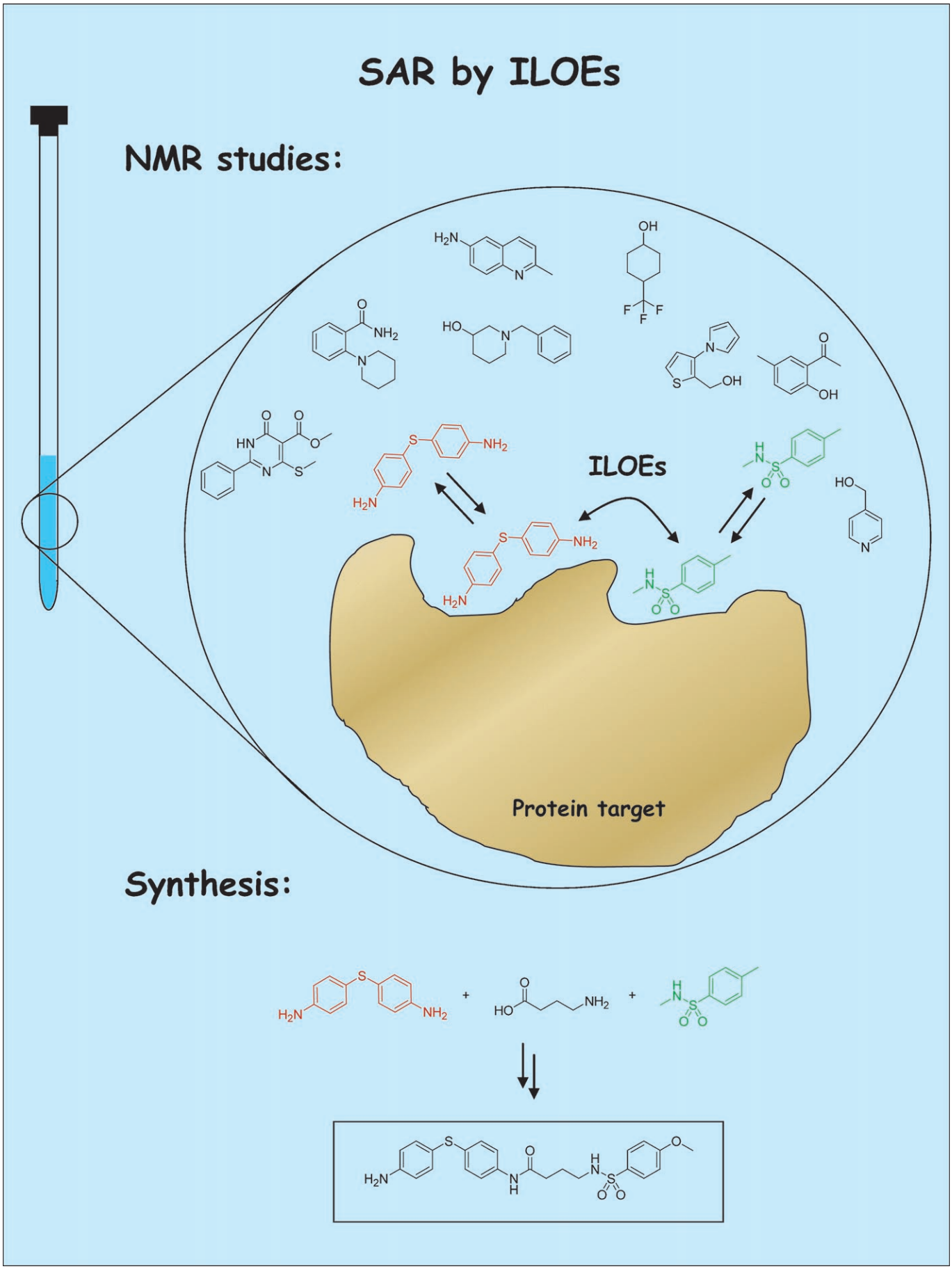

DOI: 10.1002/chem.200500636

SAR by ILOEs: An NMR-Based Approach to Reverse Chemical Genetics

Barbara Becattini and Maurizio Pellecchia*[a]

Abstract: Reverse chemical genetics is an emerging technique that makes use of small molecule inhibitors to characterize how a protein functions. In this regard, we have developed an NMR-based approach (SAR by ILOEs) that enables the identification of high affinity ligands for a given protein target without the need of a specific assay. Our approach is of general applicability and could result very powerful in reverse chemical-genetics studies, target validation, and lead discovery. We report a recent application on the design and synthesis of compounds that inhibit protein-membrane interactions.

