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Discovery of Phthalimides as Immunomodulatory and Antitumor Drug Prototypes

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Introduction

Modulation of the immune system is an emerging concept in the control of tumor growth.[1] While there are many mechanisms that underlie the role the immune system plays in tumor cells, minimizing metastasis by attenuating the expression of pro-angiogenic cytokines, or up-regulating the expression of endothelial factors that are crucial for the angiogenic process in metastasis and alternatively enhancing the antitumor immunity mediated by interferon-γ and interleukin-2 are the most significant features identified to date.[2,3] In view of this, the discovery of small immunomodulating agents is a task that is currently receiving much attention.

Among the anticancer and immunomodulatory drug candidates that have entered into clinical trials, the majority are analogues of thalidomide (Thl, **1**), such as lenalidomide (Revlimid, CC-5013) and ACTIMID (CC-4047).[4] Studies of structure–activity relationships (SARs) in the analogues and metabolites of Thl have shown that phthalimide is an essential pharmacophoric fragment.[5] Following this line of research, phthalimide has commonly been employed in the design of potential anti-inflammatory,[6] immunomodulatory,[7] antiangiogenic,[8] and antitumor[9] drug candidates. Given this promising outlook, the strategy of molecular hybridization using phthalimide as a pharmacophoric fragment has figured prominently in recent research and has given rise to many successful outcomes.[10] As an example, potent and selective histone deacetylase (HDAC) inhibitors have been designed by hybridizing two distinct structural domains: phthalimide and the hydroxamic acid subunit. This has led to the identification of various SARs and the discovery of a new potent hybrid lead compound, (*E*)-3-(1-oxo-2-(1,2-diphenylethyl)isoindolin-6-yl)-*N*-hydroxyacrylamide, which has been described as having selective toxicity against tumor cells.[11]

On the other hand, the thiosemicarbazones have figured prominently in the vast number of structural subunits used to design anticancer agents.[12] Some well-known mechanisms involving thiosemicarbazones involve the inhibition of ribonucleotide reductase,[13] alteration of DNA structure,[14] and the chelation of endogenous metals.[13] An example of this versatility was recently reported by Gottesman and co-workers, who used the molecular hybridization of the β-isatin scaffold and thiosemicarbazones. It was clearly demonstrated that the insertion of a thiosemicarbazone subunit into optimal templates leads to an improvement in the anticancer properties of β-isatins, paving the way for the discovery of potent and selective anticancer compounds such as the lead compound 1-(5′-fluoroisatin)-4-(4′-methoxyphenyl)-3- thiosemicarbazone (**2**; IC_{50} = 5.2 μM against multidrug-resistant cells that express P-glycoprotein).[15] Because of this unique pharmacological profile, the attachment of thiosemicarbazones has been employed both in the design of ligands for further complexation with transition metals[12] and during the processes of hit-to-lead or lead-to-drug conversions.[13]

Bearing in mind the molecular pharmacophores outlined above and structural requirements, we describe herein the design, synthesis, and pharmacological evaluation of 11 new potential antitumor and immunomodulatory agents. To establish an appropriate set of SARs, we first prepared phthalimides containing the thiosemicarbazide **2b** or thiosemicarbazone **4** subunits. An attempt was then made to synthesize two bioisosteres[16] of **2b**: the semicarbazide **2a** and aminoguanidine **2c** derivatives, in addition to the analogues of **2b** containing *N*-methyl or *N*-phenyl substituents. Subsequently, a short series of phthalimides bearing the thiazolin-4-one ring (compounds **3a**–**d**) was also investigated, for reasons of the bioisosteric relationship present between thiazolin-4-ones and thiosemicarbazones[17] and the significant number of thiazolin-4-ones that are active against multidrug-resistant cancer cells, as in the case of the lead compound **3**.[18] Our design incorporated the molecular hybridization approach suggested by the structural features of prototypes **1**–**3** in addition to molecular bioisosterism (Figure 1).

