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Discovery of a novel small molecule binding site of human survivin

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Abstract—Survivin is one of the most tumor-specific genes in the human genome and is an attractive target for cancer therapy. However, small-molecule ligands for survivin have not yet been described. Thus, an interrogation of survivin which could potentially both validate a small-molecule therapy approach, and determine the biochemical nature of any of survivin's functions has not been possible. Here we describe the discovery and characterization of a small molecule binding site on the survivin surface distinct from the Smac peptide-binding site. The new site is located at the dimer interface and exhibits many of the features of highly druggable, biologically relevant protein binding sites. A variety of small hydrophobic compounds were found that bind with moderate affinity to this binding site, from which one lead was developed into a group of compounds with nanomolar affinity. Additionally, a subset of these compounds are adequately water-soluble and cell-permeable. Thus, the structural studies and small molecules described here provide tools that can be used to probe the biochemical role(s) of survivin, and may ultimately serve as a basis for the development of small molecule therapeutics acting via direct or allosteric disruption of binding events related to this poorly understood target.

Survivin, a unique member of the inhibitors of apoptosis (IAP) family of proteins,[1] is highly overexpressed in many common human cancers, but undetectable in most terminally differentiated adult tissues.[1–3] Clinically, the level of expression of survivin in tumor cells is often associated with poor prognosis and shorter patient survival rates.[4,5] As with other IAP family members, survivin has been implicated in protection from apoptosis. In cell culture systems, survivin overexpression has been strongly associated with inhibition of both the intrinsic and extrinsic cell death pathways.[3,6] Consistent with this role, a number of binding partners have been proposed for survivin to exert its anti-apoptotic activities, including caspases-3[6] and -7,[7] Smac/Diablo,[8] hepatitis B X-interacting protein (HBXIP),[9] and XIAP.[10] However, interactions with caspases[6,11,12] and Smac-derived proteins and peptides[13] either cannot be confirmed or are so weak that their relevance in a cellular context can be questioned.

Survivin also appears to play an important role in cell division.[4] Survivin displays highly cell-cycle-dependent expression, with a pronounced accumulation at mitosis,[3] and has also been shown to be a member of the group of chromosomal passenger proteins, including INCENP, Aurora B kinase, and Borealin.[14,15] The chromosomal passenger proteins associate with centromeres at metaphase, central spindle microtubules at anaphase, and remain located at cytokinesis remnants at end of telophase. In particular, survivin has been shown to be important for localization of Aurora B kinase to the mitotic machinery,[16] and this complex is important in communicating lack of tension to microtubules.[17,18] In vitro knock-down of survivin gene expression results in caspase-dependent cell death as well as defects in mitotic progression such as multinucleation and polyploidy.[19–21] In vivo, mouse survivin knock-out studies show severe defects in cytokinesis, such as disrupted microtubule formation and polyploidy, leading to embryonic death. Interestingly, the survivin knock-out phenotype is

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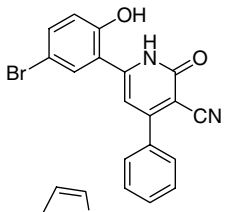
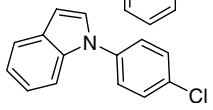
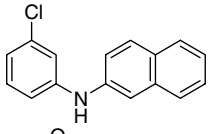
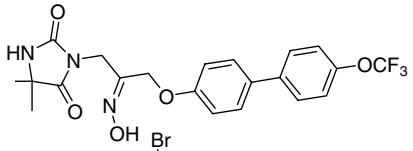
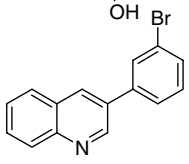
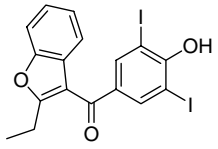
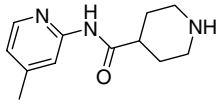
similar to that observed for INCENP, suggesting functionally related roles between these two proteins.[22] Survivin also has been shown to physically associate with the cyclin-dependent kinase cdc2 on the mitotic apparatus, and is phosphorylated at Thr34 by cdc2/cyclin B1.[23] This latter event is crucial to its activity as a cytokinesis regulator, as overexpression of non-phosphorylatable mutant survivin results in marked increases in apoptosis and polyploidy.

