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Thalidomide analogues demonstrate dual inhibition of both angiogenesis and prostate cancer

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Abstract—The identification of agents with antiproliferative activity against endothelial cells has significant value for the treatment of many angiogenesis-dependent pathologies. Herein, we describe the discovery of a series of thalidomide analogues possessing inhibitory effects against both endothelial and prostate cancer cells. More specifically, several analogues exhibited low micromolar to mid-nanomolar potency in the inhibition of human microvascular endothelial cell (HMEC) proliferation, both in the presence and absence of vascular endothelial growth factor (VEGF), with the tetrafluorophthalimido class of compounds demonstrating the greatest potency. Additionally, all the compounds were screened against two different androgen independent prostate cancer cell lines (PC-3 and DU-145). Again, the tetrafluorophthalimido analogues exhibited the greatest effect with GI_{50} values in the low micromolar range. Thalidomide was found to demonstrate selective inhibition of androgen receptor positive LNCaP prostate cancer cells. Furthermore, we showed that, as an example, tetrafluorophthalimido analogue **19** was able to completely inhibit the prostate specific antigen (PSA) secretion by the LNCaP cell line, while thalidomide demonstrated a 70% inhibition. We have also demonstrated that a correlation exists between HMEC and prostate cancer cell proliferation for this structural class. Altogether, our study suggests that these analogues may serve as promising leads for the development of agents that target both androgen dependent and independent prostate cancer and blood vessel growth.

1. Introduction

Angiogenesis is the process of new blood vessel growth, and involves the proliferation of endothelial cells in response to specific growth stimuli such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF). Angiogenesis is an important natural process occurring in the body, both in health (wound healing,[1] female reproductive cycle[2]) and disease (cancer,[3] rheumatoid arthritis,[4] diabetic retinopathy[5]). Vessel growth is controlled by a balance of endogenous inhibitors and stimulators. The growth and maintenance of solid tumors is highly dependent on neovascularization and can be regulated by compounds that interfere with either the stimulation or proliferation of endothelial cells.[6] As a result, the control of angiogenesis continues to be an attractive area for novel therapeutic agent development.[3]

One such agent is thalidomide (**1**) (Fig. 1), which was developed in the 1950′s by Chemie Grünenthal of Germany as a non-toxic sedative.[7] In addition to its sedative effects in humans, an association was reported of teratogenic limb defects from maternal thalidomide usage.[7] Aside from this serious teratogenic effect on the fetus, the drug does have therapeutic value: (1) for its immunosuppressive effect in the treatment of graft versus host disease;[8,9] (2) in the treatment of leprosy;[10,11] and (3) for inflammatory dermatoses.[12] In addition, thalidomide has significant anti-angiogenic activity, and the effects of thalidomide on corneal angiogenesis induced by vascular endothelial growth factor (VEGF) have been reported.[13] Additionally, thalidomide has demonstrated inhibitory effects on angiogenesis in the basic fibroblast growth factor (bFGF) induced rabbit corneal micropocket assay[14] and orally in mice models.[15]

Prostate cancer is the most common malignancy in American men and is the second leading cause of cancer mortality.[16] Prostate cancer, as is the case with numerous types of cancer, is dependent on the recruitment of new blood vessels to grow and metastasize. In fact, in prostate cancer, an increased microvessel density correlates to poorer prognosis.[17] As long as the cancer is confined to the prostate, it can be successfully controlled by radiation or surgery. However, in metastatic disease, few treatment options are available beyond

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androgen ablation.[18] Clinical trials of thalidomide in patients with androgen-independent prostate cancer have been reported, and they show thalidomide has modest activity in patients with metastatic prostate cancer.[19]

Inhibitors of angiogenesis can exert their effect in many ways, depending on their mechanism of action. Some inhibit endothelial cells directly, while others inhibit the angiogenesis signaling cascade or block the ability of endothelial cells to break down the extracellular matrix. Therapeutic agents that can inhibit more than one of these pathways could prove to have a distinct advantage over conventional anti-angiogenic therapies in which only one mechanism of action is targeted. On the basis of the latter statement, we have been engaged in the structural modification of thalidomide with the aim of developing novel anti-angiogenic/anti-cancer analogues that possess superior biological action relative to thalidomide.

Herein we report that substitution of the four aromatic hydrogen atoms of phthalimide analogues of thalidomide with fluorine leads to more potent angiogenic and prostate cancer cell inhibitory activity (Fig. 2). The tetrafluorophthalimides were synthesized for the following reasons. First, Ng et al. recently demonstrated that tetrafluorinated thalidomide analogues are inhibitors of angiogenesis,[20] and secondly, it has been reported that the glutarimide ring of thalidomide is not essential for antimetastatic activity against B16BL6 melanoma cells, since a completely hydrolyzed glutarimide group did not render the molecule inactive.[21] That evidence, coupled with the numerous published examples of various *N*-phenylphthalimides that possess increased TNF-α regulatory activity over thalidomide,[22–26] lends support to the notion that structural simplification of the glutarimide moiety does not produce biologically inactive analogues.

2. Results and discussion

2.1. Chemistry

The first series of target compounds **3**–**19** and **21** in Scheme 1 were synthesized by the condensation of an appropriate phthalic anhydride with an appropriate aniline or primary amine derivative in refluxing acetic acid.[27] Compounds **2** and **20** were purchased from Aldrich Chemical Company. Moderate to high yields of 34–81% were obtained without optimization of the reaction conditions (Table 1).

The second series of compounds including restricted analogue targets **22**–**27** in Scheme 2 were also generated from the condensation of an amine derivative with a phthalic anhydride derivative in refluxing acetic acid. Yields of 25–90% were obtained for these analogues (Table 2). Amine **29**, used in the preparation of **26** and **27**, was realized by conversion of the ketone precursor, 1-benzosuberone, to the oxime **28** utilizing the procedure of Ballini et al.,[28] followed by catalytic hydrogenation of **28** with 10% Pd/C and hydrogen gas (Scheme 3).

3. Biological activity studies

Thalidomide analogues **2**–**27** were first screened for their ability to inhibit the proliferation of human microvascular endothelial cells (HMEC's), both in the presence and absence of vascular endothelial growth factor (VEGF), with thalidomide as the standard. It has been demonstrated previously that inhibition of angiogenesis by thalidomide requires metabolic activation;[29] however, unactivated thalidomide was used as a standard in our proliferation assays to simply demonstrate the ability of our analogues to inhibit angiogenesis without the need for prior metabolic activation. Additionally, the above-mentioned analogues were screened for inhibitory activity against the proliferation of PC-3 and DU-145 prostate cancer cell lines. The results obtained for both of the above experiments are shown in Table 3.

In the non-fluoro-substituted phthalimide class (**2**–**10**, **20**, **22**, **24**, **26**), the most active compound against HMEC proliferation (**6**) exhibited low micromolar IC_{50} values both in the presence and absence of VEGF. Mechanistically interesting was the fact that analogue **6** demonstrated potent ability to overcome growth factor mediated proliferation of endothelial cells. Altogether,

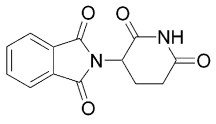


Figure 1. Thalidomide (**1**).