Keywords: chemical genetics · drug discovery · NMR spectroscopy · structure–activity relationships

Introduction

Many recent applications show the importance of NMR spectroscopy as a method to characterize protein–protein and protein–ligand interactions.[1–3] In ligand design, NMR has the enormous advantages of a very high sensitivity and a low incidence of false positives. Another important aspect of the use of NMR in drug discovery is its ability to identify fragments that bind in spatial proximity on the surface of a given protein, therefore providing the opportunity of linking them covalently to afford higher affinity bidentate compounds.[1] Since the dissociation constant of the bidentate can be approximated as the product of the dissociation constants of the individual scaffolds, it is in theory sufficient to link together two fragments that bind in the millimolar range to obtain a bidentate compound with low or sub-micromolar affinity.

We have recently reported on an NMR-based approach, named SAR by ILOEs (structure–activity relationships by *i*nter*l*igand nuclear *O*verhauser *e*ffect), which makes use of protein mediated ligand–ligand NOE interactions (ILOEs), molecular modeling, and synthetic chemistry to identify initial weak hits and convert them into bidentate compounds with higher affinity.[1,4] Combined with functional studies using the resulting ligands, the SAR by ILOEs method represents an ideal approach to reverse chemical genetics (Figure 1). Reverse chemical genetics entails selecting a protein of interest, screening for a ligand for the protein, and finally determine the eventual phenotypic alterations that the ligand induces in a cellular context.[5] Likewise, our method enables the identification of protein's hot spots by using small molecules, regardless of the knowledge of the function of the protein, and the development of a specific assay. Subsequently, such small organic molecules can be used in cellular assays to investigate the possible role of the target (Figure 1).

In particular, the approach was applied to the identification of the first inhibitor of the pro-apoptotic protein Bid and then used to characterize its function in cell.

Identification of high affinity protein binders by means of the SAR by ILOEs approach: We have recently reported on the importance of transferred ILOEs measurements to screen a library of compounds in order to identify pairs of weak binders for a target protein.[1,4] These binders represent the building blocks for higher affinity bidentate compounds (Figure 1). This NMR technique takes advantage of the difference in nuclear spin relaxation of a small molecule when bound to a large protein. In particular, since small molecules tumble rapidly in solution, the dominant ^{1}H relaxation mechanisms during a NOESY-type experiment lead to weak, positive NOEs (cross-peaks have opposite sign to that of diagonal peaks). On the contrary, when the ligand is bound to a target protein it assumes its long correlation time that translates into very strong negative NOEs (i.e., very strong positive cross-peaks in a NOESY-type experi-

[a] Dr. B. Becattini, Prof. M. Pellecchia
The Burnham Institute
10901 North Torrey Pines Rd.
La Jolla, CA 92037 (USA)
Fax: (+1) 858-713-9925
E-mail: mpellecchia@burnham.org

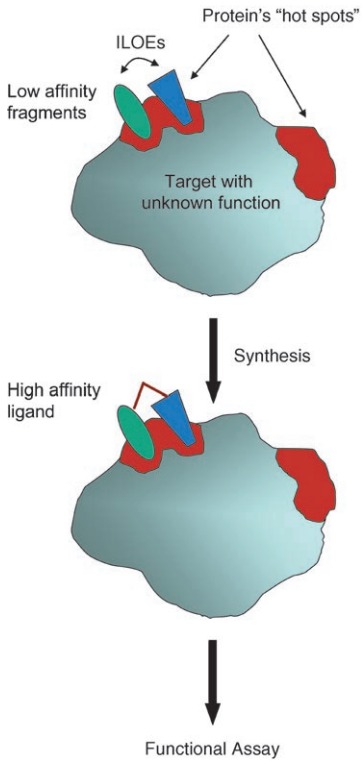

Figure 1. Reverse chemical genetics by using the SAR by ILOEs approach.