Survivin thus is an attractive therapeutic target in cancer due to both its expression in tumors compared to normal tissues and its ability to promote tumor growth and survival by multiple pathways involving multiple mechanisms. Various strategies have been used to target survivin, including antisense oligonucleotides and siRNA,[24] that have further validated survivin as a target, but small molecule inhibitors of survivin function have yet to be described. A difficulty attendant to this approach is the lack of understanding of the molecular mechanisms responsible for affecting apoptosis and mitosis. The nature of mitosis-related interactions of survivin with other chromosomal passenger proteins has yet to be elucidated, while several reports bearing on possible mechanisms of the apoptotic function of survivin have been put forward. The nature of additional regulatory interactions such as those with Hsp90 or nuclear exporters is only now becoming understood.[25,26]

Structurally, survivin has been thoroughly characterized by both X-ray crystallography [11,27,28] and solution NMR,[13] where it forms a bow-tie shaped symmetrical homodimer in solution. Each monomer contains a zinc-binding fold similar to that found in other members of the IAP family, but lacks the ring finger motif found in XIAP.[29,30] Instead, it contains a long amphipathic α-helix C-terminal to its sole BIR domain. The dimer interface is composed of a number of hydrophobic residues from its N-terminus as well as residues connecting its BIR domain to the C-terminal helix. The BIR domain contains a binding site analogous to those possessed by other IAP family members, which are known to bind to caspases and the Smac peptide. Detailed characteristics of this binding site and its interactions with substrates have been described elsewhere.[31,32]

Table 1. Ligands for survivin identified through NMR and affinity-based screening

Compound	Structure	Source	Binding site	NMR K_D (μM)
1		NMR/ ASMS	Dimer	5
2		NMR	Dimer	5
3		NMR	Dimer	<10
4		NMR	Dimer	<10
5		NMR	Dimer	10
6		ASMS	Dimer	8
7		NMR	Peptide	130

Here we demonstrate the existence of a newly discovered small molecule binding site on survivin distinct from the BIR site, and present details of an extension of this binding site through development of high-affinity ligands, including NMR structural studies of the ligand–protein interaction.

Since no biochemical mechanism of survivin is known, no robust in vitro functional assays have been established to enable a conventional high-throughput screen for lead identification. Thus, two types of affinity-based screening methods, HTS-NMR[33] and AS/MS,[34,35] were employed to identify leads for this unique target. A number of different classes of ligands for survivin were identified from the NMR screen (Table 1, **1**–**5**, **7**). Affinity selection/mass spectrometry (AS/MS) screening yielded 254 potential hits, from which two structurally interesting compounds (Table 1, **1**, **6**) were confirmed as ligands for human survivin by NMR. From an inspection of $^{1}H/^{13}C$-HSQC spectra, two distinct chemical shift perturbation patterns were observed for the leads (Fig. 1). Compound **7** shifted residue L64, similar to what is observed for Hid/Smac peptide binding, suggesting that this compound binds at or near the Hid/Smac binding site on the protein.[9] However, for the remaining hits, no shifts were observed for L64. Instead, for **1**–**6**, most of whom share a roughly similar L-shaped aromatic structural motif, a new set of residues including L98, L6, and L14, was shifted, suggesting a different binding site, with the affected residues being located near the interface of the two survivin monomers (Fig. 2a). Competition experiments between **7** and **1** indicated that