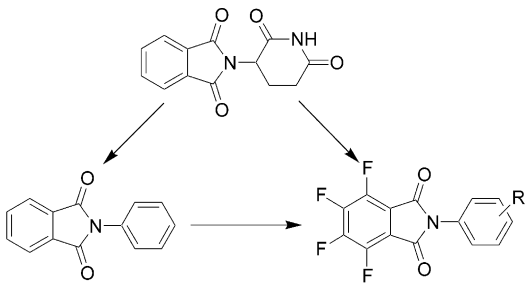
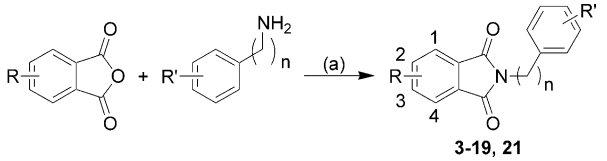
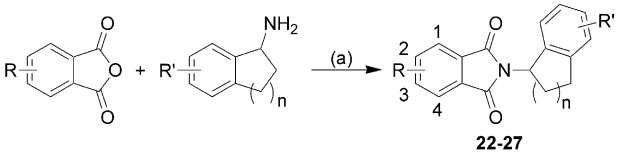


Figure 2. Design strategy of first generation analogues.



Scheme 1. Synthesis of target phthalimide analogues. Reagents: (a) acetic acid, reflux, 2–3 h.



Scheme 2. Synthesis of target phthalimide restricted analogues. Reagents: (a) acetic acid, reflux, 2–3 h.

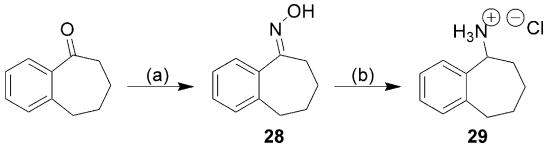
Table 1. Target phthalimides in series 1

Compd	R						
	1	2	3	4	R'	*n*	Yield (%)
2	H	H	H	H	H	0	—[a]
3	H	H	H	H	4-Cl	0	70
4	H	H	H	H	3-Cl	0	64
5	H	H	H	H	2-Cl	0	50
6	H	H	H	H	3,4-Cl	0	67
7	H	H	H	H	4-CH_3	0	77
8	H	H	H	H	3-CH_3	0	81
9	H	H	H	H	2-CH_3	0	69
10	H	H	H	H	4-OCH_3	0	71
11	F	F	F	F	H	0	70
12	F	F	F	F	4-Cl	0	45
13	F	F	F	F	3-Cl	0	51
14	F	F	F	F	2-Cl	0	35
15	F	F	F	F	3,4-Cl	0	57
16	F	F	F	F	4-CH_3	0	75
17	F	F	F	F	3-CH_3	0	78
18	F	F	F	F	2-CH_3	0	34
19	F	F	F	F	4-OCH_3	0	65
20	H	H	H	H	H	1	—[a]
21	F	F	F	F	H	1	65

[a] Commercially available from Aldrich Chemical Co.

Table 2. Target phthalimides in series 2

Compd	R						
	1	2	3	4	R′	n	Yield (%)
22	H	H	H	H	H	1	90
23	F	F	F	F	H	1	77
24	H	H	H	H	H	2	84
25	F	F	F	F	H	2	53
26	H	H	H	H	H	3	78
27	F	F	F	F	H	3	25



Scheme 3. Synthesis of amine **29**. Reagents: (a) NH_2OH-HCl, ethanol, amberlyst A-21, 24 h, rt (82%); (b) (i) 10% Pd/C, H_2; (g), ethanol, 3 days; (ii) Et_2O/HCl, (100%).

when compared to thalidomide, the non-fluoro substituted analogues (with the exception of **3**, **6**, and **7**) possessed either comparable or only slightly more potent anti-angiogenic activity with and without VEGF present.

Against prostate cancer cell lines, only compound **3** showed any appreciable increase in potency over thalidomide in the prostate cancer screen, and this effect was only seen with the DU-145 cell line. These results suggest that there is no clear advantage gained by the substitution of a phenyl ring within this class. However, this structural class does have efficacy against growth factor mediated microvessel endothelial cell proliferation.

In the tetrafluorophthalimido class (**11**–**19**, **21**, **23**, **25**, **27**), compounds **16** and **17** were the most active analogues, as they both revealed a remarkable increase in potency over thalidomide in the HMEC screen with IC_{50} values ranging from 100–330 nm (Table 3). Furthermore, all tetrafluoro-substituted analogues possessed a considerable increase in potency in anti-angiogenic activity compared to thalidomide. Similarly, in the prostate cancer screen, all tetrafluoro-analogues demonstrated significant increases in potency over thalidomide in both PC-3 and DU-145 cell lines.

Table 3. Inhibitory effects of compounds **2**–**27** on HMEC and prostate cancer cell proliferation[a]

Compd	HMEC[b] IC_{50} (μM)		Prostate cancer GI_{50} (μM)[c]	
	(+) VEGF	(−) VEGF	PC-3	DU-145
Thalidomide	>300	>300	>300	>300
2	302±21.5	242±29.0	>300	>300
3	71.1±12.8	51.6±5.81	304±16.7	74.4±27.8
4	181±11.1	155±16.9	>300	>300
5	>300	>300	>300	>300
6	10.6±6.20	15.0±4.11	>300	>300
7	71.6±31.9	68.9±35.8	>300	>300
8	167±14.6	200±4.19	>300	>300
9	187±3.54	157±9.84	>300	>300
10	208±23.6	144±27.6	321±178	>300
11	0.77±0.23	0.68±0.11	4.91	11.8
12	5.98±2.65	1.78±0.26	10.7±3.83	8.33±1.98
13	0.78±0.05	0.42±0.19	0.77	2.03
14	8.37±2.24	7.09±0.83	3.42	3.07
15	>300[d]	>300[d]	4.98	11.6
16	<0.1	0.33±0.18	17.0	12.2
17	<0.1	<0.1	1.16	5.34
18	2.40±0.21	1.73±0.36	1.77	7.57
19	2.28±0.54	1.53±0.25	2.12	3.97
20	262±35.3	62.2±26.3	>300	>300
21	0.95±0.16	0.91±0.21	0.88	1.97
22	>300	147±50.1	>300	385±225
23	1.07±0.01	2.40±0.42	1.01	0.91
24	258±17.2	306±13.9	218±19.7	116±21.1
25	0.19±0.07	0.18±0.02	1.88	1.53
26	251±87.9	28.4±2.73	>300	>300
27	0.62±0.08	0.32±0.04	0.55	1.48

[a] All experiments were run in triplicate, and the ± values represent the SEM.
[b] Human microvessel endothelial cells.
[c] Prostate cancer GI_{50} values for compounds **11**–**19**, **21**, **23**, **25**, and **27** were determined by the National Cancer Institute Developmental Therapeutics Program and all others were determined in our laboratory.
[d] The poor solubility of this compound in the biological medium may have contributed to the results obtained.