The compound series **2a**–**f** was first prepared by using microwave irradiation, in view of a recent report in which the reaction of *N*-(hydroxymethyl)phthalimide with arylamines using microwave heating under normal conditions rapidly furnishes

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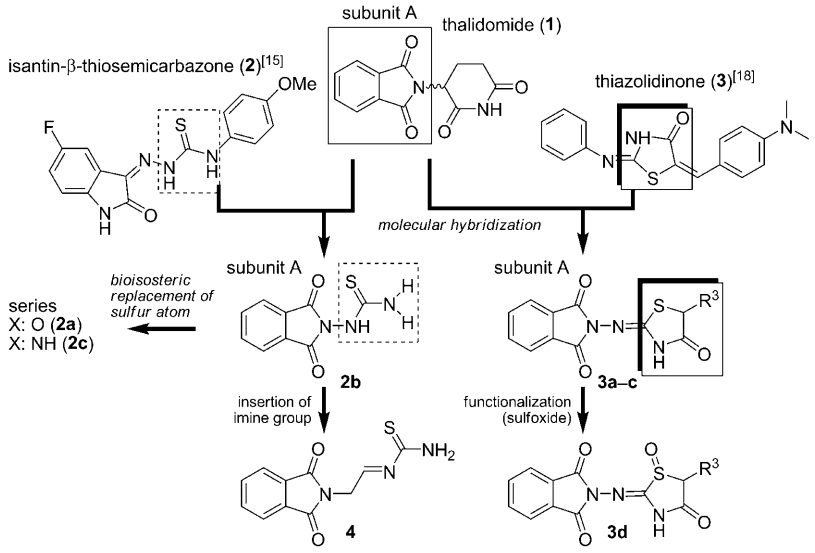
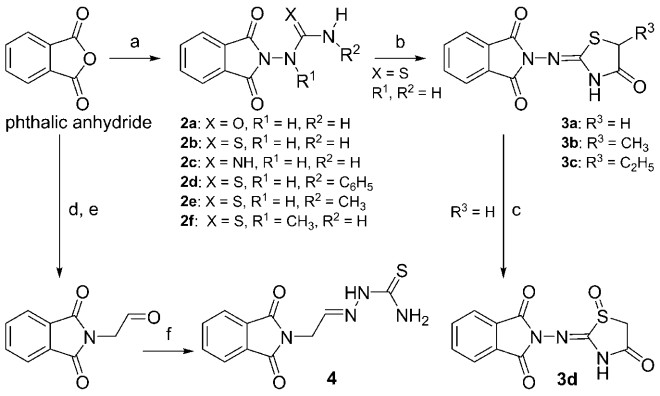


Figure 1. Our design concept of antitumor and immunomodulatory drugs exploring the molecular hybridization of prototypes **1**–**3**.

the substituted phthalimides in excellent yields.[19] However, such conditions led only to complex mixtures of by-products without the generation of desired products. Therefore, the reactions were performed with conventional heating,[20] which provided compounds **2a**–**f** in good yields in crystalline form (Scheme 1). Thiazolin-4-ones **3a**–**d** were then synthesized by



Scheme 1. Reagents and conditions: a) $NH_2NR^1C(X)NHR^2$, DMAP, DMF, reflux (3 h); b) $BrCH(R^3)CO_2H$, NaOAc, EtOH, reflux, 10 h; c) *m*CPBA, CH_2Cl_2, 0 °C, 5 h; d) aminoacetaldehyde diethyl acetal, DMAP, toluene, reflux (1 h); e) 0.1 N H_2SO_4, H_2O, reflux, 5 h; f) thiosemicarbazide, H_2SO_4 (3 drops), EtOH, reflux, 2 h.

following a reported procedure[21] that condenses thiosemicarbazide **2b** with the respective α-bromocarboxylic acids, fused sodium acetate and ethanol under reflux. Alternatively, it was possible to prepare compounds **3a**–**c** by one-pot reactions starting with phthalic anhydride. This produced yields similar to those provided by conventional two-step reactions, although more efficiently and with shorter reaction times (6 h in one-pot versus 13 h for two-step reactions).

Although the structures of **3a**–**d** appear reasonable, other possibilities, such as the tautomer **3a-l**, were also considered (scheme S1, Supporting Information).[22] An attempt to distinguish between structures **3a** and **3a-l** by ^{1}H NMR NOESY experiments was inconclusive, probably due to variations in the NH signal. Although phthalimide **2b** produced single crystals suitable for X-ray diffraction (Supporting Information), our attempt to obtain single crystals of **3a** was unsuccessful. We therefore carried out ab initio calculations using the unsubstituted derivative **3a** and its tautomer **3a-l** as models, employing the B3LYP/6-31G(d,p) level of theory (see Supporting Information). As shown in Figure 2, tautomer **3a** is 16.86 kJ mol^{-1} more stable than **3a-l**. Additional experiments corroborated this, as compound **3a** does not undergo N-acetylation under mild