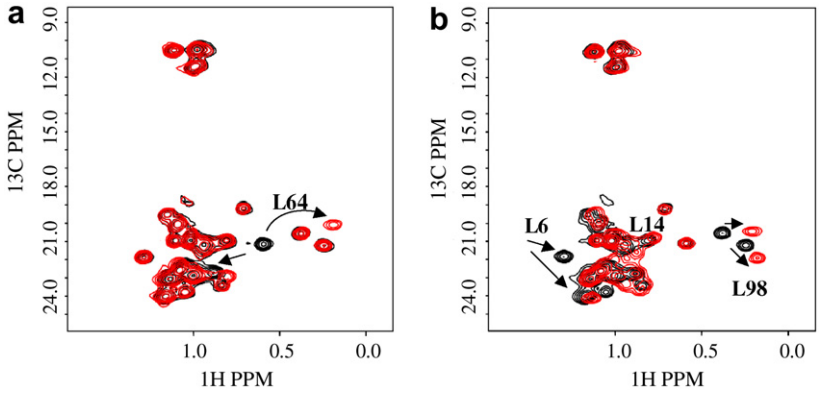


Figure 1. [^{13}C, ^{1}H]-HSQC spectra of selectively $^{13}CH_3$-labeled (Leu, Val, and Ile-δ1) survivin in the absence (black) and presence (red) of (a) acylamino pyridine hit **7** (100 mM) binding to BIR3 site, and (b) benzofuran hit **6** (100 mM) binding to dimer site. Selected chemical shift changes are indicated.

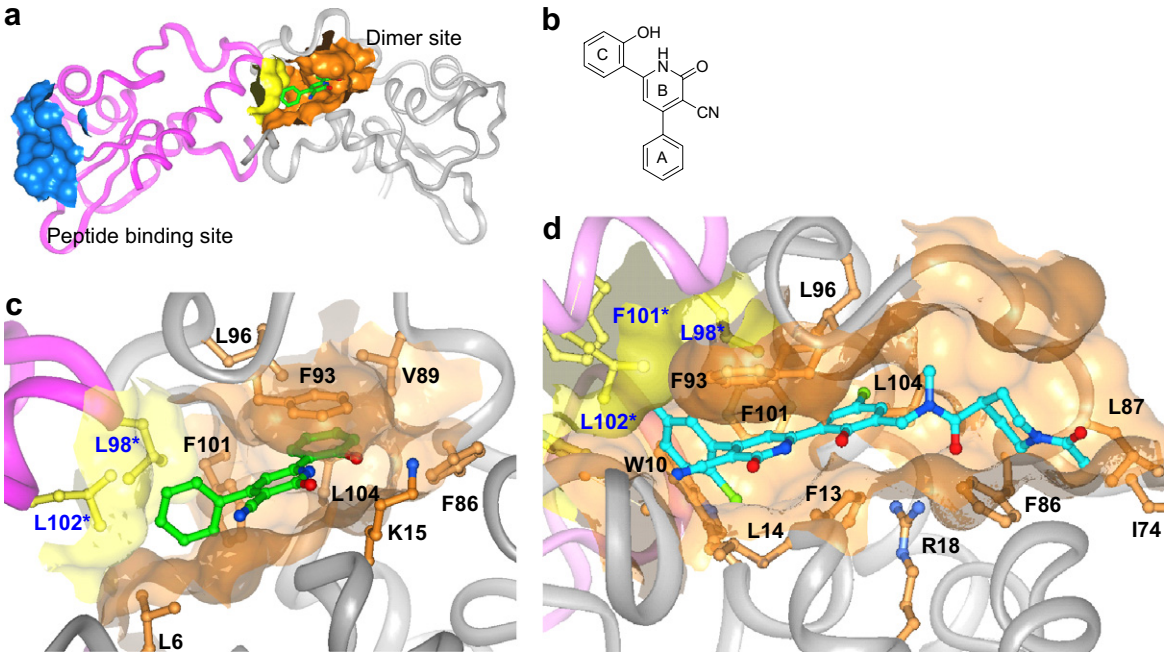


Figure 2. Details of survivin binding sites. (a) ribbon diagram of human survivin (**8** in green), with Connolly surfaces of dimer binding site (gold) and peptide binding site (blue). (b) structure of **8** with A-B-C-ring nomenclature. (c) expanded view of dimer site. Residues making important contacts with ligand are rendered. Residues belonging to adjacent survivin monomer are starred and in blue with surface in yellow. (d) NMR-derived structure of **23b** bound to survivin, with Connolly surface of binding site.

these two compounds can bind simultaneously to the protein.

