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Isosteric analogs of lenalidomide and pomalidomide: Synthesis and biological activity

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ABSTRACT

A series of analogs of the immunomodulary drugs lenalidomide (**1**) and pomalidomide (**2**), in which the amino group is replaced with various isosteres, was prepared and assayed for immunomodulatory activity and activity against cancer cell lines. The 4-methyl and 4-chloro analogs **4** and **15**, respectively, displayed potent inhibition of tumor necrosis factor-α (TNF-α) in LPS-stimulated hPBMC, potent stimulation of IL-2 in a human T cell co-stimulation assay, and anti-proliferative activity against the Namalwa lymphoma cell line. Both of these analogs displayed oral bioavailability in rat.

Thalidomide (**3**), a molecule with diverse pharmacological activities, was initially marketed as a sedative in the late 1950s and was also widely used as a treatment for morning sickness in pregnant women in the United Kingdom, Europe, Canada, and Australia. Its now well-known tragic teratogenic effects caused its withdrawal from the world market in the early 1960s. Over the years, there has been interest in thalidomide for the treatment of various hematologic malignancies,[1] solid tumors,[2] and a variety of inflammatory and autoimmune diseases.[3] Recent studies have uncovered a variety of mechanisms of thalidomide action. It was reported in 1991 that thalidomide is a selective inhibitor of tumor necrosis factor-α (TNF-α) production in lipopolysaccharide (LPS) stimulated human monocytes.[4] TNF-α is a key pro-inflammatory cytokine, and elevated levels have been linked with the pathology of a number of inflammatory and autoimmune diseases including rheumatoid arthritis, Crohn's disease, aphthous ulcers, cachexia, graft versus host disease, asthma, ARDS and AIDS.[5] Another biological activity of thalidomide is its ability to co-stimulate T cells that have been partially activated by the T cell receptor.[6] Co-stimulation results in T cell activation and facilitates an antigen-specific effector response. Taken together the immunomodulatory properties of thalidomide, which are dependent on the type of immune cell activated as well as the type of stimulus that the cell receives, provide a rationale for the mechanism of thalidomide action in the context of autoimmune and inflammatory disease states. Other pharmacologic activities of thalidomide include its inhibition of angiogenesis[7] and its anti-cancer properties.[8] In the late 1990's it was reported that thalidomide is efficacious for the treatment of multiple myeloma (MM), a hematological cancer caused by growth of tumor cells derived from the plasma cells in the bone marrow.[1] Thalidomide received FDA approval in 2006 for the treatment of newly diagnosed MM. Although the cellular pharmacology of thalidomide and its analogs had been well studied, its mechanism of action was not understood until 2010, when the molecular target of thalidomide responsible for its teratogenic effects was shown to be a protein named cereblon, that acts as a co-receptor for an E3 ubiquitin ligase complex including DNA damage binding protein 1 (DDB1) and the E3 ligase cullin 4A (CUL4A).[9] Since that initial discovery, cereblon has been reported to also mediate the anti-MM and T cell costimulatory activities of lenalidomide and pomalidomide.[10]

A medicinal chemistry program to optimize the immunomodulatory properties of thalidomide and reduce its side-effects led to the discovery of lenalidomide (**1**), which is a potent immunomodulator that is ~800 times more potent as an inhibitor of TNF-α in LPS-stimulated hPBMC.[11] In the US, lenalidomide was approved by the FDA in 2005 for low- or intermediate-1-risk myelodysplastic