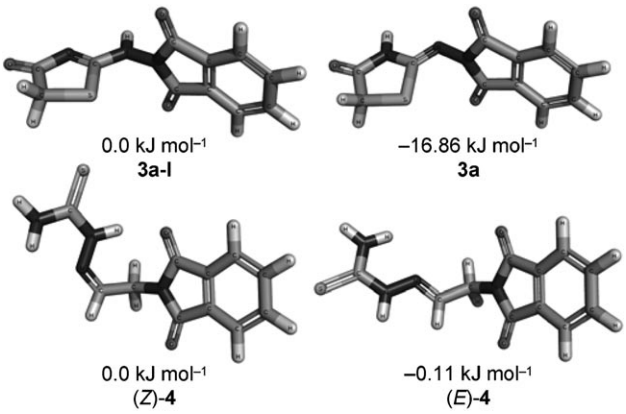


Figure 2. Optimized geometries and relative stability of the tautomers **3a** and **3a-l**, and the *Z* and *E* isomers of compound **4**, obtained by ab initio calculations using the B3LYP/6-31G(d,p) method.

conditions; this reactivity is typical of an amide NH group (scheme S1, Supporting Information). Sulfoxide derivative **3d** was prepared using a slightly modified protocol with *meta*-chloroperoxybenzoic acid (*m*CPBA) as an oxidizing agent,[23] while thiosemicarbazone **4** was obtained in three steps starting from phthalic anhydride, yielding a single product. Our quantum chemical calculations, performed with the same methodology mentioned above, revealed that the energy levels of the *E* and *Z* isomers of **4** are nearly degenerate, with the *E* isomer only 0.11 kJ mol^{-1} more stable than the *Z* isomer (Figure 2). Therefore, it seems fair to suggest that the structures proposed for **3a**–**d** are correct and that compound **4** probably adopts

the *E* configuration. The physical and chemical data for these compounds are listed in Table 1.

With their structures elucidated, all compounds were tested as immunomodulatory and anticancer agents. In an attempt to replicate the in vivo immunological aspects, we performed in vitro assays using the total spleen cell population that includes macrophages and lymphocytes.[24] To this end, spleen cells from Balb/c mice were cultured in the presence of each compound at 20 μM together with concanavalin A (Con A) or lipopolysaccharide (LPS). The stimulus with Con A was thus suitable for analyzing interleukin-2 (IL-2) production and consequent lymphocyte proliferation (Figure 3a), as well as the generation of nitric oxide (NO). Interleukin-10 (IL-10) production was only detected under LPS stimulation. In all these assays, Thl was used as the reference drug (20 μM). Regarding the production of IL-2 (a proliferative cytokine), it was observed that this was not altered by the presence of these phthalimides (Figure 3b), except for phthalimide **2b**, which decreased IL-2 production by 50% relative to LPS-treated splenocytes as an experimental control. Likewise, the proliferative effect induced by Con A was not drastically impaired by any of these phthalimides (Figure 3a). Under the same conditions, we also examined the effects of these phthalimides on the production of interferon-γ (IFN- γ), a pro-inflammatory cytokine associated with antitumor and antiviral protection.[25] Apart from compound **3c**, which altered the production of IFN-γ, we did not observe significant suppression of IFN-γ secretion by splenocytes in response to Con A (figure S3, Supporting Information).

Production of NO in splenocytes stimulated with Con A was decreased by all the phthalimides (Figure 4a), thereby enabling us to establish a set of interesting SAR data. Analysis of the phthalimides belonging to the **2a–f** series showed that the unsubstituted derivative **2a** is active in inhibiting the production of NO, but it is only half as potent as Thl. Comparison of the inhibitory activities of **2b** and its bioisostere **2a** revealed the latter to be twice as active and to have potency similar to that of Thl. More interestingly, the replacement of the S atom of **2b** by NH in **2c** led to a guanidine derivative that is almost inac-

Table 1. Physical and chemical properties of the phthalimide derivatives.

