). Yield 0.13 g, (70%); mp 140−142 °C. ^{1}H NMR (DMSO-d_6): δ 0.97 (3H, t. J = 6.5 Hz, CH_3), 0.99 (3H, t, J = 6.5 Hz, CH_3), 1.92−1.95 (4H, m, 2 × CH_2), 2.43 (3H, s, CH_3), 2.97−3.01 (4H, m, 2 × CH_2), 3.61 (4H, m, 2 × CH_2), 5.16 (2H, s, OCH_2), 5.37 (2H, s, OCH_2), 7.02−7.04 (2H, br s, 2 × NH, exchangeable), 7.45−7.48 (1H, m, ArH), 7.71−7.74 (1H, m, ArH), 8.06−8.08 (1H, m, ArH), 8.10−8.12 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.3, 15.5, 25.4, 35.5, 51.3, 56.6, 57.5, 108.8, 116.8, 117.0, 118.8, 122.6, 125.5, 127.1, 129.7, 132.2, 152.9, 156.6, 156.7. HRMS [ESI$^+$]: calcd for $C_{24}H_{31}N_5O_4$, 278.1657 [M + H − 2($OCONHC_2H_5$)]$^+$; found, 278.1660.

*(3-Methyl-6-(piperidin-1-yl)pyrrolo[2,1-a]phthalazine-1,2-diyl)-bis(methylene) bis(ethylcarbamate) (**30c**).* Compound **30c** was prepared from **29c** (0.16 g, 0.5 mmol), ethyl isocyanate (0.2 mL, 2.0 mmol), and TEA (0.3 mL, 2.0 mmol). Yield 0.16 g, (63%); mp 150−152 °C. ^{1}H NMR (DMSO-d_6): δ 0.97 (6H, t, J = 6.6 Hz, 2 × CH_3), 1.63 (2H, m, CH_2), 1.75−1.76 (4H, m, 2 × CH_2), 2.45 (3H, s, CH_3), 2.96−2.98 (4H, m, 2 × CH_2), 3.19 (4H, m, 2 × CH_2), 5.16 (2H, s, OCH_2), 5.37 (2H, s, OCH_2), 7.01−7.03 (2H, br s, 2 × NH, exchangeable), 7.48−7.51 (1H, m, ArH), 7.72−7.75 (1H, m, ArH), 7.92−7.93 (1H, m, ArH), 8.07−8.09 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.3, 15.5, 24.6, 25.9, 35.5, 52.6, 56.6, 57.3, 109.3, 116.6, 117.4, 119.2, 122.8, 125.0, 126.1, 126.4, 129.6, 132.5, 155.6, 156.5, 156.6. HRMS [ESI$^+$]: calcd for $C_{25}H_{33}N_5O_4$, 468.2611 [M + Na]$^+$; found, 490.2431.

*(6-([1,4′-Bipiperidin]-1′-yl)-3-methylpyrrolo[2,1-a]phthalazine-1,2diyl)bis(methylene) bis(ethylcarbamate) (**30d**).* Compound **30d** was prepared from **29d** (0.10 g, 0.25 mmol), ethyl isocyanate (0.1 mL, 1.0 mmol), and TEA (0.15 mL, 1.0 mmol). Yield 0.085 g, (85%); mp 172−173 °C. ^{1}H NMR (DMSO-d_6): δ 0.99 (6H, t, J = 6.7 Hz, 2 × CH_3), 1.39−1.41 (2H, m, CH_2), 1.51−1.55 (4H, m, 2 × CH_2), 1.77−1.81 (2H, m, CH_2), 1.81−1.87 (2H, m, CH_2), 2.44−2.46 (1H, m, CH), 2.46 (3H, s, CH_3), 2.51−2.53 (4H, m, 2 × CH_2), 2.82−2.87 (2H, m, CH_2), 2.97−3.01 (4H, m, 2 × CH_2), 3.64−3.66 (2H, m, CH_2), 5.18 (2H, s, OCH_2), 5.38 (2H, s, OCH_2), 6.99−7.10 (2H, br s, 2 × NH, exchangeable), 7.49−7.52 (1H, m, ArH), 7.74−7.77 (1H, m, ArH), 7.93−7.95 (1H, m, ArH), 8.09−8.10 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 8.8, 15.0, 24.6, 26.1, 27.4, 35.0, 49.7, 50.9, 56.1, 56.9, 61.8, 108.9, 116.1, 116.9, 118.7, 122.4, 124.5, 125.6, 126.0, 129.1, 132.1, 154.6, 156.0, 156.1. . HRMS [ESI$^+$]: calcd for $C_{30}H_{42}N_6O_4$, 551.3290 [M + H]$^+$; found, 551.3337.

