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Dimeric Macrocyclic Antagonists of Inhibitor of Apoptosis Proteins for the Treatment of Cancer

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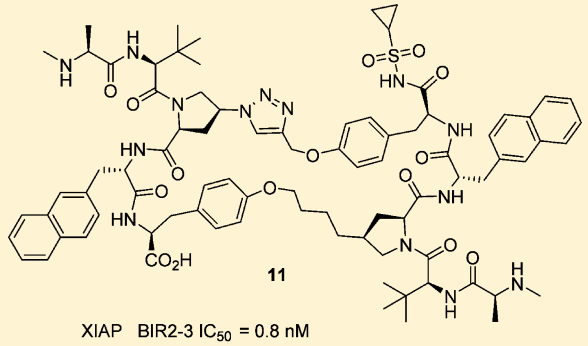
Supporting Information

ABSTRACT: A series of dimeric macrocyclic compounds were prepared and evaluated as antagonists for inhibitor of apoptosis proteins. The most potent analogue **11**, which binds to XIAP and c-IAP proteins with high affinity and induces caspase-3 activation and ultimately cell apoptosis, inhibits growth of human melanoma and colorectal cell lines at low nanomolar concentrations. Furthermore, compound **11** demonstrated significant antitumor activity in the A875 human melanoma xenograft model at doses as low as 2 mg/kg on a q3d schedule.



KEYWORDS: *Inhibitor of apoptosis proteins (IAPs), dimeric macrocyclic antagonists, cancer*

The ability of tumor cells to evade apoptosis, or programmed cell death, is a hallmark of human cancers.[1] In addition to cancer cell survival, defects in apoptotic pathways (i.e., extrinsic and intrinsic) may also contribute to tumor progression and chemo-resistance. The inhibitor of apoptosis proteins (IAPs), which includes the X-linked inhibitor of apoptosis protein (XIAP) and two cellular inhibitor of apoptosis proteins (cIAP-1 and cIAP-2), are a family of pro-oncogenic proteins that can inhibit apoptosis by regulating initiator and effector caspases.[2] XIAP directly binds to and inhibits caspase-3, -7, and -9 through its second and third baculovirus IAP repeat (BIR2 and BIR3) domains, resulting in inhibition of apoptosis.[3] While not direct inhibitors of caspases, cIAPs are involved in NF-κB signaling and play important roles in regulating caspase-8 activation.[4] Elevated expression of XIAP and cIAPs has been reported in many cancers and is associated with poor prognosis.[5]

The antiapoptotic activities of the IAP proteins can be neutralized by the mitochondrial protein Smac/Diablo (second mitochondrial activator of caspases/direct IAP binding protein with a low isoelectric point), which binds to the BIR domains of the IAP proteins through its N-terminal AVPI tetrapeptide binding motif.[6] Importantly, it has been demonstrated that the AVPI peptide itself, or small molecule AVPI mimetics, can potently antagonize the IAP proteins and elicit antitumor activity *in vivo*.[7−10] Furthermore, dimeric AVPI mimetics, which can bind to both the BIR2 and BIR3 domains and thus achieve much higher binding affinity for the IAP proteins, were shown to be significantly more potent at inducing apoptosis in multiple cancer cell lines. Several Smac mimetics that bind selectively to the BIR domains of XIAP and cIAPs have entered clinical trials and shown encouraging results.[11]

We recently reported the discovery of dimeric macrocyclic IAP antagonist **1** (Table 1) via the generation of "milla-molecular" libraries.[12,13] Macrocyclization has been exploited extensively as a means of conformationally restricting peptides and thereby improving their proteolytic stability, target affinity, and cell penetration.[14−16] We have shown that compound **1** binds to both cIAP and XIAP proteins with high affinities and is active in cellular assays despite its peptidic nature and high molecular weight. One drawback of compound **1** is that it is about 10-fold less potent in cell-based proliferation assays than other nonmacrocyclic IAP antagonists we have recently disclosed.[17,18] As a result, significantly higher doses are required to achieve similar levels of efficacy in mouse tumor xenograft models. In an effort to improve the *in vitro* and *in vivo* potencies of this millamolecular series with the ultimate goal of identifying a clinical candidate, we systematically designed and synthesized a series of analogues of **1**. Herein, we report