Compd	Formula[a]	M_r [Da]	Yield [%][b]	mp [°C]	R_f[c]	Clog *P*[d]
2a	$C_9H_7N_3O_3$	205.17	57	203–205	0.71	−0.42
2b	$C_9H_7N_3O_2S$	221.23	61	264–268	0.87	0.39
2c	$C_9H_8N_4O_2$	204.19	55	287–291	0.74	−0.84
2d	$C_{15}H_{11}N_3O_2S$	297.33	62	261–263	0.40	2.26
2e	$C_{10}H_9N_3O_2S$	235.26	45	231–233	0.66	0.74
2f	$C_{10}H_9N_3O_2S$	235.26	90	216–218	0.82	0.63
3a	$C_{11}H_7N_3O_3S$	261.25	50	221–223	0.84	0.56
3b	$C_{12}H_9N_3O_3S$	275.28	65	229–230	0.79	0.93
3c	$C_{13}H_{11}N_3O_3S$	289.31	99	178–179	0.87	1.37
3d	$C_{11}H_7N_3O_4S$	277.26	42	264–265	0.76	0.03
4	$C_{11}H_{10}N_4O_2S$	262.29	75	221–222	0.80	1.35

[a] Analytical data for C, H, N, and S are within ±0.4% of calculated values. [b] Isolated products. [c] Solvent: EtOAc/CH_2Cl_2 (8:2); spots were visualized under UV light. [d] Determined with ALOGPS 2.1.[26]

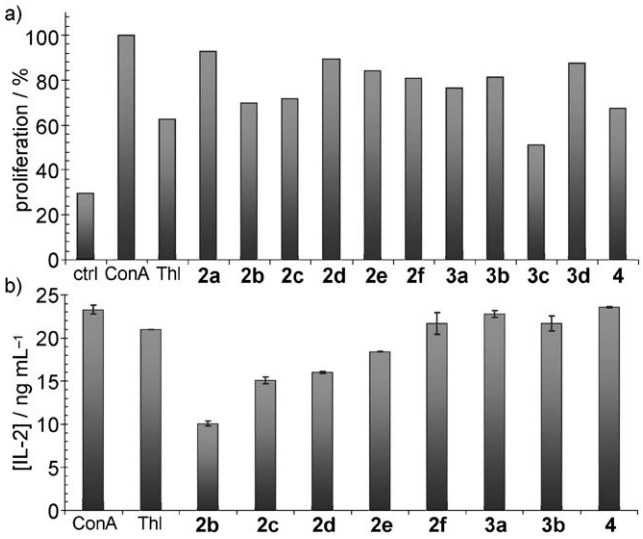


Figure 3. a) Proliferation response of Balb/c mouse splenocytes stimulated with Con A (2.5 μg mL^{-1}) alone and in conjunction with phthalimides or Thl (each at 20 μM); the cell proliferation induced by Con A was monitored by the percentage of AlamarBlue reduction after 62 h treatment with the tested compounds; data are the average of three replicates. b) Effect of phthalimides and Thl (each at 20 μM) on the production of IL-2 by Balb/c mouse splenocytes (5×10^6) stimulated with Con A (5 μg mL^{-1}); IL-2 content in the supernatant from splenocyte culture was measured after 24 h by sandwich ELISA (BD Bioscience); data are the mean ± SD of values obtained in triplicate.

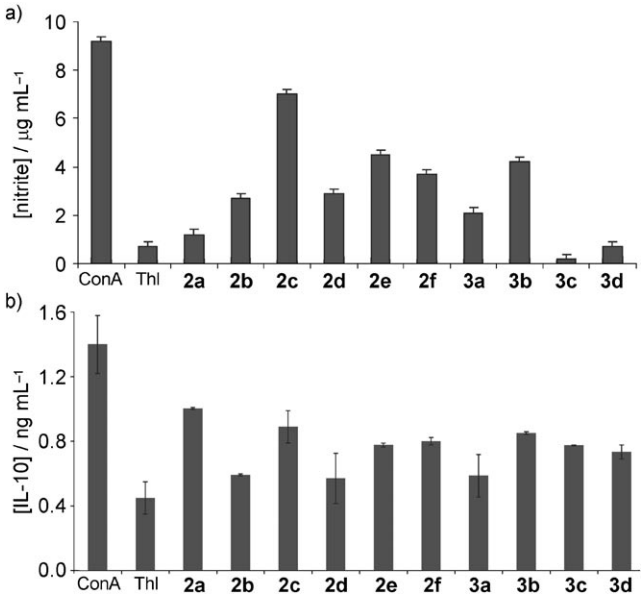


Figure 4. a) Effect of phthalimides and Thl (each at 20 μM) on NO production by Balb/c mouse splenocytes (5×10^6) stimulated with Con A (5 μg mL^{-1}); the content of NO in the supernatant of splenocyte culture was determined with the Griess reagent after incubation for 72 h; absorbance was measured at λ 540 nm; nitrite concentration was calculated from a NaNO standard curve, and data are the mean ± SD of values obtained in triplicate. b) Effect of phthalimides and Thl (each at 20 μM) on the production of IL-10 by Balb/c mouse splenocytes (5×10^6) stimulated with LPS (5 μg mL^{-1}); IL-10 content in the supernatant from cultured cell was measured after 72 h by sandwich ELISA (BD Bioscience); data are the mean ± SD of values obtained in triplicate.