*(6-(Dimethylamino)-3-methylpyrrolo[2,1-a]phthalazine-1,2-diyl)bis(methylene)bis-(isopropylcarbamate) (**31a**).* Compound **31a** was prepared from **29a** (0.10 g, 0.5 mmol), isopropyl isocyanate (0.2 mL, 2.0 mmol), and TEA (0.3 mL, 2.0 mmol). Yield 0.1 g, (46%); mp

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J. Med. Chem. 2019, 62, 2404−2418

154−156 °C. ^{1}H NMR (DMSO-d_6): δ 1.01 (12H, d, J = 6.5 Hz, 4 × CH_3), 2.46 (3H, s, CH_3), 2.92 (6H, s, $N(CH_3)_2$), 3.56 (1H, q, J = 6.5 Hz, CH), 3.61 (1H, q, J = 6.5 Hz, CH), 5.16 (2H, s, OCH_2), 5.37 (2H, s, OCH_2), 6.92−6.95 (2H, br s, 2 × NH, exchangeable), 7.48−7.51 (1H, m, ArH), 7.72−7.75 (1H, m, ArH), 7.99−8.00 (1H, m, ArH), 8.07−8.09 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.3, 23.0, 43.0, 56.4, 109.3, 116.5, 117.3, 119.1, 122.8, 125.0, 126.0, 126.9, 129.6, 132.5, 155.5, 155.8, 156.0. HRMS [ESI$^+$]: calcd for $C_{24}H_{33}N_5O_4$, 456.2611 [M + Na]$^+$; found, 478.2431.

*(3-Methyl-6-(pyrrolidin-1-yl)pyrrolo[2,1-a]phthalazine-1,2-diyl)-bis(methylene)bis-(isopropylcarbamate) (**31b**).* Compound **31b** was prepared from **29b** (0.16 g, 0.5 mmol), isopropyl isocyanate (0.2 mL, 2.0 mmol), and TEA (0.3 mL, 2.0 mmol). Yield 0.11 g, (65%); mp 160−162 °C. ^{1}H NMR (DMSO-d_6): δ 1.01 (12H, d, J = 6.0 Hz, 4 × CH_3), 1.94 (4H, m, 2 × CH_2), 2.44 (3H, s, CH_3), 3.61 (6H, m, 2 × CH_2, 2 × CH), 5.16 (2H, s, OCH_2), 5.37 (2H, s, OCH_2), 6.92−6.96 (2H, br s, 2 × NH, exchangeable), 7.44−7.47 (1H, m, ArH), 7.70−7.73 (1H, m, ArH), 8.06−8.07 (1H, m, ArH), 8.10−8.11 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.4, 23.0, 25.4, 42.7, 51.3, 56.5, 108.8, 116.8, 117.0, 118.8, 122.6, 124.3, 125.5, 127.1, 132.1, 152.9, 155.9. HRMS [ESI$^+$]: calcd for $C_{26}H_{35}N_5O_4$, 482.2767 [M + Na]$^+$; found, 504.2587.