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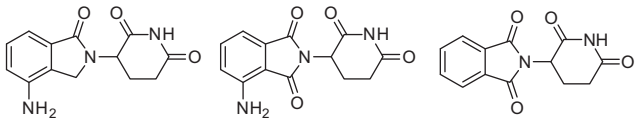
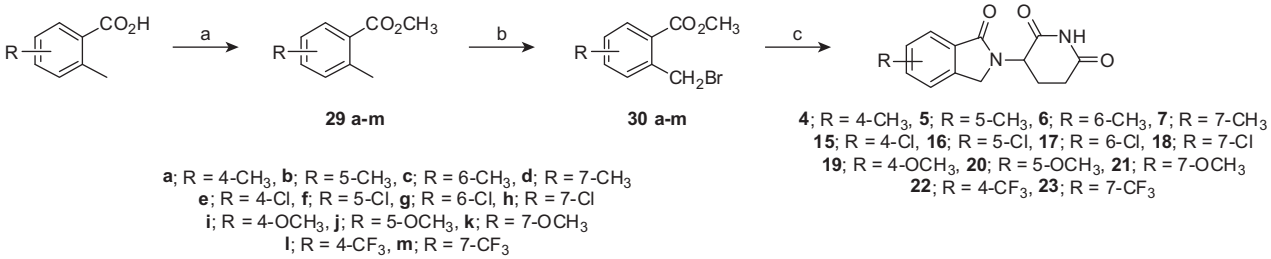


Figure 1. Structures of lenalidomide (**1**), pomalidomide (**2**), and thalidomide (**3**).

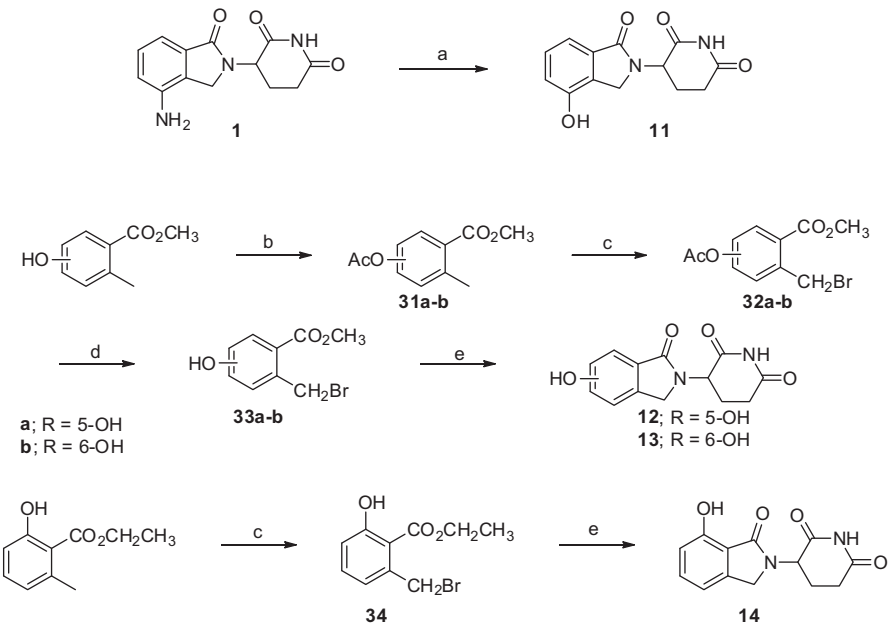
syndromes (MDS) associated with a chromosome 5q31 deletion, with or without additional cytogenetic abnormalities, and in 2006 for the treatment of patients with multiple myeloma (MM) who have received at least one prior therapy. Lenalidomide possesses direct tumoricidal effects that have been linked to cereblon binding, resulting in inhibition of expression of tumor oncogenes such as *IRF4* and *C-MYC* with the attendant induction of tumor suppressor genes,[12] cell cycle arrest,[13]actin polymerization and the relocalization of membrane proteins leading to cytoskeletal reorganization,[14] and inhibition of autocrine cytokines.[15] In multiple myeloma, lenalidomide treatment leads to caspase activation and resultant tumor cell apoptosis.[16] Lenalidomide also has profound immunomodulatory properties, including restoration of immune synapse formation,[17] augmentation of natural killer cell cytotoxicity,[18] and inhibition of regulatory T cells.[19] These actions result in an immune-mediated tumor cell killing that is in direct contrast to the immune suppression which typifies conventional chemotherapy. Lenalidomide also possesses anti-angiogenic properties and decreases paracrine secretion of key mediators of angiogenesis.[20] In addition to its approved indications of MM and MDS, clinical studies have also demonstrated that lenalidomide has efficacious activity in chronic lymphocytic leukemia (CLL)[21] and non-Hodgkin's lymphoma (NHL).[22]