Compounds **1**–**6** are relatively insoluble and were not suitable for structural studies. However, a soluble, desbromo analog of **1**, compound **8** (Fig. 2b), exhibiting a K_D of 75 μM for survivin, was amenable for NMR study. Structural study of the survivin/**8** complex revealed details of a binding site that is located at the interface of the two survivin monomers (Fig. 2a and c). A total of 24 intermolecular NOEs were observed between **8** and survivin, which defined extensive hydrophobic contacts. In particular, the bottom or A-ring of **8** packs deeply into a pocket formed by the side chain of residues F101 and L6 from one monomer and residue L102* and L98* from the adjacent monomer. The top phenol or C-ring packs against the side chains of residues L96, F93, V89, L104, and F86. Compared to the apo-protein, a large side-chain rearrangement of F93 occurs, and results in exposure of a hydrophobic pocket on the protein which is blocked by F93 in the apo-protein. There is no rearrangement of the protein backbone in this region as a result of small-molecule binding. In addition, the N-terminus of the protein, which is disordered in the apo form, becomes more ordered in the complex and exhibits NOEs to ring A of the ligand. A hydrogen bonding network of the B- and C-ring functional groups probably serves to lock the two rings into a coplanar arrangement. Also of note is that the stoichiometry of binding is one ligand per survivin monomer, thus the complex exists as a doubly occupied dimer. Subsequent compound complexes showed similar features.

The functional role of this dimer interface site is currently unknown. However, several pieces of evidence suggest that the dimer interface site is probably involved in binding to other proteins that regulate survivin function in vivo. First, the number and variety of chemical structures that bind to this pocket are indicative of a biologically relevant protein binding site that can be targeted with small molecule drugs. The NMR hit rate for this binding site was 0.35%, which is comparable to other druggable protein targets, and much higher than the rate of 0.01% observed for the BIR site.[36] Also, the hit rate for the XIAP BIR3 site was 0.09%, again much higher than for the corresponding survivin domain. Finally, the large conformational rearrangements that are observed upon ligand binding are similar to those observed for other protein–protein interaction sites[37] including Bcl-xL,[38] IL-2,[39] MDM-2,[40] and IgG domains.[41]

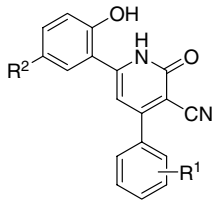
As there is, again, no clearly defined biochemical function, for survivin, and no known endogenous ligand for the dimer site, a variety of biophysical techniques were investigated in order to quantitatively measure ligand binding. NMR can monitor ligand binding over a wide affinity range, but cannot quantitate K_D values of less than ~10 μM. Initially a variety of fluorophores were attached to analogs of **8** to serve as probes in fluorescence polarization competition assays, but high non-specific binding precluded their routine use. Alternatively, the intrinsic tryptophan fluorescence of survivin was monitored, but compound interference was a significant problem. Fortunately, most of the pyridinone analogs exhibit relatively high fluorescence (with excitation and emission wavelengths of 450 and 530 nm, respectively) increasing in intensity upon complex formation. Compound concentrations as low as 100 nM could be utilized on standard instrumentation, allowing for the quantitation of binding affinities down to the 20 nM range (see Supplementary Material). This assay was utilized for the lead optimization described below.[42] As this assay is non-competitive, we also verified the site of binding by NMR for all compounds.

Initial work focused on separate studies of modifications to each of the three rings of **1**. Synthesis was normally carried out by a one-pot procedure.[43] We found that the central pyridone ring did not tolerate modification of any kind, and the top, C-ring phenol likewise was required for good binding. Our hypothesis, later verified by NMR structure, was that the two groups form a hydrogen bonding network, via the 2-hydroxypyridine central ring tautomer, which serves to lock the two rings into a near-coplanar arrangement. Surprisingly, we also found the cyano group to be necessary for binding, in spite of NMR structures providing no indication of a specific interaction with the protein. Thus, replacement with carboxamide, methyl, halogen, or hydrogen all greatly diminished binding (data not shown). Substitution elsewhere on the C-ring tolerated substitution at positions *ortho* and *para* to the hydroxy group, in keeping with structure-based prediction. *Para* substituents are completely buried by protein; we found various alkyl groups and halogens to be roughly equal in affinity to that of the bromo group of **1**. NMR structures suggested that larger substituents such as phenyl resulted in compounds being pushed out toward solvent to some degree, with still larger groups resulting in a loss of affinity (**9**–**12**).