*(3-Methyl-6-(piperidin-1-yl)pyrrolo[2,1-a]phthalazine-1,2-diyl)-bis(methylene)bis-(isopropylcarbamate) (**31c**).* Compound **31c** was prepared from **29c** (0.16 g, 0.5 mmol), isopropyl isocyanate (0.2 mL, 2.0 mmol), and TEA (0.3 mL, 2.0 mmol). Yield 0.13 g, (68%); mp 141−143 °C. ^{1}H NMR (DMSO-d_6): δ 1.02 (12H, d, J = 5.6 Hz, 4 × CH_3), 1.64 (2H, m, CH_2), 1.77 (4H, m, 2 × CH_2), 2.46 (3H, s, CH_3), 3.20 (4H, m, 2 × CH_2), 3.59−3.60 (2H, m, 2 × CH), 5.18 (2H, s, OCH_2), 5.39 (2H, s, OCH_2), 6.95 (2H, br s, 2 × NH, exchangeable), 7.49−7.52 (1H, m, ArH), 7.73−7.76 (1H, m, ArH), 7.93−7.94 (1H, m, ArH), 8.08−8.10 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.3, 23.0, 23.8, 24.6, 25.9, 42.7, 52.6, 56.2, 57.4, 109.7, 116.9, 117.7, 119.4, 121.3, 126.1, 126.8, 129.9, 132.7, 155.6, 156.4. HRMS [ESI$^+$]: calcd for $C_{27}H_{37}N_5O_4$, 496.2924 [M + Na]$^+$; found, 518.2744.

*(6-([1,4′-Bipiperidin]-1′-yl)-3-methylpyrrolo[2,1-a]phthalazine-1,2diyl)bis(methylene) bis(isopropylcarbamate) (**31d**).* Compound **31d** was prepared from **29d** (0.10 g, 0.25 mmol), isopropyl isocyanate (0.1 mL, 1.0 mmol), and TEA (0.15 mL, 1.0 mmol). Yield 0.079 g, (79%); mp 161−163 °C. ^{1}H NMR (DMSO-d_6): δ 1.01−1.04 (14H, m, 4 × CH_3 and CH_2), 1.39−1.42 (2H, m, CH_2), 1.51−1.55 (4H, m, 2 × CH_2), 1.79−1.81 (2H, m, CH_2), 1.83−1.87 (2H, m, CH_2), 2.44−2.46 (1H, m, CH), 2.47 (3H, s, CH_3), 2.54−2.58 (2H, m, CH_2), 2.84−2.89 (2H, m, CH_2), 3.61−3.68 (4H, m, 2 × CH and CH_2), 5.19 (2H, s, OCH_2), 5.40 (2H, s, OCH_2), 6.72−6.96 (2H, br s, 2 × NH, exchangeable), 7.50−7.53 (1H, m, ArH), 7.75−7.77 (1H, m, ArH), 7.95−7.97 (1H, m, ArH), 8.10−8.12 (1H, m, ArH). ^{13}C NMR (DMSO-d_6): δ 9.3, 23.0, 23.7, 25.1, 26.6, 27.9, 42.7, 50.2, 51.4, 56.4, 57.2, 67.4, 109.4, 116.5, 117.4, 119.2, 122.8, 125.0, 126.1, 126.5, 129.6, 132.5, 155.1, 155.8, 156.0, 157.2. HRMS [ESI$^+$]: calcd for $C_{32}H_{46}N_6O_4$, 579.3659 [M + H]$^+$; found, 579.3666.

Cytotoxicity Assay. The antiproliferative activities of the newly synthesized compounds were analyzed in the human lymphoblastic leukemia cell line CCRF-CEM, corresponding vincristine-resistant subcell line (CCRF-CEM/VBL), and human solid tumor cell lines, including HCT-116 colon cancer cells, H460 NSCLC cells, H526 SCLC, and PaCa S1 pancreatic cancer cells as previously described.[67] Briefly, 3000 cells were seeded in each well of a 96-well plate and treated with the newly synthesized compounds at various concentrations. After a 72 h incubation period, the cell proliferation was determined by inoculation with PrestoBlue Cell Viability Reagent (Thermo Fisher Scientific, USA), incubation at 37 °C for 1−3 h, and (except for the H526 cells) the absorbance of each well was read at 570 and 600 nm with a microplate reader. The cell proliferation of H526 cells was determined by a fluorescence microplate reader system using an excitation wavelength of 515 nm and emission wavelength of 615 nm. The IC_{50} values were determined from the dose-effect relationship at six or seven concentrations of each test compound using CompuSyn software (version 1.0.1; CompuSyn, Inc., Paramus, NJ) by Chou and Martin, which is based on the median-effect principle and plot.[68]

DNA Interstrand cross-linking Assay. As previously described, the DNA cross-linking activities of compounds **29d** and **23b** were determined by alkaline gel electrophoresis.[42] Briefly, after incubation of plasmid DNA (pEGFP-N1) with various concentrations of the test compounds at 37 °C for 2 h, the single-stranded (SS) DNA and cross-linked strands were electrophoretically separated on an alkaline gel.