Compounds **16** and **17** possess para and meta methyl groups on the phenyl ring attached to the imide nitrogen respectively (Table 1). Replacement of the *para* methyl group of **16** with a *para* chloro group (**12**) results in a slight loss in activity, raising the IC_{50} to the low micromolar range. Replacement of the *meta* methyl group of **17** with a *meta* chloro group (**13**) results in only a modest decrease in activity to the high nanomolar range. Both methyl and chloro groups add similar contributions to the overall lipophilicity of the compound; therefore, the electron donating properties of the methyl group may play a more critical role in the increased activity of compounds **16** and **17**. The unsubstituted tetrafluoro-analogue (**11**) shows activity similar to that of compounds **12** and **13**, lending more support to the electrostatic effect of the methyl group. Com-

pound **19**, incorporating a *para* methoxy group, also has similar activity to **12** and **13**, possibly due to the methoxy groups moderately inductive electron withdrawing properties.

Tetrafluoro-analogue **21**, with a methylene insertion between the phenyl group and the imide nitrogen, also shows significant anti-angiogenic and anti-prostate cancer activity in the high nanomolar to low micromolar range. Additionally, the tetrafluoro-substituted restricted analogues **23**, **25**, and **27** show similar to slightly increased anti-angiogenic and anti-prostate cancer activity relative to both **21** and the tetrahydro counterparts **22**, **25**, and **26**. Consequently, it was found that restricting the rotation of the phenyl ring in **21** does not prove to be detrimental to the compounds in vitro potency (Figs 3 and 4).

In order to broaden the activity spectrum of the tetrafluorophthalimido class, we selected one tetrafluoro analogue to study the effect against the androgen dependent LNCaP prostate cancer cell line. Analogue **19** was chosen for this study because of its consistent potency profile across all four biological experiments presented in Table 3. As shown in Table 4, analogue **19**, with an IC_{50} of 15.6 μM, proved to be of equal potency as thalidomide at inhibiting the proliferation of the LNCaP cells. Further, this data confirms that thalidomide has selective activity towards androgen receptor positive prostate cancer (LNCaP IC_{50} 25.3±21.1 μM, Table 4) in comparison to androgen receptor negative prostate cancer ($IC_{50} > 300$ μM for DU-145 and PC-3, Table 3). This finding is different from studies on angiogenesis where thalidomide must be metabolically activated.[29] Our data suggests that thalidomide will be (1) directly effective in androgen receptor positive prostate cancer cell lines, (2) does not need to be activated for the LNCaP subtype, or (3) androgen receptor positive prostate cancer has a metabolic profile that is different from androgen receptor negative prostate cancer. Further studies should be warranted in these areas.

We have also found that PSA secretion can be inhibited by thalidomide treatment on a per cell basis. Thalidomide showed a 70% inhibition in the amount of PSA per cell at its IC_{50} concentration for the androgen receptor positive LNCaP cell line (Table 4). We demonstrate, as an example, that the tetrafluorophthalimido analogue **19** showed a 100% inhibition of PSA secretion at its IC_{50} concentration. Altogether, the prostate cancer inhibition profile for analogue **19** is considerably better than thalidomide with demonstrated effectiveness against both androgen positive and negative prostate cancer and PSA secretion.

Unfortunately for the prostate cancer patient, a poor prognosis has been correlated with increased microvessel density.[17] We have found a significant correlation exists between HMEC and PC-3 proliferation (Plots A and B in Fig. 3), even in the presence of the vascular growth factor. This is significant and suggests that our compounds have ability to overcome this growth sti-

Table 4. Effect of **19** on LNCaP proliferation and PSA secretion[a]

Compd	LNCaP IC_{50} (μM)	%PSA/cell[b]
Thalidomide	25.3±21.1	30
19	15.6±1.37	0

[a] All experiments were run in triplicate, and the ±values represent the SEM.
[b] %PSA per cell was measured relative to control at the IC_{50} dose of drug. Controls were with (0.2%) DMF vehicle.

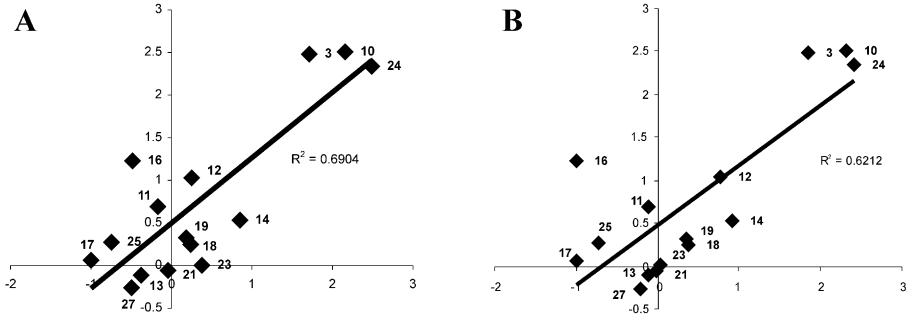


Figure 3. Correlation plots of (A): log [PC-3 IC_{50}] versus log [(−)VEGF IC_{50}]; and (B): log [PC-3 IC_{50}] versus log [(+)VEGF IC_{50}].

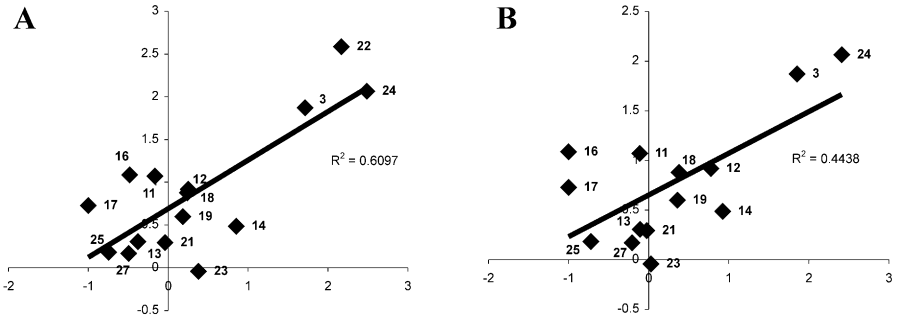


Figure 4. Correlation plots of (A): log [DU-145 IC_{50}] versus log [(−)VEGF IC_{50}]; and (B): log [DU-145 IC_{50}] versus log [(+)VEGF IC_{50}].

mulus pathway. In less aggressive DU-145 cells, our compounds demonstrated a lesser ability to inhibit in the presence of VEGF (Plot B in Fig. 4). This suggests that our compounds have a much greater efficacy against the more aggressive androgen independent prostate cancer phenotype.

4. Conclusion

Our study has identified new thalidomide analogues with dual anti-angiogenic/anti-prostate cancer activity (analogues **16**, **17**, **21**, **23**, **25**, and **27**). The preliminary SAR with these compounds revealed the importance of tetrafluorination of the phthalimide core, in addition to *para* or *meta* methyl substitution on the opposite phenyl ring. Furthermore, methylene insertion between the phenyl ring and the imide nitrogen in the tetrafluorophthalimido class does not abrogate in vitro activity, nor does restricting the rotation of the freely rotatable phenyl group.