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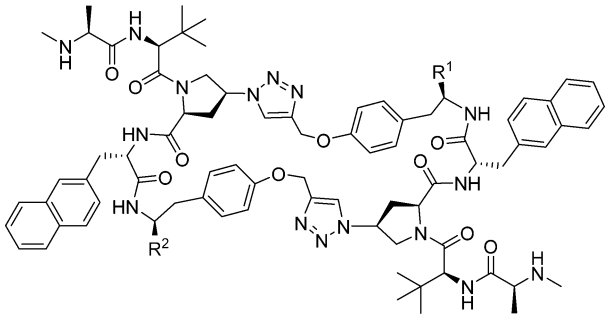
Table 1. Biological Activities of IAP Antagonists[a]



compd	R^1	R^2	XIAP BIR2-3 IC_{50} (nM)	A875 IC_{50} (nM)[c]
1	COOH	COOH	1.4	73
2	COOMe	COOH	3.7	310
3	COOMe	COOMe	32	690
4			1.8	79
5		COOH	1.8	39
6[b]		COOH	1.1	83

[a]IC_{50} values are an average of three independent experiments unless otherwise noted. [b]$N = 1$. [c]Inhibition of cell growth in A875 cancer cell line in the presence of TNF.

the synthesis, structure−activity relationship, and *in vivo* evaluation of a series of macrocyclic dimeric IAP antagonists with improved potency and pharmacokinetic properties.

Compound **1** is a potent antagonist of XIAP BIR2-3 protein (IC_{50} = 1.4 nM, Table 1) and inhibits the proliferation of A875 human melanoma cells with an IC_{50} of 73 nM. On the basis of our predicted binding model and previous SAR, we hypothesized that compound **1** occupies the same binding pocket as the AVPI peptide on the surface of the BIR2-3 protein (Figure 1). In this model, the C-terminal carboxylic acids are solvent exposed and do not contribute significantly to binding potency. In contrast to this prediction, however, the mono- and bis-methyl esters analogues **2** and **3** are significantly less potent than **1** in both the XIAP BIR2-3 FRET binding assay[19] and the A875 antiproliferation assay (IC_{50} = 310 and 690 nM, respectively, Table 1). Several additional analogues of **1**, where the carboxylic acids were replaced with nonacidic primary or secondary amide groups, also gave poor biochemical and cellular activities (data not shown). These results lead us to postulate the acid moieties may be important for conformational reasons. Compounds with the acid moieties may be able to more easily adopt the conformation required for binding simutaneously to the BIR2 and BIR3 proteins. Consistent with this hypothesis, replacing one or both of the carboxylic acid groups with similarly acidic cyclopropyl acylsulfonamide or tetrazole moieties was well tolerated. The bis-cyclopropyl acylsulfonamide **4** is equipotent to **1** in both biochemical (XIAP BIR2-3 IC_{50} = 1.8 nM) and cellular antiproliferation assay (A875 IC_{50} = 79 nM), whereas monocyclopropyl acylsulfonamide analogue **5** gave similar biochemical potency but improved cellular potency (A875 IC_{50} = 39 nM).[20]

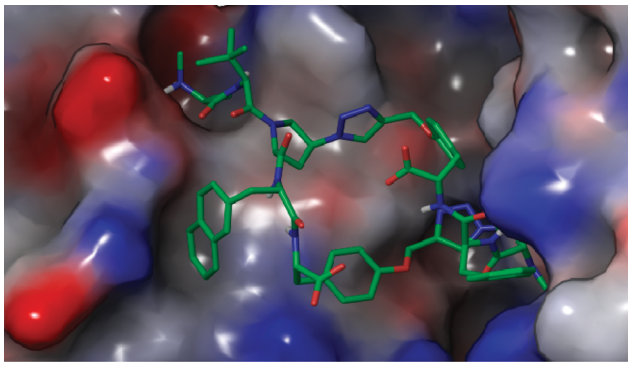


Figure 1. Binding model of compound **1** in the Bir2-3 domains of XIAP protein. Carbon atoms of **1** are in green. Oxygen and nitrogen atoms are highlighted in red and blue, respectively. The protein surface is represented by electrostatic potential.