tive at the tested dose. On the one hand, replacing the R^2 H atom of **2b** with a phenyl group in **2d** led to equipotent compounds. Replacement of hydrogen by methyl groups (compounds **2e** and **2f**), on the other hand, led to a steady decrease in potency to inhibit NO production.

Regarding the inhibition of NO by phthalimides containing the thiazolin-4-one ring (compounds **3a**–**d**), it was possible to draw up an interesting set of SAR data. On the one hand, replacement of the thiosemicarbazide subunit of **2b** with the thiazolin-4-one ring in **3a** led to equipotent compounds. On the other hand, with the exception of thiazolin-4-one **3c**, which was as potent as Thl in decreasing NO levels, the other thiazolin-4-ones displayed low efficacy in inhibiting NO production. Although tested in triplicate, NO was not detected in spleen cells treated with thiosemicarbazone **4** at 20 μM. In general, the inhibition of NO displayed by these compounds is attractive, because NO plays a pivotal role in tumor growth and inflammatory processes.[10d] As proof of this, the modulation of NO may be directly associated with different cell activities, including angiogenesis.[10a]

Additional experiments were performed involving the measurement of IL-10, as this cytokine controls the overproduction of pro-inflammatory molecules such as NO, tumor necrosis factor-α (TNF-α), and IFN-γ. Figure 4b shows that the tested phthalimides decrease IL-10 levels to a significant degree, although not to the extent observed with Thl. There was a decrease in IL-10 levels in the case of all the phthalimides, with inhibition of 30–57% relative to cells treated with LPS alone, while the value for suppression of IL-10 by Thl was 70%. This attenuation in IL-10 secretion is in accordance with our earlier observation that these compounds inhibit NO synthesis.

It is possible to draw some conclusions in light of these findings. First, these compounds share immunosuppressive properties to some degree and achieve this by maintaining low IL-10 production and suppressing NO synthesis; however, this comes with the notable advantage that these compounds do not affect the immune response elicited by T-lymphocytes. Second, the results for lymphocyte proliferation, IL-2 and IFN-γ syntheses suggest that these phthalimides do not possess general immunosuppressive properties, a characteristic that is highly desirable in new immunomodulatory drug candidates. These findings provide evidence that these phthalimides act on the immune system, halting the spread of tumor metastases. It is also plausible that these compounds may sustain antitumor immunity dependent on T-cells.

In view of the way these compounds act on the immune system, it is reasonable to investigate their anticancer properties. These phthalimides were thus first evaluated in vitro against four tumor cell lines: MDA/MB-435 (breast), HL-60 (leukemia), HCT-8 (colon), and SF-295 (glioblastoma). Table 2 summarizes the IC_{50} values for these compounds, using doxorubicin (Dox) as the control drug and Thl as the reference phthalimide. Apart from sulfoxide derivative **3d**, which is capable of inhibiting the growth of all four cell lines tested, all the others were inactive ($IC_{50} > 300$ μM). In the same assay, Thl was not able to inhibit the proliferation at the highest dose of 300 μM. Likewise, using mouse erythrocytes, neither these phthalimides nor Thl led to hemolytic action, even at the highest concentration of 200 μg mL^{-1} (data not shown), confirming that these compounds do not have in vitro antiproliferative effects and do not act on the cell membranes of erythrocytes.

Table 2. In vitro cytotoxic activity of compounds against human tumor cells.

Compd	IC_{50} [μM][a] MDA/MB-435	HCT-8	SF-295	HL-60
2a	>300	>300	223 (147–225)	216 (119–320)
2b	>300	>300	>300	>300
2c	>300	>300	>300	>300
2d	>300	>300	>300	176 (156–196)
2e	>300	>300	>300	>300
2f	>300	>300	>300	>300
3a	>300	>300	>300	>300
3b	>300	>300	>300	>300
3c	>300	>300	>300	>300
3d	16 (13–17)	31.6 (24–41)	10 (8–13)	8.0 (5.6–11.3)
4	>300	>300	>300	>300
Dox[b]	2.5 (2.5–5.0)	2.5 (2.5–5.0)	5.7 (4.7–6.2)	5.0 (2.5–5.0)
Thl[c]	>300	>300	>300	121 (91–162)

[a] Values measured after incubation for 72 h; 95% confidence interval obtained from at least three independent experiments; SD values in parentheses. [b] Doxorubicin. [c] Thalidomide.