The presence of the fluorine substituents on the phthalimide core drastically increases the biological potency of the aforementioned analogues. Since the fluorine atom is a close steric mimic of hydrogen; incorporation of fluorine into drugs often improves their biological potency.[30] Such improvements have been attributed to either the enhanced lipophilicity of fluorinated compounds or to stereoelectronic changes imparted to compounds by the strongly electronegative fluorine substitution resulting in more effective binding to their respective target(s). Additionally, the fluorinated compounds presented herein represent analogues that would be resistant to the phenyl metabolism that has been reported for thalidomide.[31]

The evidence presented herein indicates the dramatically increased activity and dual acting nature of the tetrafluorophthalimido analogues, and therefore offers a unique opportunity for utilizing these fluorinated analogues as leads in the search for novel therapeutic agents for prostate cancer. These compounds demonstrate increased effectiveness against prostate cancer in comparison to thalidomide and show ability to inhibit both phenotypes. In particular, the fluorinated analogues seem to be more active against the androgen independent PC-3/DU-145 cells, and it is precisely this androgen independent prostate cancer that proves to be more fatal to patients because of a current lack of effective treatment strategies. Further, the use of these compounds could be extended towards evaluating them towards other cancers in which poor patient prognosis correlates to increased microvessel density.

5. Experimental

5.1. Materials

Reagents were purchased from Aldrich or Lancaster and were used as received. Solvents used were previously dried by distillation.

5.2. Analytical procedures

Melting points were determined in open capillary tubes on an Electrothermal melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were measured at 25 °C on compounds in solution in $CDCl_3$ or DMSO-d_6 on a GE 300 MHz spectrometer. APCI mass spectra were obtained on a Finnagan LcQ Classic. The University of Illinois at Urbana-Champaign Mass Spectrometry Laboratory performed the EI analyses. IR spectra were recorded on a Nicolet Impact 400D spectrophotometer. Elemental analyses were performed by Atlantic Microlab, and microanalytical data were within ±0.4% of the calculated figures. Thin-layer chromatography was done on precoated aluminum silica gel plates (silica gel 60 F_{254}).

5.3. General procedure for preparation of phthalimide derivatives

The appropriate phthalic anhydride and amine were stirred under reflux in acetic acid for 2–4 h. The solution was poured into water, and the resulting precipitate filtered off by suction and recrystallized from absolute ethanol.

5.3.1. *N*-(4-Chlorophenyl)-phthalimide (3). Compound **3** was prepared from phthalic anhydride (5.0 g/33.8 mmol) and 4-chloroaniline (4.31 g/33.8 mmol): mp 189–192 °C (lit.[27] 193–194 °C); ^{1}H NMR ($CDCl_3$) δ 7.4–7.5 (m, 4H, Ar*H*), 7.7–8.0 (m, 4H, Ar*H*); ^{13}C NMR ($CDCl_3$) δ 167.5, 135.1, 134.3, 132.1, 130.7, 129.8, 128.2, 124.4; APCI *m*/*z* (rel intensity) 258.3 (M^+, 100).

5.3.2. *N*-(3-Chlorophenyl)-phthalimide (4). Compound **4** was prepared from phthalic anhydride (5.0 g/33.8 mmol) and 3-chloroaniline (4.31 g/33.8 mmol/3.58 mL): mp 160–163 °C (lit.[32] 164 °C); ^{1}H NMR ($CDCl_3$) δ 7.3–7.5 (m, 4H, Ar*H*), 7.7–8.0 (m, 4H, Ar*H*); ^{13}C NMR ($CDCl_3$) δ 167.3, 135.1, 133.3, 132.0, 130.5, 128.7, 127.1, 125.1, 124.4; APCI *m*/*z* (rel intensity) 258.3 (M^+, 100).

5.3.3. *N*-(2-Chlorophenyl)-phthalimide (5). Compound **5** was prepared from phthalic anhydride (300 mg/2.03 mmol) and 2-chloroaniline (260 mg/2.03 mmol/0.21 mL): mp 143–145 °C (lit.[33] 143 °C); ^{1}H NMR ($CDCl_3$) δ 7.2–8.0 (m, 8H, Ar*H*); ^{13}C NMR ($CDCl_3$) δ 166.2, 134.1, 132.8, 131.5, 130.3, 130.1, 129.2, 127.4, 123.6, 123.2; APCI *m*/*z* (rel intensity) 256.7 (100), 258.6 (M^+, 60).

5.3.4. *N*-(3,4-Dichlorophenyl)-phthalimide (6). Compound **6** was prepared from phthalic anhydride (920 mg/6.2 mmol) and 3,4-dichloroaniline (1.0 g/6.2 mmol): mp 196–198 °C (lit.[34] 198 °C); ^{1}H NMR ($CDCl_3$) δ 7.3–7.6 (m, 3H, Ar*H*), 7.7–8.0 (m, 4H, Ar*H*); ^{13}C NMR ($CDCl_3$) δ 167.1, 135.3, 131.9, 131.2, 128.7, 126.0, 124.5; APCI *m*/*z* (rel intensity) 292.2 (M +, 100), 294.2 (65).

5.3.5. *N*-(4-Methylphenyl)-phthalimide (7). Compound **7** was prepared from phthalic anhydride (1.4 g/9.3 mmol) and p-toluidine (1.0 g/9.3 mmol/1.03 mL): mp 204–206 °C (lit.[35] 201–203 °C); ^{1}H NMR ($CDCl_3$) δ 2.4 (s,

3H, CH_3), 7.3 (s, 4H, ArH), 7.7–8.0 (m, 4H, ArH); ^{13}C NMR ($CDCl_3$) δ 167.9, 138.7, 134.8, 132.4, 130.3, 129.5, 127.0, 124.2, 21.7; APCI m/z (rel intensity) 238.2 ($M+$, 100).

5.3.6. *N*-(3-Methylphenyl)-phthalimide (8). Compound **8** was prepared from phthalic anhydride (300 mg/2.03 mmol) and m-toluidine (220 mg/2.03 mmol/0.22 mL): mp 177–180 °C (lit.[36] 175–178 °C); ^{1}H NMR ($CDCl_3$) δ 2.4 (s, 3H, CH_3), 7.2–7.5 (m, 4H, ArH), 7.8–8.0 (m, 4H, ArH); ^{13}C NMR ($CDCl_3$) δ 167.0, 138.8, 134.0, 131.4, 131.1, 128.7, 128.6, 126.9, 123.4, 21.0; APCI m/z (rel intensity) 237.6 (M^+, 100).