On the basis of these promising results, we decided to determine if the acid isosteres had improved pharmacokinetic (PK) properties. As shown in Table 2, following a 1 mg/kg IV bolus injection, bis-cyclopropyl acylsulfonamide **4** demonstrated reduced clearance and enhanced exposure (AUC_{0-7} = 2350 nM h) relative to compound **1**. Monocyclopropyl acylsulfonamide **5** provided lower clearance, lower steady state volume of distribution, and higher exposure than both **1** and **4** (AUC_{0-7} = 5850 nM h) at the same dose. Thus, in addition to maintaining an optimal level of cellular potency, the acylsulfonamide acid isosteres also benefited from improved PK properties relative to the initial lead **1**.

Table 2. Pharmacokinetic Parameters of Select Compounds in Mice Following a 1 mg/kg IV Dose[a,b]

cmpd	$T_{1/2}$ (h)	CL (mL/min/kg)	Vss (L/kg)	AUC_{0-7} (nM h)
1	0.85	7.1	0.3	1550
4	0.90	3.6	0.3	2350
5	1.0	1.5	0.1	5850

[a]Vehicle: 12% hydroxypropyl-β-cyclodextrin saline solution. [b]Data reported as an average of two male mice.

We next sought to evaluate the effect of the triazole linkers on cellular potency, using monoacylsulfonamide **5** as reference. Previous investigations of dimeric IAP antagonists revealed that the length, rigidity, and polarity of the linker can have a profound effect on biochemical binding and cell permeability.[21,22] The relatively polar and rigid triazole linker was selected in the initial optimization to take advantage of the efficiency of constructing macrocycles using azide−alkyne click chemistry.[23] However, our predicted binding model indicated that both triazole groups in **1** occupy hydrophobic pockets on the surface of the XIAP BIR2-3 proteins (Figure 1), suggesting

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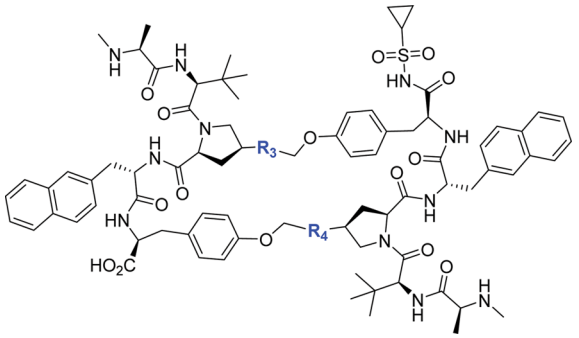
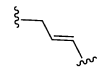
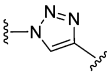
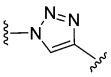
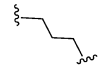
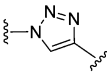
that a less polar and nonaromatic linker may be tolerated. We explored ring closing metathesis (RCM), which is compatible with acylsulfonamide-containing peptide substrates to provide access to analogues **7** and **8**, where one or both of the triazoles were replaced with less polar but similarly rigid propenyl groups. Both **7** and **8** displayed comparable biochemical and cellular activities to the bis-triazole analogue **5**, thus demonstrating the triazole linker was not required for binding or cell permeability. Macrocyclization was also shown to be essential for potent *in vitro* activity, as the macrocycle **8** was greater than 20-fold more potent than the corresponding uncyclized compound **9** in the biochemical binding and antiproliferation assays (see Table 3).

Table 3. Biological Activities of IAP Antagonists[a]



compd	R^3	R^4	XIAP BIR2-3 IC_{50} (nM)	A875 IC_{50} (nM)[c]
7[b]			1.7	23
8			1.1	51
9			24	>1000
10			1.3	15
11			0.8	19

[a]IC_{50} values are an average of three independent experiments unless otherwise noted. [b]$N = 1$. [c]Inhibition of cell growth in A875 cancer cell line in the presence of TNF.

To remove the potentially labile allyl ether functionality, reduction of the alkene groups provided bis- or monopropyl-linked analogues **10** and **11**. Binding data showed that despite increased conformational flexibility, both **10** and **11** bind to XIAP BIR2-3 proteins with IC_{50} values in the low single-digit nanomolar range. Both compounds also displayed approximately a 5-fold improvement in cellular potency relative to compound **1** (A875 IC_{50} = 15 and 19 nM, respectively).