These preliminary observations are not inconsistent, because in order to effect antitumor activity, Thl and its analogues undergo biological metabolism.[9a] To ascertain this, the antitumor properties of selected compounds were investigated in mice bearing 180 sarcoma (Figure 5). In this case, our in vivo results were more promising. Three of the most potent NO inhibitors

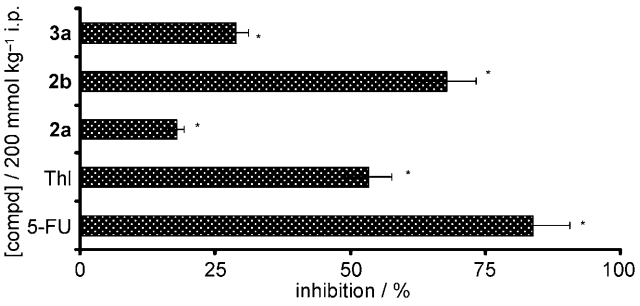


Figure 5. Antitumor effects of phthalimides in mice transplanted with S-180 sarcoma. Results are expressed as the mean value ± SEM for $n = 8$ animals. Percent inhibition was obtained by comparison with the control group, which received only vehicle (data not shown); $^*p < 0.01$ relative to control by ANOVA followed by Student Newman–Keuls test.

that decreased IL-10 levels (**2a**–**b** and **3a**) were first screened for general toxicity in mice treated intraperitoneally with these compounds at a single dose of 200 μmol kg^{-1}. The animals were monitored for signs of general toxicity, including behavior and feeding, until 72 h post-treatment. This experiment showed that such compounds are neither lethal in mice nor cause tissue damage. The same dose was therefore used for in vivo antitumor evaluation. In the model using male Swiss mice bearing the solid S-180 sarcoma, compounds **2a**, **2b**, and

3a were administered intraperitoneally for seven consecutive days after 24 h of tumor transplant. Figure 5 shows that, in contrast to semicarbazide **2a**, which is almost inactive, its bioisostere thiosemicarbazide **2b** is a potent antitumor agent and is endowed with potency similar to that of Thl. The bioisosteric exchange of thiosemicarbazide **2b** with thiazolin-4-one **3a** decreased the antitumor activity, with **3a** being half as potent as **2b**.

From these in vivo results, some general conclusions can be drawn. The bioisosteric relationship between the semicarbazide **2a** and thiosemicarbazide **2b** suggests that the thiocarbonyl group of derivative **2b** plays an important role in this compound's antitumor properties. Moreover, the weak antitumor activity of compounds **2a** and **2c** could be due, in part, to their low calculated log *P* values[26] (Table 1), as poor lipophilicity is often associated with problems in permeating the cell membrane. With regard to the antitumor mechanism of compound **2b**, it cannot be confirmed whether the anticancer activity is a result of direct modulation of the immune system. However, in view of prior knowledge that the processes of immunomodulation and antiangiogenesis are closely related and are both inhibited by Thl, it is reasonable to argue that modulation of cytokines or NO is, at the very least, involved to some extent in the tumor inhibition provided by these phthalimides.

In summary, a new set of phthalimides was designed in which bioisosterism and molecular hybridization were explored. We were able to identify phthalimide **2b** as the most potent anticancer and immunomodulatory agent of this series. Compound **2b** did not show cytotoxicity against splenocytes at a concentration of 20 μM, which was the dose used in the immunological assays. In other words, it is an immunomodulatory drug at concentrations that do not overtly affect mammalian cells. Despite the fact that **2b** is as effective as Thl, one drawback is that it proved to be roughly half as potent as 5-fluorouracil (5-FU) as an antitumor drug. This warrants further optimization studies with a view to improving the antitumor properties of this prototype.

Experimental Section

The Supporting Information details the synthesis of the compounds outlined in Scheme 1. Pharmacological and computational studies conducted can also be found in the Supporting Information. CCDC 757441 (**2b**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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Keywords: antitumor agents · hydrazones · immunomodulation · molecular hybridization · phthalimides

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