Likewise, A-ring substitution found only hydrophobic groups to be preferred. Both 2,4- and particularly 2,5-disubstitution were favored, with Cl and Me preferred at the 2 position, and Cl, Me, and CF_3 at the 4 and 5 positions, resulting in a roughly tenfold improvement in affinity. Larger groups at the bottom of the pocket were not tolerated, in keeping with the complexed NMR structure with **8**. The cumulative result of these investigations resulted in a number of compounds in the 300–550 nM binding range (Table 2).

We next sought to access the well-defined, though relatively shallow cleft adjacent to the C-ring, formed largely by residues E40, I74, F86, L87, and K91. The substituents of **17** were chosen for their unreactivity toward the reaction conditions necessary for further elaboration. Experimentation with various substituents *ortho* to the C-ring phenol led to use of an aminomethyl group as a substrate for high-throughput organic synthesis (HTOS). Fully elaborated compounds **22**–**26** were synthesized from **17** as in Scheme 1. Iodination and carbonylation gave **20**,[44] which could easily be condensed with a variety of amines or primary amides.[45] Subse-

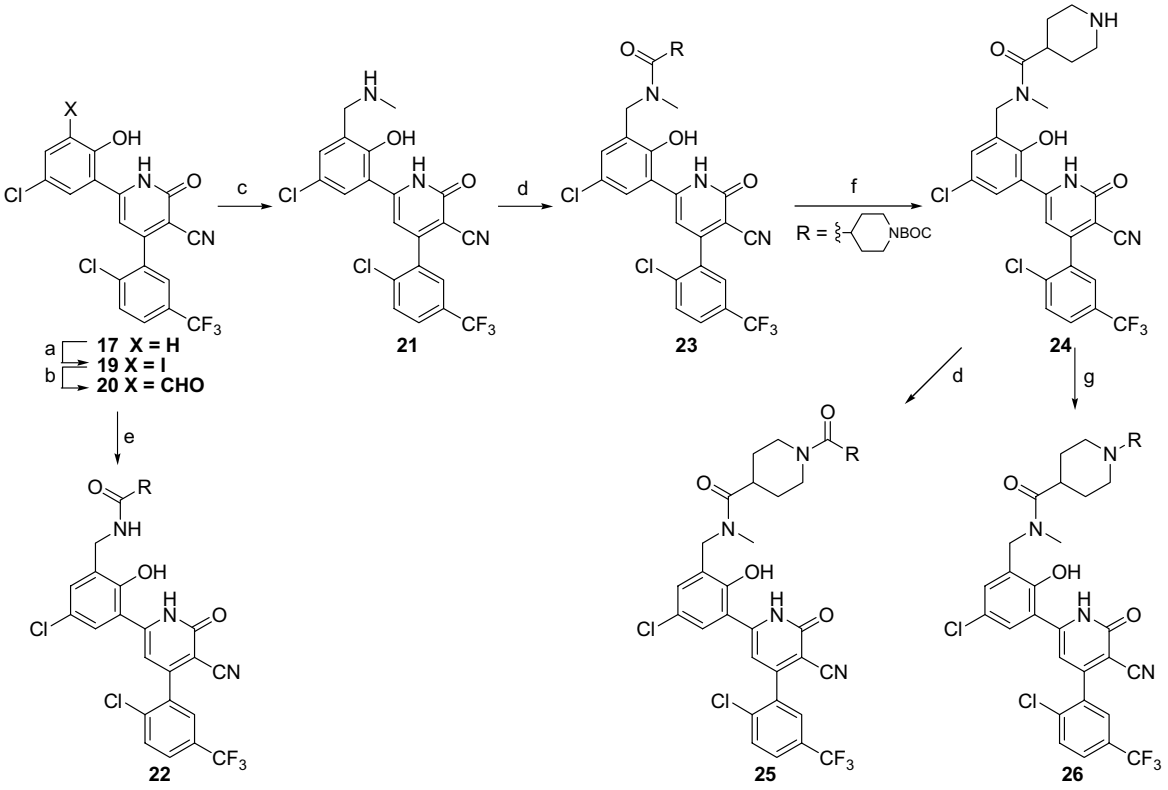
Table 2. SAR of survivin compound A- and C-rings