Structural optimization of thalidomide, **3** also led to the discovery of pomalidomide (**2**), which was 10-fold more potent than lenalidomide as a TNF-α inhibitor and IL-2 stimulator.[11] Pomalidomide is currently undergoing late-stage clinical development for treatment of multiple myeloma and myeloproliferative neoplasm-associated myelofibrosis.[23] In clinical trials for multiple myeloma, pomalidomide has been shown to be effective in overcoming resistance to lenalidomide and thalidomide, as well as the proteosome inhibitor bortezomib.[24]

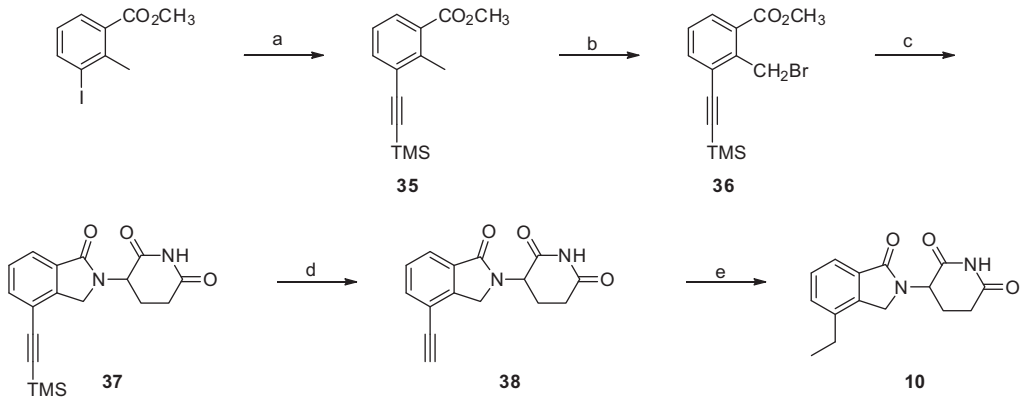
Agents **1**, **2**, and **3** are structurally similar, with all three sharing the common α-(isoindolinone-2-yl)-glutarimide core structure (Fig. 1). A previous report described the activity of positional isomers of **1** and **2**.[10] It was shown that analogs possessing an amino group residing at the 4-position of the isoindolinone or isoindolinedione (phthalimide) ring displayed potent inhibition of TNF-α with the other isomers showing much reduced activity. If the amino group was moved to the 5-position of the phthalimide, or to the 5-, 6-, or 7-positions of the isoindolinone ring, the resulting analogs had IC_{50}s of ⩾100 μM as TNF-α inhibitors. In light of the clinical success of lenalidomide and pomalidomide, a more detailed



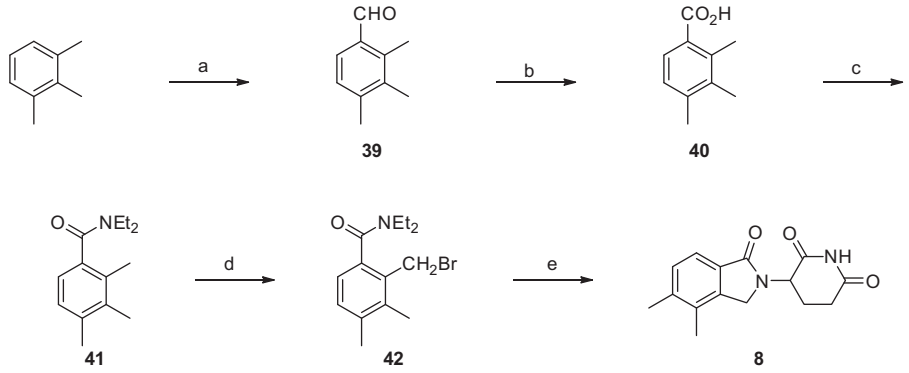
Scheme 1. Reagents and conditions: (a) CH_3I, $NaHCO_3$, DMF or $HC(OCH_3)_3$, 79–99%; (b) NBS, AIBN, MeCN or CCl_4,15–80%; (c) α-aminoglutarimide HCl, TEA, DMF, 31–47%.