Encouraged by the excellent cellular potency of compounds **10** and **11**, we evaluated their physiochemical properties to select a compound for full *in vitro* and *in vivo* characterization. In particular, we aimed to identify a compound with sufficient aqueous solubility compatible with intravenous administration. We found that in this series, aqueous solubility correlates well with lipophilicity and overall charge of the peptide. Compounds that are more lipophilic and net neutral are in general less soluble. Accordingly, the most lipophilic compound **10**, while among the most potent compounds tested in biochemical and cellular assays, has greatly diminished aqueous solubility (<0.01 mg/mL at pH 7.4) relative to compounds **1** and **11** (0.13 and 0.05 mg/mL, respectively, at pH 7.4). On the basis of its *in vitro* potency and aqueous solubility, compound **11** was selected for further characterization in additional biological assays.

We previously described a solid-phase synthesis of compound **1** using an on-resin cyclization promoted by a Cu-mediated azide−alkyne click reaction.[13] Synthesis of compound **11** on resin, however, was significantly more challenging, especially on the scale required to provide sufficient material for additional profiling. Since the key RCM reaction could only be performed in solution and the alkene-containing amino acid building blocks required to assemble the linear peptide precursors were difficult to access, a solution based method was developed to prepare compound **11**. Starting from commercially available 2-naphthyl alanine **12**, coupling to the known 4-substituted prolines **13a** and **13b** furnished compounds **14a** and **14b**. Successive incorporation of *tert*-leucine and *N*-methylalanine building blocks using EDC-HOAt as the coupling reagents furnished **15a** and **15b** after hydrolysis. HATU-mediated amide bond coupling of **15a** and **15b** with substituted tyrosine derivatives **16** and **18** provided pentapeptides **17** and **19**, respectively. Cycloaddition of **17** and **19** in the presence of $CuSO_4$, followed by RCM and TFA deprotection, furnished compound **8**. Compound **8** was then converted to **11** via a straightforward hydrogenation reaction. Multiple batches of compound **11** were prepared on the 400−500 mg scale using this approach (see Scheme 1).

Compound **11** was evaluated in additional FRET-based binding assays and a cell-free caspase-3 rescue assay[24] (Table 4). FRET assays confirmed binding of compound **11** to the isolated BIR3 domain of the XIAP protein as well as the BIR2-3 domain of cIAP-1 protein with IC_{50} values in the low single-digit nanomolar range. Consistent with the mechanism of action of antagonizing XIAP to relieve inhibition of effector caspases, **11** was found to increase caspase-3 activity in a dose-dependent manner, with an IC_{50} of 9.4 nM.

Compound **11** was further profiled to establish its *in vitro* safety and ADME properties (Table 4). Despite the increased lipophilicity of the acylsulfonamide substituent and alkyl linker, compound **11** possesses a favorable inhibitory profile toward human cytochrome P450 (CYP) isoforms, has very low potential for CYP induction based on the human PXR transactivation assay, and showed almost no propensity for hERG inhibition. Plasma protein-binding of **11** was measured by equilibrium dialysis. No significant difference in plasma protein binding was observed in mouse, rat, dog, or human plasma. Additionally, compound **11** demonstrated excellent metabolic stability in liver microsomes ($t_{1/2}$ = 84 min in rat and >120 min in mouse, dog, and human liver microsomes). Finally, PK properties of compound **11** were assessed in mouse, rat, and dog following a single IV dose. Consistent with the predicted hepatic clearance from liver microsome metabolic stability assays, compound **11** demonstrated short to moderate half-lives and very low clearance across species.

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Scheme 1. (a) EDCI, HOAt, NMM, DMF; (b) TFA, DCM; (c) Boc-*t*-*L*-Leucine, EDCI, HOAt, NMM, DMF; (d) TFA, DCM; (e) Boc-*N*-methyl-*L*-Alanine, EDCI, HOAt, NMM, DMF; (f) aq. LiOH, THF; (g) HATU, NMM, DMF; (h) $CuSO_4$, sodium ascorbic acid, THF/*t*BuOH/H_2O; (i) Hoveyda-Grubbs II catalyst, DCE, 70 °C; (j) TFA, DCM; (k) Pd/C, H_2, MeOH