5.3.7. *N*-(2-Methylphenyl)-phthalimide (9). Compound **9** was prepared from phthalic anhydride (300 mg/2.03 mmol) and o-toluidine (220 mg/2.03 mmol/0.22 mL): mp 184–186 °C (lit.[37] 182–183 °C); ^{1}H NMR ($CDCl_3$) δ 2.3 (s, 3H, CH_3), 7.2–7.5 (m, 4H, ArH), 7.7–8.0 (m, 4H, ArH); ^{13}C NMR ($CDCl_3$) δ 167.0, 136.2, 134.0, 131.6, 130.8, 129.1, 128.4, 126.5, 126.2, 123.4, 17.7; APCI m/z (rel intensity) 237.7 (M^+, 100).

5.3.8. *N*-(4-Methoxyphenyl)-phthalimide (10). Compound **10** was prepared from phthalic anhydride (1.2 g/8.1 mmol) and p-anisidine (1.0 g/8.1 mmol): mp 162–164 °C (lit.[38] 162 °C); ^{1}H NMR ($CDCl_3$) δ 3.8 (s, 3H, CH_3), 7.0 (d, $J=8.85$ Hz, 2H, ArH), 7.3 (d, $J=8.86$ Hz, 2H, ArH), 7.7–8.0 (m, 4H, ArH); ^{13}C NMR ($CDCl_3$) δ 168.1, 159.8, 134.8, 132.3, 128.4, 124.8, 124.2, 115.0, 56.0; APCI m/z (rel intensity) 254.2 (M^+, 100).

5.3.9. *N*-Phenyl-tetrafluorophthalimide (11). Compound **11** was prepared from tetrafluorophthalic anhydride (200 mg/0.91 mmol) and aniline (80 mg/0.91 mmol/0.08 mL): mp 206–208 °C (lit.[39] 202 °C); ^{1}H NMR ($CDCl_3$) δ 7.3–7.6 (m, 5H, ArH); ^{13}C NMR ($CDCl_3$) δ 161.0, 146.2, 145.0, 143.1, 141.6, 130.1, 129.0, 128.5, 126.1, 113.9; APCI m/z (rel intensity) 296.2 (M^+, 100).

5.3.10. *N*-(4-Chlorophenyl)-tetrafluorophthalimide (12). Compound **12** was prepared from tetrafluorophthalic anhydride (200 mg/0.91 mmol) and 4-chloroaniline (120 mg/0.91 mmol): mp 260–263 °C; ^{1}H NMR (DMSO-d_6) δ 7.4 (d, $J=8.67$ Hz, 2H ArH), 7.6 (d, $J=8.66$ Hz, 2H, ArH); ^{13}C NMR (DMSO-d_6) δ 161.7, 146.1, 144.3, 142.5, 140.9, 133.3, 129.8, 129.2, 114.0; EI m/z (rel intensity) 329.0 (M^+, 100), 331.2 (36). Anal. ($C_{14}H_4ClF_4NO_2$) C, H, N; C: calcd, 51.01; found, 50.81; H: calcd, 1.22; found, 1.44; N: calcd, 4.25; found, 4.18.

5.3.11. *N*-(3-Chlorophenyl)-tetrafluorophthalimide (13). Compound **13** was prepared from tetrafluorophthalic anhydride (400 mg/1.8 mmol) and 3-chloroaniline (230 mg/1.8 mmol/0.19 mL): mp 182–184 °C; ^{1}H NMR (DMSO-d_6) δ 7.4–7.5 (m, 2H, ArH), 7.6–7.7 (m, 2H, ArH); ^{13}C NMR (DMSO-d_6) δ 161.6, 146.0, 144.4, 142.6, 133.1, 132.3, 130.8, 128.8, 127.3, 126.2; EI m/z (rel intensity) 329.0 (M^+, 100), 331.2 (35). Anal. ($C_{14}H_4ClF_4NO_2$) C, H, N; C: calcd, 51.01; found, 50.99; H: calcd, 1.22; found, 1.08; N: calcd, 4.25; found, 4.27.

5.3.12. *N*-(2-Chlorophenyl)-tetrafluorophthalimide (14). Compound **14** was prepared from tetrafluorophthalic anhydride (400 mg/1.8 mmol) and 2-chloroaniline (230 mg/1.8 mmol/0.19 mL): mp 161–163 °C; ^{1}H NMR (DMSO-d_6) δ 7.5–7.7 (m, 3H, ArH), 7.7–7.8 (m, 1H, ArH); ^{13}C NMR (DMSO-d_6) δ 161.1, 146.3, 144.7, 132.1, 131.8, 131.3, 130.2, 128.5, 128.2; EI m/z (rel intensity) 329.0 (M^+, 10), 294.0 (100). Anal. ($C_{14}H_4ClF_4NO_2$) C, H, N; C: calcd, 51.01; found, 51.02; H: calcd, 1.22; found, 1.10; N: calcd, 4.25; found, 4.20.

5.3.13. *N*-(3,4-Dichlorophenyl)-tetrafluorophthalimide (15). Compound **15** was prepared from tetrafluorophthalic anhydride (200 mg/0.91 mmol) and 3,4-dichloroaniline (150 mg/0.91 mmol): mp 206–209 °C; ^{1}H NMR (DMSO-d_6) δ 7.4 (m, 1H, ArH), 7.7–7.9 (m, 2H, ArH); ^{13}C NMR (DMSO-d_6) δ 161.4, 146.1, 144.5, 142.6, 141.0, 131.6, 131.4, 131.2, 130.8, 129.2, 127.7, 113.9; EI m/z (rel intensity) 362.9 (M^+, 100), 364.4 (68). Anal. ($C_{14}H_3Cl_2F_4NO_2$) C, H, N; C: calcd, 46.19; found, 45.98; H: calcd, 0.83; found, 0.96; N: calcd, 3.85; found, 3.85.

5.3.14. *N*-(4-Methylphenyl)-tetrafluorophthalimide (16). Compound **16** was prepared from tetrafluorophthalic anhydride (200 mg/0.91 mmol) and p-toluidine (100 mg/0.91 mmol/0.1 mL): mp 234–236 °C; ^{1}H NMR ($CDCl_3$) δ 2.4 (s, 3H, CH_3), 7.2–7.4 (m, 4H, ArH); ^{13}C NMR ($CDCl_3$) δ 161.2, 146.5, 145.0, 143.0, 138.7, 129.6, 127.4, 125.9, 20.9; EI m/z (rel intensity) 309.0 (M^+, 100). Anal. ($C_{15}H_7F_4NO_2$) C, H, N; C: calcd, 58.26; found, 58.48; H: calcd, 2.28; found, 2.31; N: calcd, 4.53; found, 4.58.

5.3.15. *N*-(3-Methylphenyl)-tetrafluorophthalimide (17). Compound **17** was prepared from tetrafluorophthalic anhydride (400 mg/1.8 mmol) and m-toluidine (190 mg/1.8 mmol/0.20 mL): mp 188–191 °C; ^{1}H NMR (DMSO-d_6) δ 2.3 (s, 3H, CH_3), 7.2–7.3 (m, 3H, ArH), 7.4–7.5 (t, $J=7.45$, 7.66 Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6) δ 161.9, 143.1, 140.9, 138.7, 130.9, 129.5, 129.0, 127.8, 124.5, 20.9; EI m/z (rel intensity) 309.1 (M^+, 100). Anal. ($C_{15}H_7F_4NO_2$) C, H, N; C: calcd, 58.26; found, 58.08; H: calcd, 2.28; found, 2.27; N: calcd, 4.53; found, 4.59.