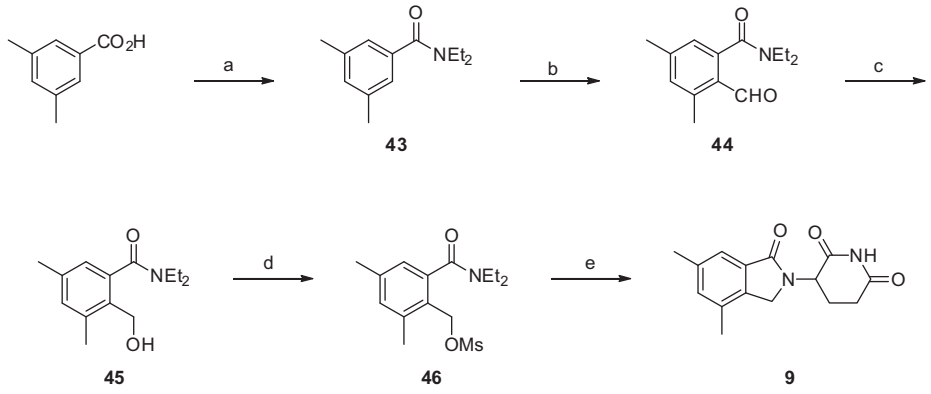
Scheme 2. Reagents and conditions: (a) $NaNO_2$, HCl, H_2O, 2.4%; (b) Ac_2O, 58–95%; (c) NBS, CCl_4, hυ, 63–69%; (d) NH_4OAc, MeOH, H_2O or Amberlyst 15, 63–73%; (e) α-aminoglutarimide HCl, TEA, DMF or MeCN, 16–47%.



Scheme 3. Reagents and conditions: (a)TMSA, $Pd(PPh_3)_4$, CuI, TEA, 85%; (b) NBS, $(PhCO_2)_2$, PhH, 89%; (c) α-aminoglutarimide HCl, TEA, DMF, 49%; (d) CsF, DMF, 85%; (e) 10% Pd-C, H_2, DMF, 97%.



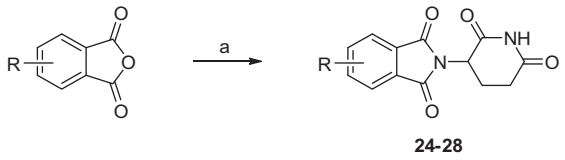
Scheme 4. (a) Cl_2CHOCH_3, $TiCl_4$, CH_2Cl_2, 90%; (b) $KMnO_4$, Me_2CO, H_2O; (c) (i) $(COCl)_2$, CH_2Cl_2, (ii) Et_2NH, 71%; (d) (i) *s*-BuLi, THF, (ii) $B(OMe)_3$, (iii) Br_2, 96%; (e) (i) α-aminoglutarimide HCl, TEA, MeCN, (ii) AcOH, 72%.



Scheme 5. (a) (i) $(COCl)_2$, CH_2Cl_2, (ii) Et_2NH, 96%; (b) (i) *s*-BuLi, TMEDA, THF, (ii) DMF, 50%; (c) $NaBH_4$, MeOH, 97%; (d) MsCl, TEA, CH_2Cl_2, 74%; (e) (i) α-aminoglutarimide HCl, TEA, MeCN, (ii) AcOH, 52%.

examination of the structure–activity relationships (SAR) of this remarkable class of compounds is herein reported. The present study reports the results of isosteric replacement of the amino moiety of **1** and **2**, and presents the synthesis and activity of these analogs of **1** and **2**.