Cell cycle Analysis. The effects of the test compounds on cell cycle progression were analyzed by flow cytometry as previously described.[38] Briefly, 1.0 × 10^5 H460 cells were seeded in each well of a 6-well plate and incubated at 37 °C in a 5% CO_2 humidified atmosphere overnight. The cells were then incubated with various concentrations of compound **29d** for different times. At the end of the incubation period, the attached cells were trypsinized, fixed in ice-cold 70% EtOH, and stored at −20 °C overnight. The cells were then stained with 4 μg/mL propidium iodide (PI) in phosphate-buffered saline (PBS) containing 0.1 mg/mL RNase A and 1% Triton X-100 and subjected to the flow cytometry analysis (FACScan flow cytometer, Becton Dickinson, San Jose, CA). The cell cycle phase distribution was analyzed with ModFit LT 3.0 software (Verity Software House, Topsham, ME) based on the DNA histograms.

Apoptosis Assay. As previously described,[39] H460 cells were treated with compound **29d** or cisplatin for 24, 48, and 72 h. Apoptotic cell death was determined using an Annexin V-FITC Apoptosis Detection Kit (eBioscience, San Diego, CA, USA) and flow cytometer according to the manufacturer's instructions. Annexin V-positive cells, including the bottom right and top right quadrants, represented the early and late apoptotic populations, respectively.

Western Blotting Analysis. The inhibition of VEGFR-2 phosphorylation was analyzed by western blotting analysis as previously described.[59] The following primary antibodies were used: anti-VEGFR-2 (Abcam) and anti-p-VEGFR-2 (Abcam).

Tube Formation Assay. EA.hy926 cells were treated with compound **29d** or vatalanib at various concentrations for 24 h. After the treatment period, an aliquot of cells (8 × 10^3 cells) was suspended in 100 μL of medium containing 1% fetal bovine serum (FBS) and seeded onto a 96-well plate (ibidi, Munich, Germany) precoated with 10 μL of Matrigel Matrix (Corning, MA, USA) 1 h before seeding at 37 °C. The tube formation ability was then assayed after a 24 h incubation period.[69] The tube formation was determined by microscopy. The total number of nodes was calculated and averaged by counting the branch points of tube-like structures in three random fields.

Transwell Migration Assay. A Boyden chamber system was used to evaluate cell migration.[70] Gelatin (10 μg/mL) was added to each well of a Transwell plate (Corning-Costar, Corning, ME; 8 μm pore size), and then, the membranes were allowed to dry at 37 °C for 1 h. Afterward, 5 × 10^3 cells were suspended in 100 μL of an FBS-free medium and seeded in the upper chamber of a Transwell plate. The lower chamber was filled with medium containing 5% FBS. After incubating for 12 h, the cells on the top side of the Transwell membrane were removed using cotton swabs. The cells trapped on the bottom side of the membrane were fixed with methanol and stained with a solution of 4,6-diamidino-2-phenylindole (10 μg/mL; Invitrogen) for 20 min. The numbers of cells from eight different fields on each membrane were counted using a fluorescence microscope.

Liposomal Encapsulation of 29d (29dL). To overcome the poor solubility, liposomal **29d** (**29dL**) was prepared by a combination of the modified dehydration-rehydration method and repeated extrusion according to the following procedure.[71] SPC, DSPC, CHO, and PEG-2000 (molar ratio 50:45:4:1) were dissolved in chloroform and transferred to a round-bottomed flask. Compound **29d** was dissolved in the reaction mixture (4 mg/mL). The solvent was removed by rotary evaporation under reduced pressure. The resulting dry lipid film was hydrated at 60 °C in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) and dispersed by hand shaking. The suspension was frozen and thawed 10 times and then extruded through polycarbonate membrane filters (Costar,

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J. Med. Chem. 2019, 62, 2404−2418