5.3.16. *N*-(2-Methylphenyl)-tetrafluorophthalimide (18). Compound **18** was prepared from tetrafluorophthalic anhydride (400 mg/1.8 mmol) and o-toluidine (190 mg/1.8 mmol/0.19 mL): mp 143–145 °C; ^{1}H NMR (DMSO-d_6) δ 2.1 (s, 3H, CH_3), 7.3–7.4 (m, 4H, ArH); ^{13}C NMR (DMSO-d_6) δ 161.8, 145.9, 144.5, 136.5, 130.9, 129.9, 129.7, 129.0, 126.8, 17.3; EI m/z (rel intensity) 309.1 (M^+, 100). Anal. ($C_{15}H_7F_4NO_2$) C, H, N; C: calcd, 58.26; found, 58.54; H: calcd, 2.28; found, 2.17; N: calcd, 4.53; found, 4.56.

5.3.17. *N*-(4-Methoxyphenyl)-tetrafluorophthalimide (19). Compound **19** was prepared from tetrafluorophthalic anhydride (200 mg/0.91 mmol) and p-anisidine (110 mg/0.91 mmol): mp 215–218 °C; ^{1}H NMR ($CDCl_3$) δ 3.8 (s, 3H, CH_3), 7.0 (d, $J=9.05$ Hz, 2H, ArH), 7.3 (d, $J=9.05$ Hz, 2H, ArH); ^{13}C NMR ($CDCl_3$) δ 161.3, 159.4, 146.5, 145.1, 145.0, 143.0, 141.5, 141.4, 127.4, 122.6, 114.3, 55.2; EI m/z (rel intensity)

325.2 (M^+, 100). Anal. ($C_{15}H_7F_4NO_3$) C, H, N; C: calcd, 55.40; found, 55.52; H: calcd, 2.17; found, 2.20; N: calcd, 4.31; found, 4.33.

5.3.18. *N*-Benzyltetrafluorophthalimide (21). Compound **21** was prepared from tetrafluorophthalic anhydride (400 mg/1.8 mmol) and benzylamine (190 mg/1.8 mmol/0.20 mL): mp 179–181 °C; ^{1}H NMR ($CDCl_3$) δ 4.8 (s, 2H, CH_2), 7.3–7.5 (m, 5H, Ar*H*); ^{13}C NMR ($CDCl_3$) δ 161.8, 146.2, 144.9, 134.8, 128.5, 128.4, 128.0, 42.0; EI *m*/*z* (rel intensity) 309.1 (M^+, 100). Anal. ($C_{15}H_7F_4NO_2$) C, H, N; C: calcd, 58.26; found, 58.19; H: calcd, 2.28; found, 2.20; N: calcd, 4.53; found, 4.51.

5.3.19. 2-Indan-1-yl-isoindole-1,3-dione (22). Compound **22** was prepared from phthalic anhydride (100 mg/0.675 mmol) and 1-aminoindan (90 mg/0.675 mmol/0.09 mL): mp 173–176 °C; ^{1}H NMR (DMSO-d_6) δ 1.8–1.9 (m, 1H, NCHCH_2CH$_2$), 2.3–2.4 (m, 1H, NCHCH_2CH$_2$), 2.7–2.9 (m, 2H, NCHCH$_2$CH_2), 5.4–5.5 (q, J = 8.09 Hz, 1H, NC*H*), 7.1–7.7 (m, 7H, Ar*H*), 8.6 (d, J = 8.47 Hz, 1H, Ar*H*); ^{13}C NMR (DMSO-d_6) δ 169.4, 168.9, 145.1, 143.7, 139.6, 132.1, 131.6, 130.1, 130.0, 128.7, 128.2, 127.2, 125.3, 54.9, 33.4, 30.7; APCI *m*/*z* (rel intensity) 263.8 (M^+, 100). Anal. ($C_{17}H_{13}NO_2$–1H_2O) C, H, N; C: calcd, 72.58; found, 72.50; H: calcd, 5.37; found, 5.39; N: calcd, 4.98; found, 5.00.

5.3.20. 4,5,6,7-Tetrafluoro-2-indan-1-yl-isoindole-1,3-dione (23). Compound **23** was prepared from tetrafluorophthalic anhydride (400 mg/1.8 mmol) and 1-aminoindan (240 mg/1.8 mmol/0.23 mL): mp 166–168 °C; ^{1}H NMR (DMSO-d_6) δ 2.3–2.5 (m, 2H, NCHCH_2CH$_2$), 2.9–3.0 (m, 1H, CHNCH$_2$CH_2), 3.1–3.2 (m, 1H, NCHCH$_2$CH_2), 5.7 (t, J = 7.45, 8.05 Hz, 1H, NC*H*), 7.1–7.3 (m, 4H, Ar*H*); ^{13}C NMR (DMSO-d_6) δ 162.3, 143.5, 139.9, 128.0, 126.5, 124.7, 123.6, 54.8, 30.5, 29.0; EI *m*/*z* (rel intensity) 335.0 (M^+, 10), 116.0 (100). Anal. ($C_{17}H_9F_4NO_2$) C, H, N; C: calcd, 60.90; found, 60.78; H: calcd, 2.71; found, 2.68; N: calcd, 4.18; found, 4.16.

5.3.21. 2-(1,2,3,4-Tetrahydronaphthalen-1-yl)-isoindole-1,3-dione (24). Compound **24** was prepared from phthalic anhydride (3.02 g/0.0204 mol) and 1,2,3,4-tetrahydro-1-naphthylamine (3.0 g/0.0204 mol): mp 129–132 °C (lit.[40] 127–128 °C); ^{1}H NMR (DMSO-d_6) δ 1.7–1.9 (m, 1H, C*H*), 2.0–2.2 (m, 2H, CH_2), 2.3–2.5 (m, 1H, C*H*), 2.8–3.1 (m, 2H, CH_2), 5.3 (dd, J = 5.39 Hz, 1H, C*H*), 6.9–7.2 (m, 4H, Ar*H*), 7.7–7.9 (m, 4H, Ar*H*); ^{13}C NMR (DMSO-d_6) δ 168.6, 138.2, 135.8, 135.5, 132.4, 129.9, 127.6, 127.0, 126.4, 124.1, 49.6, 29.8, 28.3, 22.8; APCI *m*/*z* (rel intensity) 278.0 (M^+, 55), 131.2 (100).