Scheme 1 depicts the standard synthetic pathway used to access isoindoline analogs of **1**. Appropriately substituted *o*-methyl benzoic acids were converted to the corresponding methyl esters and then subjected to NBS-mediated benzylic radical bromination. The generated benzyl bromide benzoate esters are set up for S_N2 displacement/cyclization upon treatment with α-aminoglutarimide in the presence of base. The synthesis of hydroxy-substituted analogs **11**–**14** is shown in Scheme 2. The 4-hydroxy analog **11** could be obtained in low yield by diazotization/hydrolysis of **1**. During the synthesis of the 5- and 6-positional isomers, it was found that the bromination was hindered by the free phenol but after protection as the respective acetates, the bromination performed adequately and generated the requisite benzyl bromides in 63–69% yield. Deprotection of the phenol and reaction with α-aminoglutarimide delivered the targeted analogs **12**–**13**. For synthesis of the 7-hydroxy isomer it was found that when the ethyl ester was used, phenol protection was unnecessary. To



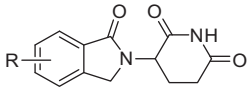
Scheme 6. (a) α-Aminoglutarimide HCl, NaOAc, HOAc, 27–82%.

prepare the 4-ethyl analog **10**, methyl 3-iodo-2-methylbenzoate was converted to the TMS-protected acetylene under Sonogashira conditions in high yield (Scheme 3). After benzylic bromination and reaction with α-aminoglutarimide, the TMS group was removed with CsF and the triple bond was hydrogenated to provided the targeted ethyl analog. For the preparation of the 4,5-dimethyl analog **8** (Scheme 4), 1,2,3-trimethylbenzene was formylated using α,α-dichloromethyl methyl ether in the presence of $TiCl_4$,[25] and the resulting benzaldehyde was oxidized to the carboxylic acid using $KMnO_4$. Following conversion to the diethyl amide, directed lateral lithiation and quench with trimethylborate followed by bromine treatment delivered the desired benzyl bromide **42** in 96% yield. After reaction with α-aminoglutarimide and ring closure, the product was recrystallized from acetic acid, providing the product in 72% yield from the benzyl bromide **42**. Scheme 5 shows the synthesis of 4,6-dimethylisoindoline **9**. 3,5-Dimethylbenzoic acid was converted to the diethyl amide **43**, and directed ortho lithiation followed by DMF quench gave the *o*-formyl amide in 50% yield. The formyl group was reduced with sodium borohydride and the resulting benzyl alcohol was mesylated under standard conditions to yield mesylate **46**. Reaction of **46** with α-aminoglutarimide and recrystallization from acetic acid gave the targeted analog in 52% combined yield. Finally, the phthalimide analogs **24**–**28** were easily accessed in a single step from commercially available appropriately substituted phthalic anhydrides by treatment with α-aminoglutarimide in a medium of sodium acetate in acetic acid (Scheme 6).

In this publication we will report on three assays used to test these novel analogs for biological activities characteristic of lenalidomide (**1**) and pomalidomide (**2**): inhibition of TNF-α levels in LPS-stimulated human PBMC, potentiation of IL-2 levels in anti-CD3 stimulated human T cells, and anti-proliferative activity against Namalwa CSN.70 cells.[26] The Namalwa cells are a chromosome 5 deleted B cell Burkitt's lymphoma cell line, selected for screening as a measure of the compounds' anti-cancer activity since it has previously been observed to be the cell line most responsive to lenalidomide from among a panel of hematopoietic tumor cell lines with and without chromosome 5 deletions.[27] As noted above, the molecular target(s) of lenalidomide was until recently unknown and there is still no standard quantitative biochemical assay for intrinsic activity. The assays used to measure bioactivity in this study are cellular assays with TNF inhibition and IL-2 stimulatory assay using primary human cells that confer direct human relevance to the assays. As a result, the ability to reach the target is a prerequisite for activity, and low activity in the assays may reflect either poor intrinsic activity or inability of the compound to reach the target.