Compound	R^1	R^2	K_D (μM)
1	—	Br	5.7
8	—	—	75
9	2,5-Cl	Ph	0.70
10	2,5-Cl	*c*-C_5H_9	0.51
11	2,5-Cl	*i*-Bu	7.56
12	2,5-Cl	*c*-C_6H_{11}	2.98
13	2,4-Me	Br	0.70
14	2,5-Me	Br	1.17
15	2,5-Cl	Br	0.81
16	2-Cl, 5-CF_3	Br	0.49
17	2-Cl, 5-CF_3	Cl	0.52
18	2-Cl, 5-Ph	Br	>4.3

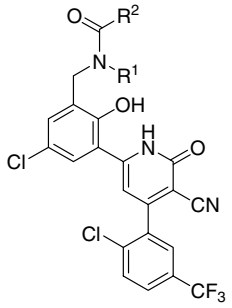
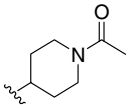
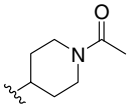
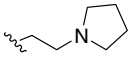
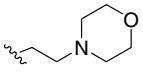
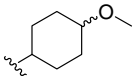
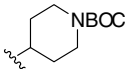
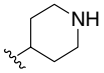
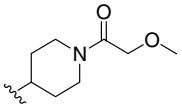
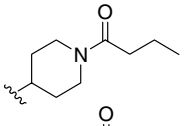
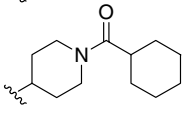
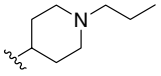
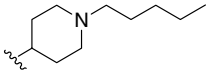
quent coupling of **21** gave a series of amides **23**; the Boc-protected amide from this group was deprotected and amides **25** and amines **26** were again made largely via parallel synthesis. All compounds **22**–**26** were characterized by ^{1}H NMR and MS. Both **21** itself and tertiary amines derived from it exhibited reduced affinity, but many *N*-methyl amides bound at least as well as the core structure **9** (Table 3). In addition, some analogous secondary amides (**22a**,**b**) were also synthesized; these were roughly equal in affinity and more water-soluble than their *N*-methyl counterparts, but were highly crystalline, and insoluble in organic solvents. Placement of an amine (**23c**,**d**) two or three carbon atoms away from the amide carbonyl improved affinity by another order of magnitude. Other compounds such as **23e**, which situate heteroatoms in a similar location, were somewhat less potent. The secondary amine **24** bound no better than **9**, or the cyclohexyl amide **23a**. However, we employed **24** in a second round of HTOS, and many resulting tertiary amines such as **26a** were an order of magnitude more potent. Also, a number of amides, such as **25a**,**b**, exhibited similar affinity gains. Small alkyl or heteroalkyl groups were tolerated in both of these series, and small alkyl branching or cycloalkyl groups, such as in **25c**, were only slightly less potent, but much larger, or longer alkyl groups such as in **26b** had a deleterious effect on binding.

We obtained an NMR-derived structure for **23b** bound to the extended cleft (Fig. 2d). NOE data indicate that the pyridinone core of **23b** binds in a similar location to compound **8** with minor changes to accommodate the amide linker. The hydrogen bonding network of the B- and C-rings is again



Scheme 1. Reagents and conditions: (a) NIS, CH_3CN, Δ; (b) $Pd(OAc)_2$, dppf, Et_3N, Et_3SiH, DMF, CO, 80 °C; (c) $MeNH_2$·HCl, $NaBH(OAc)_3$, $(CH_2Cl)_2$; (d) RCO_2H, polymer-supported-DCC, HOBt·H_2O, DIPEA, DMA; (e) $RCONH_2$, TFA, Et_3SiH, toluene, 100 °C; (f) aq HCl, dioxane; (g) RCHO, $NaBH(OAc)_3$ $(CH_2Cl)_2$.