5.3.22. 4,5,6,7-Tetrafluoro-2-(1,2,3,4-tetrahydronaphthalen-1-yl)-isoindole-1,3-dione (25). Compound **25** was prepared from tetrafluorophthalic anhydride (400 mg/1.8 mmol) and 1,2,3,4-tetrahydro-1-naphthylamine (260 mg/1.8 mmol/0.26 mL): mp 160–162 °C; ^{1}H NMR (DMSO-d_6) δ 1.7–2.2 (m, 4H, NCHCH_2CH_2CH$_2$), 2.9–3.1 (m, 2H, NCHCH$_2$CH$_2$CH_2), 5.3–5.4 (q, J = 5.44, 4.64, 5.84 Hz, 1H, NC*H*), 7.0–7.2 (m, 4H, Ar*H*); ^{13}C NMR (DMSO-d_6) δ 162.4, 137.4, 134.0, 129.1, 126.9, 126.1, 125.8, 49.5, 28.8, 27.2, 21.7; EI *m*/*z* (rel intensity) 349.0 (M^+, 5), 130.0 (100). Anal. ($C_{18}H_{11}F_4NO_2$) C, H, N; C: calcd, 61.90; found, 61.65; H: calcd, 3.17; found, 3.16; N: calcd, 4.01; found, 3.97.

5.3.23. 2-(6,7,8,9-Tetrahydro-5H-benzocyclohepten-5-yl)-isoindole-1,3-dione (26). Compound **26** was from phthalic anhydride (250 mg/1.69 mmol) and amine 29 (272 mg/1.69 mmol): mp 196–198 °C; ^{1}H NMR (DMSO-d_6) δ 1.2–1.3 (m, 1H), 1.5–2.0 (m, 5H), 2.8–2.9 (bs, 2H), 5.1–5.2 (t, J = 8.47, 8.67 Hz, 1H, NC*H*), 7.1–7.8 (m, 7H, Ar*H*), 8.8 (d, J = 7.32 Hz, 1H, Ar*H*); ^{13}C NMR (DMSO-d_6) δ 168.9, 168.4, 143.7, 141.6, 139.7, 132.1, 130.1, 130.0, 128.8, 127.2, 126.8, 125.7, 53.1, 36.0, 35.4, 29.9, 28.0; APCI *m*/*z* (rel intensity) 292.4 (M^+, 100). Anal. ($C_{19}H_{17}NO_2$–1H_2O) C, H, N; C: calcd, 73.77; found, 73.80; H: calcd, 6.19; found, 6.17; N: calcd, 4.53; found, 4.57.

5.3.24. 4,5,6,7-Tetrafluoro-2-(6,7,8,9-tetrahydro-5H-benzocyclohepten-5-yl)-isoindole-1,3-dione (27). Compound **27** was from tetrafluorophthalic anhydride (400 mg/1.8 mmol) and amine **29** (360 mg/1.8 mmol): mp 190–192 °C; ^{1}H NMR (DMSO-d_6) δ 1.2–1.3 (m, 1H), 1.7–1.8 (m, 1H), 1.9–2.0 (m, 2H), 2.1–2.2 (m, 2H), 2.8–2.9 (m, 2H), 5.3–5.4 (m, 1H, NC*H*), 6.9–7.2 (m, 4H, Ar*H*); ^{13}C NMR (DMSO-d_6) δ 162.7, 145.9, 144.4, 144.3, 142.4, 142.2, 140.9, 140.7, 140.4, 139.0, 130.0, 127.2, 126.1, 123.9, 54.8, 35.3, 32.6, 30.0, 26.9; EI *m*/*z* (rel intensity) 363.0 (M^+, 15), 144.0 (100). Anal. ($C_{19}H_{13}F_4NO_2$) C, H, N; C: calcd, 62.81; found, 62.60; H: calcd, 3.61; found, 3.51; N: calcd, 3.86; found, 3.84.

5.3.25. 6,7,8,9-Tetrahydrobenzocyclohepten-5-one oxime (28). Amberlyst A-21 ion-exchange resin (1.25 g) was added in one portion to a stirring solution of 1-benzosuberone (1.0 g/6.24 mmol/0.93 mL) and hydroxylamine hydrochloride (870 mg/12.48 mmol) in 20 mL of ethanol. The mixture was stirred at room temperature for 24 h. The mixture was then filtered to remove the resin and concentrated to give a yellow/white solid. Ether (60 mL) was added and the solution was filtered to remove the excess hydroxylamine hydrochloride. The ether solution was then dried (Na_2SO_4) and evaporated to afford 28 as a light brown solid (82%). No further purification was necessary: mp 97–99 °C (lit.[41] 108 °C); ^{1}H NMR ($CDCl_3$) δ 1.6–1.8 (m, 4H), 2.7–2.8 (m, 4H), 7.1–7.5 (m, 4H, Ar*H*), 9.3 (bs, 1H, NO*H*); ^{13}C NMR ($CDCl_3$) δ 162.4, 139.1, 135.6, 129.0, 128.5, 127.0, 126.2, 31.5, 25.6, 25.5, 21.1; APCI *m*/*z* (rel intensity) 175.8 (M^+, 100).

5.3.26. 6,7,8,9-Tetrahydro-5H-benzocyclohepten-5-yl-amine-hydrochloride (29). Compound **28** (900 mg/5.14 mmol) was dissolved in 20 mL of absolute ethanol, and 10% Pd/C (50 mol%) was added. The mixture was stirred under an atmosphere of H_2 (g) for 3 days. The used Pd/C was removed by filtration through Celite and the solvent was removed in vacuo. The resulting clear viscous oil/solid suspension was taken up in ether, and 6 N HCl was added to precipitate the amine as the hydrochloride salt (100%). No further purification was necessary: mp 268–271 °C (lit.[42] 265–268 °C); ^{1}H NMR (DMSO-

d_6) 0.8–0.9 (m, 1H), 1.1–1.5 (m, 4H), 1.6–1.7 (m, 1H), 2.2–2.4 (m, 2H), 3.9 (m, 1H, NC*H*), 6.6–6.8 (m, 4H, Ar*H*), 8.3 (bs, 3H, NH_3); ^{13}C NMR ($CDCl_3$) δ 141.1, 137.8, 130.0, 128.0, 126.4, 124.4, 54.1, 35.2, 32.8, 28.3, 26.8; APCI *m*/*z* (rel intensity) 144.9 (100), 197.9 (M^+, 20).

6. Biological methods

6.1. Endothelial cell culture

HMEC cells were grown in Gibco MCDB-131 media supplemented with 2 mM L-glutamine (Gibco), 10% fetal bovine serum (Gibco). The culture medium also contained 50,000 IU/L penicillin (Gibco), 50 mg/L streptomycin (Gibco), and 20 μg/L epidermal growth factor (Sigma). Cells were routinely grown in filter-capped Nunc T25 and T75 flasks and kept at 37±0.5 °C in a humidified (90±1%) incubator under an air/CO_2 (95%/5%) atmosphere.

Cells were re-fed 3 times per week and passaged once per week. Upon passaging, cells received a 30±3 second rinse with phosphate buffered saline pH 7.4, and were then treated with Trypsin EDTA (0.05% Trypsin, 0.53 mM EDTA) (Gibco) for 2–7 min. The receiving cell surface was previously treated at room temperature with heated (37±0.5 °C) 2% bovine gelatin serum (Sigma) for 20–30 min, and then allowed to stand at room temperature for ≥30 min. Cell cultures were re-fed every 24±3 h prior to seeding.