Biological activity data for isoindolinone analogs of **1** are shown in Table 1. Compound **1** itself inhibited TNF-α with IC_{50} of 100 nM and showed stimulation of T cells with an EC_{50} of 150 nM. Compared to its activity in these assays of immunomodulation, **1** displayed similar potency in the Namalwa anti-proliferation assay, with IC_{50} of 360 nM. Isosteric replacement of the amino group in **1** with a methyl group (i.e., **4**) provided a 10-fold enhancement of TNF-α and IL-2 activity (IC_{50} and EC_{50} were both 10 nM), and a similar improvement in Namalwa anti-proliferative activity as well (IC_{50} 13 nM). Thus the heteroatom lone pair and H-bond donating ability do not appear to be a prerequisite for these biological activities. Moving the methyl group to the 5-, 6-, or 7-position resulted in a significant drop-off in activity measured by the TNF-α and IL-2 assays. This finding is in agreement for the SAR reported previously for **1**;[10] namely that substitution was required at the 4-position of the benzo ring for the most potent activity and that positional isomers showed decreased activity. Compounds **8** and **9** possess an additional methyl substitution while still having the 4-methyl substituent on the benzo ring of **4**, and these compounds also showed reduced activity with limited if any effects at 10 μM in all three

Table 1
TNF-α inhibition, IL-2 stimulation, and Namalwa anti-proliferative activity of lenalidomide (**1**) and isoindolinone analogs **4**–**23**

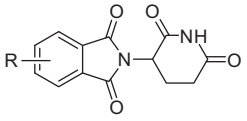


1, 4-23

Compd	Substitution	TNF-α IC_{50} (μM)[a]	IL-2 EC_{50} (μM)[a]	Namalwa IC_{50} (μM)[a]
1	4-NH_2	0.10	0.15	0.36
4	4-CH_3	0.010	0.010	0.013
5	5-CH_3	>10	>10	>10
6	6-CH_3	>10	>10	>10
7	7-CH_3	>10	>10	>10
8	4,5-$(CH_3)_2$	>10	>10	>10
9	4,6-$(CH_3)_2$	>10	>10	>10
10	4-C_2H_5	3.5	1.1	>10
11	4-OH	0.055	0.045	0.18
12	5-OH	>1	>1	>1
13	6-OH	>1	>1	>1
14	7-OH	>1	>1	>1
15	4-Cl	0.079	0.021	0.067
16	5-Cl	>1	>1	>1
17	6-Cl	>1	>1	>1
18	7-Cl	>1	>1	>1
19	4-OCH_3	1.2	0.65	>1
20	5-OCH_3	>1	>1	>1
21	7-OCH_3	>1	>1	>1
22	4-CF_3	>1	>1	>1
23	7-CF_3	>1	>1	>1

[a] IC_{50} and EC_{50}values are the means of at least three independent determinations.

Table 2
TNF-α inhibition, IL-2 stimulation, and Namalwa anti-proliferative activity of pomalidomide (**2**), thalidomide (**3**) and phthalimide analogs **24**–**28**

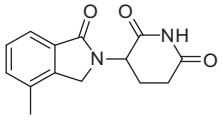
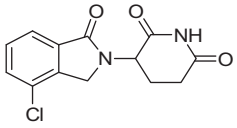
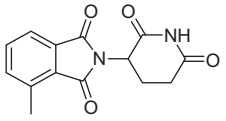


2,3, 24-28

Compd	Substitution	TNF-α IC_{50} (μM)[a]	IL-2 EC_{50} (μM)[a]	Namalwa IC_{50} (μM)[a]
2	4-NH_2	0.013	0.0080	0.030
3	—	77	>10	nd
24	4-OH	>1	>1	>1
25	4-CH_3	0.10	0.057	nd
26	4-Cl	>1	>1	>1
27	4-OCH_3	>1	>1	>1
28	4-C_2H_5	2.9	>10	>10

nd = not determined.
[a] IC_{50} and EC_{50}values are the means of at least three independent determinations.