Cambridge, MA) with 0.8 μm pores 6 times, 0.6 μm pores 6 times, 0.4 μm pores 8 times, and 0.2 μm pores 10 times using a high-pressure extrusion equipment (Lipex Biomembranes, Vancouver, Canada) at 60 °C. The product solution was stored at 4 °C. The concentration of liposomal **29dL** was determined with HPLC. Briefly, chromatographic separation was performed (Agilent Technologies) on a RP-18 column (4.6 × 150 mm, 5 A) using a detection wavelength of 254 nm. Compound **29d** without liposomal encapsulation was used as the standard. The isocratic mobile phase (acetonitrile/MeOH/H_20 (0.5% TFA) (45/50/5)) was used at a flow rate of 0.5 mL/min for 15 min. The retention time of **29d** was 4.6 min. In our preparations (n = 3), the liposomes contained 3.12−2.94 mg/mL of compound **29d** (encapsulation rate, 76−70%). The stability analysis revealed approximately 10% decay of **29dL** occurred during storage at 4 °C for 3 weeks.

Therapeutic Efficacy in Animals. The therapeutic efficacy of liposomal **29dL** against H526 cells was determined following a previously described protocol.[39] All animal studies followed the guidelines approved by the Institutional Animal Care and Use Committee. Male athymic nude mice bearing the nu/nu gene (5 weeks old) were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and housed for 1 week before experimental manipulation. An aliquot of H526 cells (2×10^7 cells in 100 μL of PBS) was subcutaneously implanted into the dorsal flank of each mouse.

When the tumor size reached approximately 100 mm^3, the mice were randomized into several groups and subjected to different treatments. Liposomal **29d** (10 mg/kg) was iv administered via the tail vein. Vatalanib was administered orally at a dose of 100 mg/kg. Cisplatin, obtained from Across and dissolved in 5% dextrose solution, was iv injected at a dose of 4 mg/kg. The vehicle was 5% dextrose solution. Tumor volume was measured using a caliper and calculated by the following formula: (length × $width^2$)/2.

Immunohistochemical Staining. To further study the expression of CD31 and phospho-γ-H2AX in tumor sections derived from xenografts treated with **29dL**, vatalanib and cisplatin at different time intervals, H526 cells were subcutaneously inoculated into the dorsal flank region of nude mice. When the average tumor size reached approximately 300 mm^3, the mice were randomized and treated with vehicle, **29dL**, vatalanib, and cisplatin according to the protocol described above. On days 3, 6, and 9, three mice from each group were sacrificed, and their tumors were excised, fixed with 10% formalin, and subjected to paraffin sectioning. Immunohistochemical staining was performed by using a Novolink Polymer Detection System (Leica Biosystems, Wetzlar, Germany) according to the manufacturer's instructions. The primary antibodies (Abcam, Cambridge, MA) used were anti-CD31 (#ab28364) and anti-p-γH2AX (#ab22551). After immunohistochemical processing, tissue sections were scanned by a Panoramic 250 Flash II whole slide scanner, and the expression levels of CD31 and p-γH2AX in the tumor tissue sections were determined by image analysis software. For quantification, the staining intensities of 15 randomly selected fields from three tumor sections were determined using 3DHISTECH Panoramic viewer software (3DHISTECH Ltd., Budapest, Hungary).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.8b01689.

Representative histogram of cell cycle progression analysis; induction of apoptotic death; inhibition of cell proliferation; suppression of cell migration; ^1H and ^{13}C NMR spectra, EMS−MS spectra, and HPLC chromatograms of new compounds; DEPT, COSY, HMQC, and HMBC NMR spectral characteristics; and NMR data for compound 29d (PDF)

Molecular formula strings (CSV)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Ac_2O, acetic anhydride; CL, cross-linking; DCM, dichloromethane; DMAD, dimethyl acetylenedicarboxylate; DMF, dimethylformamide; EtBr, ethidium bromide; IC_{50}, half maximal inhibitory concentration; iv inj, intravenous injection; LAH, lithium aluminum hydride; MeI, methyl iodide; MTD, maximum tolerated dose; NaH, sodium hydride; PBS, phosphate-buffered saline; PI, propidium iodide; QD, once per day; SAR, structure−activity relationship; SCLC, small-cell lung cancer; TEA, triethylamine; THF, tetrahydrofuran; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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