Table 3. Survivin affinities for analogs of **22–26**

Compound			K_D (μM)
	R^1	R^2	
23a	Me		0.62
22a	H		0.88
23b	Me		0.086
22b	H		0.048
23c	Me		0.044
23d	Me		0.076
23e	Me		0.20
23f	Me		0.26
24	Me		0.53
25a	Me		0.064
25b	Me		0.060
25c	Me		0.10
26a	Me		0.037
26b	Me		0.22

apparent. NOEs were observed between the amide *N*-methyl and residues Q92, V89, K90, F93, and L96, and between the piperidine acetyl methyl and residues A41, I74, and L87, positioning this group in the extended cleft adjacent to the C-ring. Also of note is that the HSQC spectrum for **23b** shows perturbation of L64, which is part of the BIR binding site. This result was reproduced by other compounds in Table 3. It is possible that compounds with sufficient bulk may be able to allosterically disrupt the BIR site, most likely through an effect on the intervening zinc finger motif.

A few other structural features are of note. The exocyclic amide bond of **23b** potentially forms a hydrogen bond to the terminal guanidine of R18, with an approximate distance of 3.0 Å. The amide *N*-methyl group fits snugly into a small pocket formed by the residues linking the BIR-domain C-terminus to the long terminal α-helix. We did find that larger amide *N*-alkyl groups depressed binding (data not shown). It is also apparent that larger terminal *N*-alkyl groups such as those of **26b** will extend beyond the end of the cleft and into solvent, accounting for the reduced affinity of long-chain compounds in the binding assay.

In order to evaluate the potential of these compounds as probes of intracellular processes, several of their physical properties were evaluated. Amines were determined to have very low aqueous solubilities. However, both **23b** and other amides among the group containing **25** possessed solubilities of >32 μM by nephelometry, and **23b** and **25a** were soluble to 300 and 138 μM, respectively, at pH 7.4 by the shake-flask method. In addition, several representative compounds in Tables 2 and 3 were shown to readily pass through cell membranes, and were highly associated with the cytosol in a red blood cell uptake assay. Compound **23b** had a $[Cell]_{free}$/[Extracellular] ratio of 1.80, while the ratio for **23c** was 2.61. Thus, compounds such as **23b**, **25a**, and **25b** should be amenable to use as probes in experiments designed to uncover the various mechanisms whereby survivin exerts its biological effects.

Though survivin has been implicated in both apoptosis and cell cycle regulation, neither function has been associated with a specific binding event; while the BIR binding site was initially assumed to play some role, that remains to be confirmed. A related but separate question concerns whether small molecules are likely to be found that can bind with biologically meaningful affinity. We have identified a second potential binding site on the survivin surface that appears to possess a far greater propensity for small-molecule binding than the BIR site. In preliminary experiments, these ligands do not exert the broad phenotypic consequences associated with survivin knock-down through antisense or siRNA approaches (e.g., apoptosis, polyploidy), though global ablation of survivin should be expected to exert a greater effect than inhibition of selected pathways. Nonetheless, further study of this binding site using these or other ligands should lead to better understanding of the biochemical roles of survivin.

In addition, the structural studies of these protein–ligand complexes provide a framework for interpreting biochemical studies with putative binding partners. We hope that the work presented here stimulates renewed interest in determining the biochemical behavior of this difficult but important target.

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Supplementary data

Experimental details of protein preparation, NMR and ASMS screening, NMR structure determination, and structure calculations; and representative curves for the fluorescence assay. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.03.042.

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gamma globulin (BGG), 3% DMSO, 0.5% Pluronic-F68, and 1 mM dithiothreitol (DTT). Fluorescence intensity was measured by LJL Biosystems with excitation wavelength at 450/55 nm and emission wavelength at 530/25 nm. K_D values were derived assuming a two-component binding equilibrium.

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