Table 3
PK parameters in fasted male CD-IGS rats after oral administration in 0.5% CMC/0.25% Tween 80 suspension formulation in a volume of 5 mL/kg

Compd	Structure	Dose (mg/kg)	C_{max} (ng/mL)	AUC (ng-h/mL)	T_{max} (h)
4		10	4600	29,000	2.0
15		10	2800	24,000	1.7
25		10	440	2200	2.5

assays. This result demonstrates that not only is there a requirement for the 4-substitution in this series, but there is an additional requirement for no methyl group at the 5- or 6-positions for optimal activity in these analogs. In compound **10**, the methyl group of **4** was homologated to an ethyl group. Surprisingly, this relatively minor modification resulted in >100-fold decline in potency. Evidently there is a strict threshold for some property of the group at site, violation of which results in a dramatic decline in observed potency.

In addition to the methyl group, the use of other amine bioisosteres was also investigated. The 4-hydroxy analog **11** and the 4-chloro analog **15** both showed activity that was intermediate between **1** and **4**. Like **1** and **4**, these analogs also possessed improved Namalwa IC_{50}s that were similar to their TNF IC_{50} and IL-2 EC_{50}, suggesting that they are relatively non-selective among these activities or that the activities are linked. As was the case with the methyl series, positional isomers in which the hydroxy or chloro group was moved around the benzo ring displayed less activity, with IC_{50} or EC_{50} >10 μM in all three assays. Methoxy analog **19** was roughly ten-fold less active than the hydroxy compound **4**; its activity was similar to that observed for the ethyl analog **10**, which is in accord with the SAR discussed above in two ways. First, adding an additional carbon to the atom bound to the 4-position of the phenyl ring was detrimental; and second, the similar potency of the ethyl and methoxy analogs illustrates that it is not important whether the atom at the 4-position of the benzo ring is carbon or a heteroatom. The results for the trifluoromethyl analog **22** showed reduced activity compared to methyl compound **4**. Although **22** is a closely related structural analog of lenalidomide, it showed significantly less activity. The reason for its reduced potency is not currently understood.

Table 2 presents biological activity data for pomalidomide (**2**), thalidomide (**3**), and for phthalimide analogs **24**–**28**. In a previous report,[10] we reported that pomalidomide is approximately 10-fold more potent than **2** as a TNF-α inhibitor. As shown in Table 2, it also has 10-fold greater potency in the IL-2 and Namalwa assays, with IC_{50} or EC_{50} values of ~10 nM in all three assays. However, the new phthalimide analogs **24**–**26** were less active than the corresponding isoindolinone analogs **4**, **11**, and **15**. Of the three, only the methyl analog was active at sub μM concentrations. The reversal of relative activity of the isoindolinone and phthalimide analogs is puzzling. It is possible that differences in cellular permeability or another physicochemical property takes on increasing importance in the case of the phthalimides. However, whether or not the reduced activity of **24**–**26** reflects the compounds' intrinsic activity, it is clear that these compounds are much less potent. Determination of the intrinsic activity awaits the availability of a quantitative biochemical assay.

Compounds **4**, **15**, and **25** were selected for pharmacokinetic evaluation in rat (Table 3). All compounds were administered

orally at a dose of 10 mg/kg.[28] The two isoindolinones **4** and **15** displayed good oral exposure while the phthalimide **25** had only moderate exposure. The methyl isoindolinone **4** achieved a C_{max} of 4600 ng/mL at 2 h, and had an AUC of 29,000 ng-h/mL. Chloro isoindolinone **15** had a similar AUC (24,000 ng-h/mL), although C_{max} was somewhat lower (2800 ng/mL). On the other hand, the methyl phthalimide **25** showed a ~10-fold lower C_{max} and AUC.