7. Assay proliferation

Cells were seeded into a previously gelatin-treated 96-well Nunc culture plate at approximately 5×10^3 cells per well in MCDB-131 media as described above in either the presence or absence of 10 ng/mL vascular endothelial growth factor (BD Biosciences). Drug dosing occurred at 24±3 h, with redosing at 72±3 and 120±3 h. Drugs were dissolved in DMF at 375 mM and diluted to yield final concentrations in half log steps between 300 and 0.1 μM with a constant DMF concentration of 0.2%.

7.1. Crystal violet staining

Cells were fixed at 144±3 h with 1% gluteraldehyde in PBS (Gibco) for 15 (±30 s) min and then stained with 5 mg/L crystal violet (Fisher Scientific) in sterilized purified water for 15 min (±30 s). No less than three rinses (3–5) were performed to remove excess concentrated stain with PBS (as mentioned above). The resulting dilute stain was then removed through passive diffusion in tap water immersion. At ≥3 h under minimal light conditions cells received 250 μL/well Sorenson's solution: 8.967 g trisodium citrate (Fisher Scientific), 19.5 mL 1 N HCl, 480 mL distilled water, in 500 mL 90% Ethanol. Cells were shaken for 15 min at low speed and read using a Thermo Labsystems Multiskan Ascent plate reader at 492 nm immediately after completion on the plate shaker (<1 min).

7.2. IC_{50} Determination

A logarithmic concentration curve was made using concentrations decreasing in half log steps between 300 and 0.1 μM, with each concentration tested in triplicate both in the absence and in the presence of VEGF (as described above). Negative controls were tested twice in triplicate. Average absorbance values were divided by average negative control values after subtracting average blank values from both and plotted versus concentration. A linear regression was obtained and an IC_{50} value was calculated from that regression. For each concentration, high and low absorbance values were calculated by adding and subtracting, respectively, the standard deviation. The resultant values were plotted versus concentration to obtain high and low linear regressions of which high and low values of the IC_{50} were determined. The absolute values of the deviations from the original IC_{50} were averaged to determine the standard deviation of the IC_{50}. The percent error represents the standard error of the mean (SEM).

7.3. Prostate cancer cell culture

Du145, PC3 and LNCaP cells were grown in Gibco T-Media (custom formulation) supplemented 5% fetal bovine serum (Gibco) and 50,000 IU/L penicillin, 50 mg/L streptomycin (Gibco). Cells were routinely grown in filter-capped Nunc T25 and T75 flasks and kept at 37±0.5 °C in a humidified (90±1%) incubator under an air/CO_2 (95%/5%) atmosphere.

Cells were re-fed three times per week and passaged once per week. Upon passaging, cells received a 30±3 second rinse with phosphate buffered saline pH 7.4, and were then treated with 0.025% Trypsin without EDTA (Gibco) for 2–7 min. Cell cultures were re-fed every 24±3 h prior to seeding.

7.4. Assay proliferation

Cells were seeded into a 96-well Nunc culture plate in T-Media as described above at approximately 1×10^3 cells per well for Du145 and PC3 lines, and approximately 2×10^3 cells per well for LNCaP's. Drug dosing occurred at 24±3 h, with redosing at 72±3 and 120±3 h. Drugs were dissolved in DMF at 375 mM and diluted to yield final concentrations in half log steps between 300 and 0.1 μM with a constant DMF concentration of 0.2%.

7.5. PSA ELISA

Using PSA ELISA kits from Yes Biotech, 50 μL media samples from each test well (including all controls and blanks) of the androgen dependent cell line, LNCaP, were added to the antibody pre-coated Microtiter Plate as well as standard samples all in duplicate at 144±3 h, to which 50 μL of kit sample diluent was then added. Plates were covered and shaken at moderate speed for 2 min±20 s and then incubated as described above for 30 min±1 min. The ELISA plate was then rinsed with approximately 300 μL of distilled water per well five times. After rinsing, 100 μL of kit conjugate was added

to each well, the plate was covered, and incubated as described immediately above. Rinsing was repeated as described immediately above and then 50 μL of kit substrate solution A was added to each well followed by 50 μL of kit substrate solution B. Plates were covered, shaken at moderate speed for 30 ± 3 s and incubated as described immediately above for 15 min ± 1 min. 100 μL of kit stop solution was then added to each well and the plate was covered and shaken at moderate speed for 5 min ± 30 s. Plates were immediately (< 1 min) read using a Thermo Labsystems Multiskan Ascent plate reader at 450 nm.

7.6. Crystal violet staining

Cells were fixed at 144 ± 3 h with 1% gluteraldehyde in PBS (Gibco) for 15 min (± 30 s) and then stained with 5 mg/L crystal violet (Fisher Scientific) in sterilized purified water for 15 min (± 30 s). No less than three rinses (3–5) were performed to remove excess concentrated stain with PBS (as mentioned above). The resulting dilute stain was then removed through passive diffusion in tap water immersion. Cells received 250 μL/well Sorenson's solution: 8.967 g trisodium citrate (Fisher Scientific), 19.5 mL 1 N HCl, and 480 mL distilled water in 500 mL 90% Ethanol, at ≥3 h under minimal light conditions. Cells were then shaken for 15 min at low speed and read using a Thermo Labsystems Multiskan Ascent plate reader at 492 nm immediately after completion on the plate shaker (< 1 min).

7.7. PSA determination

A standard curve was plotted of ng PSA versus. $O.D._{450\ nm}$ using the 8 provided standards (subtracting the blank from all subsequent values) and a linear trendline was fitted to the values with $b = 0$ and acceptable R^2 values ≥99.9%. The O.D. values obtained for all ELISA test wells were then compared to the trendline equation after subtraction of the ELISA standard blank. The average mass value of the cell blanks was then subtracted from the averages of each set of test wells (twice in triplicate) with acceptable standard deviations between duplicates ≤0.1% of the actual value. The resultant normalized masses were then divided by the negative control average normalized mass to give a mass to mass PSA percent of control. That value was divided by the corollary crystal violet ratio (described below): (average test $O.D._{492\ nm}$ −blank)/(average negative control $O.D._{492\ nm}$ −blank). To calculate final values representing PSA secreted per cell, we plotted these ratios as percent of control. This normalized the values such that all values equal to one had no observable net effect on PSA secretion, and all values deviating from one had a respective correlation with increase or decrease in PSA secretion.

7.8. IC_{50} Determination

A logarithmic concentration curve was made using concentrations decreasing in half log steps between 300 and 0.1 μM, with controls and each concentration tested in triplicate (as described above). Average absorbance values were divided by average negative control values after subtracting average blank values from both and plotted versus concentration. A linear regression was obtained and an IC_{50} value was calculated from that regression. For each concentration, high and low absorbance values were calculated by adding and subtracting, respectively, the standard deviation. The resultant values were plotted versus concentration to obtain high and low linear regressions of which high and low values of the IC_{50} were determined. The absolute values of the deviations from the original IC_{50} were averaged to determine the average SEM.

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