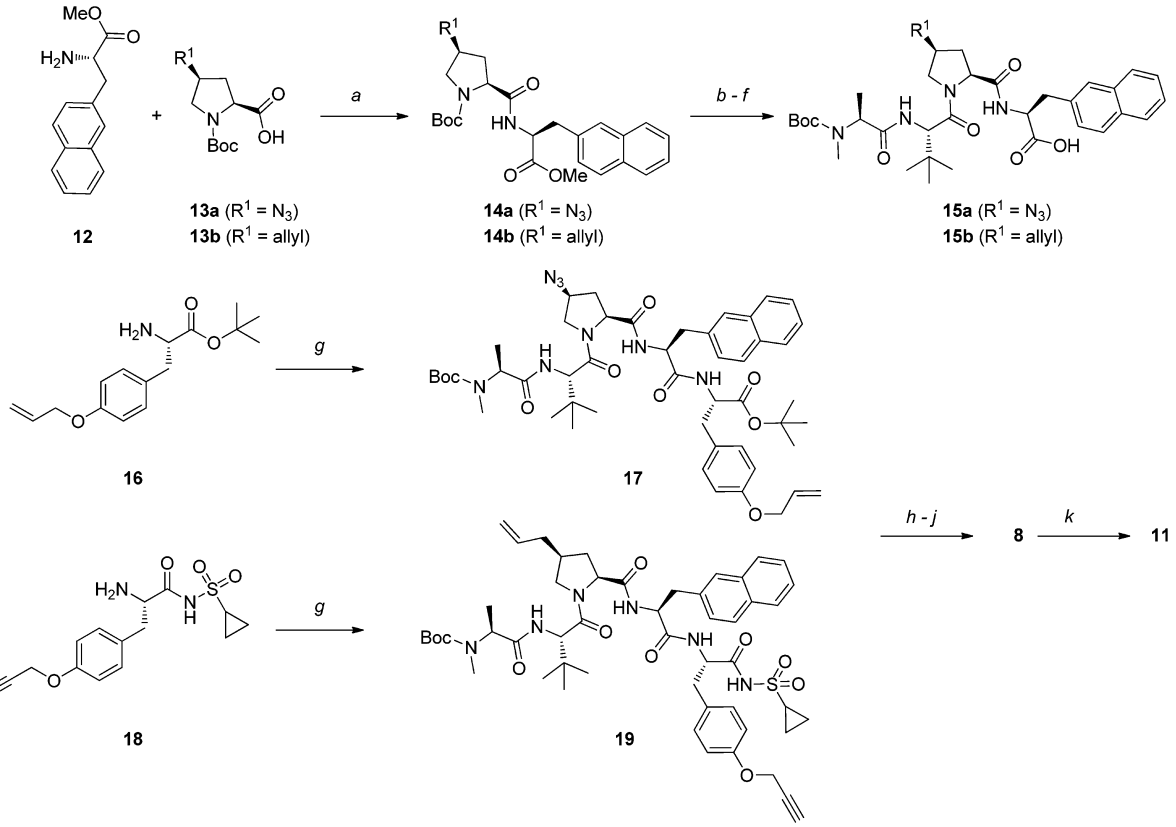

Table 4. Summary of IAP Biological Data and ADME Properties for Compound 11[a]

assay	results
XIAP BIR3 IC_{50}	3.5 nM
XIAP BIR2-3 IC_{50}	0.8 nM
cIAP-1 BIR2-3 IC_{50}	3.0 nM
Caspase-3 Rescue EC_{50}	9.4 nM
human CYP 1A2, 2B6, 2C8, 2C9, 2D6, 3A4 IC_{50}	>20 μM
PXR-TA EC_{50}	>50 μM
hERG inhibition at 30 μM	9.0%
protein binding (% free): mouse, rat, dog, human	5.8, 1.9, 2.4, 7.5
IV PK: $T_{1/2}$ (h), CL (mL/min/kg), AUC_{0-7} (nM h)	
mouse (1 mg/kg):	1.7, 2.1, 4490
rat (1 mg/kg):	1.0, 6.2, 1710
dog (0.3 mg/kg):	4.7, 0.4, 8430

[a] IC_{50} and EC_{50} values are an average of three experiments.

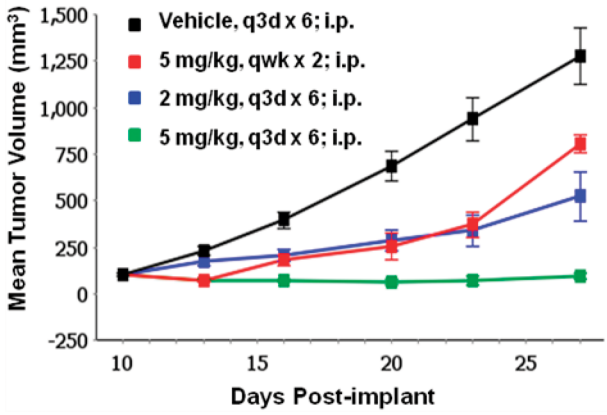

Figure 2. Antitumor activity of **11** in the A875 xenograft model in mice. Compounds were administered intraperitoneally (i.p.) every 3 days for six doses or weekly for two doses beginning on day 10.