In summary, the isosteric replacement the amino moiety of lenalidomide and pomalidomide resulted in the identification of several potent analogs with drug-like physicochemical properties. The concept of isosterism was used to guide compound design. The amino group of lenalidomide could be replaced with methyl or chloro moieties to provide new analogs with potent activity and good pharmacokinetic performance in a rodent model. Lenalidomide has demonstrated a variety of pharmacological activities and has been used successfully to treat thousands of patients; the identification and characterization of other analogs may lead to other therapeutics. The clinical activity of thalidomide in oncology and inflammatory diseases suggest that pre-clinical study of the new analogs may uncover differences in the profile of biological activities, differentiate new compounds, and point to areas of clinical activity for compounds in these series.[8] Future publications will expand on the SAR reported here.

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26. Assay procedures: TNF-α inhibition assay: PBMC (2×10^5 cells) were plated in 96-well flat-bottom Costar tissue culture plates in triplicate. Cells were stimulated with LPS at 1 ng/ml final in the absence or presence of compounds were dissolved in DMSO and further dilutions were done in culture medium immediately before use. The final DMSO concentration in all assays was 0.25%. Compounds were added to cells 1 h before LPS stimulation. Cells were incubated for 18–20 h at 37 °C in 5% CO_2 and supernatants were then collected, diluted with culture medium and assayed for TNF α levels by ELISA. IL-2 production by T cells: Tissue culture 96-well flat-bottom plates were coated with anti-CD3 antibody OKT3 at 5 μg/ml in PBS, 100 μl per well, incubated at 37 °C for 3–6 h, then washed four times with complete medium 100 μL/well just before T cells were added. Cultures were incubated at 37 °C 5% CO_2 for 2–3 days, and supernatants analyzed for IL-2 by ELISA (R&D Systems). IL-2 levels were normalized to the amount produced in the presence of 10 μM CC-4047, and EC_{50} values were calculated using non-linear regression, sigmoidal dose–response, constraining the top to 100% and bottom to 0%, allowing variable slope. Namalwa CSN.70 del 5 Burkitt's Lymphoma proliferation assay: Namalwa CSN.70 cells (DSMZ Cell Bank; Germany) were plated in 96-well plates at 6000 cells/75 μL medium (RPMI-1640 10% FCS 1% Pen/Strep) per well. All samples were done in triplicate. Cells were incubated at 37 °C in a humidified incubator at 5% CO_2 for 72 h. One microcurie of ^{3}H-thymidine was added to each well and cells were incubated again at 37 °C in a humidified incubator at 5% CO_2 for an additional 6 h. The cells were harvested onto UniFilter GF/C filter plates (Perkin Elmer) using a cell harvester (Tomtec) and the plates were allowed to dry overnight. Microscint 20 (Packard) (25 μl/well) was added and plates were analyzed in TopCount NXT (Packard). Each well was counted for one minute. The percent inhibition of cell proliferation was calculated by averaging all triplicates and normalizing to the DMSO control (0% inhibition).
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28. CD-IGS rats (male, jugular vein-cannulated (JVC), weight range: 200–225 g) supplied by Charles River Laboratories were used in this study. Test articles were administered as a single po administration at 10 mg/kg in a 0.5% CMC/0.25% Tween 80 suspension formulation, in a volume of 5 mL/kg. Serial blood samples were obtained via the cannulae at specified time points: 30 min, 1, 2, 4, 9, and 24 h post-dose, deposited into heparinized vacutainer tubes and stored on ice after collection. The harvested whole blood was then centrifuged at ~9000 rpm for ~10 min within 0.5 h to separate plasma. An aliquot of plasma was then transferred to a 1.5 mL Eppendorf vial, an equal volume of Sorensen's buffer (250 mM citrate buffer, pH 1.5) was added, samples were vortexed and then placed on dry ice. Following terminal sample collection, all samples were transferred to a −20 °C freezer until processing. The pre-diluted plasma samples (50 μL) were processed by addition of 2.5 volumes of methanol containing internal standard (proprietary compound), filtration of the mixture through a Captiva™ 96-well filtration plate (0.45 μm; Varian, Inc., Palo Alto, CA), and an aliquot of the filtrate was injected into a liquid chromatography/tandem mass spectrometer (LC/MS/MS).