In vivo efficacy studies were conducted with **11** in A875 human tumor xenografts implanted in athymic mice. Gratifyingly, compound **11** demonstrated robust antitumor activity (Figure 2). Intraperitoneal administration of **11** at 2 mg/kg every 3 days for six doses resulted in 67% tumor growth inhibition (TGI). Complete tumor stasis (>100% TGI) was observed throughout dosing at 5 mg/kg. Compound **11** was also efficacious when administered twice on a weekly schedule at 5 mg/kg (TGI = 80%). Importantly, compound **11** was well tolerated. No overt toxicity (weight loss or morbidity) was observed. These results compare favorably to those of **1**, where a 50 mg/kg dose on a more frequent q3d schedule was required to achieve similar efficacy.

Compound **11** was further profiled against a panel of human colorectal cancer cell lines, including those that harbor the multidrug-resistant (MDR) phenotype. Significantly, compound **11** displayed robust activities in these tumor cell lines regardless of their MDR status. For example, compound **11** was highly potent at inhibiting cell growth in both the parental HCT116 cell line and its MDR positive variant HCT116/VM46[25] (IC_{50} = 92 and 11 nM, respectively). This is in

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contrast to several previously reported nonmacrocyclic IAP antagonists reported by us and others, which were shown to be susceptible to the MDR phenotype.[17,26]

In summary, a potent dimeric macrocyclic IAP antagonist **11** with improved *in vitro* and *in vivo* activities was identified through systematic optimization of the carboxylic acid and linker regions of compound **1**. Despite its high molecular weight and large number of lipophilic aromatic rings, compound **11** demonstrated desirable *in vitro* pharmacology, safety, and PK parameters. Compound **11** was found to be efficacious at a much lower dose compared to compound **1** when evaluated in the A875 xenograft model. Furthermore, given the large number of tumors that are susceptible to the MDR phenotype, the intriguing potency of this novel series of IAP antagonists in MDR-positive cell lines may provide a distinct advantage for this target class. Further profiling of compound **11** in additional MDR-positive cell lines both *in vitro* and *in vivo* is ongoing and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the synthesis of all new analogues and procedures for IAP binding assays, A875 proliferation assay, caspase rescue assay, pharmacokinetic experiments, and *in vivo* efficacy experiments. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00091.

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

IAP, inhibitor of apoptosis protein; XIAP, X-linked inhibitor of apoptosis protein; cIAP, cellular inhibitor of apoptosis protein; BIR, baculovirus inhibitor of apoptosis repeat; Smac, second mitrochondria-derived activator of caspases; MDR, multidrug resistance; CL, clearance; Vss, steady-state volume of distribution; AUC, area under the curve; EDCI, *N*-(3-(dimethylamino)propyl)-*N*-ethylcarbodiimide hydrochloride; HOAt, 1-hydroxy-7-azabenzotriazole; NMM, *N*-methylmorpholine; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid, *N*-Boc-*L*-*tert*-leucine, (*S*)-2-((*tert*-butoxycarbonyl)amino)-3,3-dimethylbutanoic acid; rt, room temperature; Boc-*N*-methyl-*L*-alanine, (*S*)-2-((*tert*-butoxycarbonyl)(methyl)amino)-propanoic acid; DMF, dimethylformamide; DCM, dichloromethane; *t*BuOH, *tert*-butanol; THF, tetrahydrofuran; DCE, dichloroethane; hERG, human ether-à-go-go-Related Gene; PXR-TA, pregnane X receptor-transactivation; TGI, tumor growth inhibition; PK, pharmacokinetic

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(12) Bristol-Myers Squibb refers to cyclic and acyclic compounds with a molecular weight between 600 and 6000 Da as millamolecules. Millamolecules, especially macrocyclic millamolecules, are ideal for pursuing the so-called "undruggable targets" such as complex protein−protein interactions.

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