ment). In the case of a rapid exchange between free and bound state (namely when $k_{off} \gg 1/T_1$, the longitudinal relaxation rate), this effect can be observed also when the target is present in a sub-stoichiometric amount (~10:1 to 100:1 ligand/protein ratio). Therefore, in a mixture of compounds in presence of a sub-stoichiometric amount of target, only those compounds that bind appreciably to the protein will exhibit strong negative NOEs, whereas non-binders will show no NOEs or at most very weak positive ones. Moreover, if two or more ligands bind simultaneously in adjacent sites on the protein surface, strong negative ligand–ligand NOEs (ILOEs) can also be observed.[1,4,6–9] While the observation of ligand–ligand NOEs has been reported already in the past,[6–9] its application in the design and synthesis of novel ligands was only very recently reported by us.[1,4] In fact, we have realized that the detection of this kind of interactions can also be realized in complex mixtures, since NMR spectra of individual compounds are readily available.[1,4] However, since ligand–ligand magnetization transfers are more efficient for larger proteins, because of smaller rotational correlation times, we can ease the detection of ILOEs by "increasing" the molecular weight (M_W) of a given protein. In practice, ILOE effects could be observed with proteins of $M_W \gg$ 20–30 kDa. A common way to artificially increase the M_W of a given protein target is to express it as a GST fusion.[1] To exclude the possibility of unspecific binding to GST, the experiment should be repeated in presence of GST alone. The enhancement of the ILOE effect in GST fusion proteins was recently illustrated for the Bir2 domain of XIAP (~10 kDa), in which pairs of compounds binding on the surface of the protein could be readily identified using GST-Bir2 (M_W ~72 kDa).

Compounds that display ligand–ligand interactions can then be covalently linked to obtain bidentate derivatives with increased affinity for the target. The library is usually tested in mixtures of compounds (100 μM to 1 mM each) and in presence of smaller amounts of target (1–10 μM). Typical NOESY spectra are measured with mixing times of 300 to 600 milliseconds to maximize the detection of trNOEs and ILOEs. Analysis of the data and subsequent deconvolution of the spectra allow the identification of weak ligands by means of positive trNOEs cross-peaks. Similarly, pairs of compounds that bind to the protein in close proximity (less than 5 Å) are identified by detecting intermolecular NOEs (ILOEs) serving as building blocks for producing linked compounds. Eventual protein-mediated spin-diffusion effects can also be ruled out by using QUIET-NOESY[9,10] techniques or per-deuterated protein. ILOE build-up measurements also provide a rough estimation of inter-nuclear distances that are used to determine the relative orientation of the two ligands for linker design.

Library and linker design: To minimize the number of compounds to be tested, a small but diverse library of compounds that represent the most common scaffolds found in drugs can be used. Since the compounds have very simple structures, a few hundred derivatives are sufficient to represent the diverse frameworks. In our first application we mainly selected molecules that presented esters, carboxylic acids, amines or alcohol functionalities as these could serve as starting point for fragment linking. This principle is schematically illustrated in Figure 2 in which we report scaffolds of general structures for each functional group (representing both aromatic and aliphatic derivatives), a selection of possible linkers, and potential bidentate derivatives corresponding to the two fragments. While the systematic synthesis of several possible bidentate compounds is the most sensitive route to achieve an optimally linked compound, the number of possible candidates could be narrowed by virtual docking analysis if the three-dimensional structure of the protein target is known.[1,4]

In our implementations, the most recurring bond used to link the fragments turned out to be the peptidic one. Several methods can be employed, involving traditional procedures (EDC and DMAP in DMF),[11] resin-bound reagents (such as *N*-cyclohexylcarbodiimide-*N*′-propylmethyl polystyrene PS-CDI from Argonaut and SiliaBond Carbodiimide Si-DCT from Silicycle), microwave irradiation and/or a combination of the last two.[12] Resin-bound reagents have the great advantage of providing the final compound in pure form by using a simple filtration as purification protocol. A possible limit of such protocol may be the low solubility of the starting materials in CH_2Cl_2, which represents the solvent of choice for these procedures, and the relatively high cost of the resins. An alternative interesting method involves the use of microwave irradiation to shorten reaction times

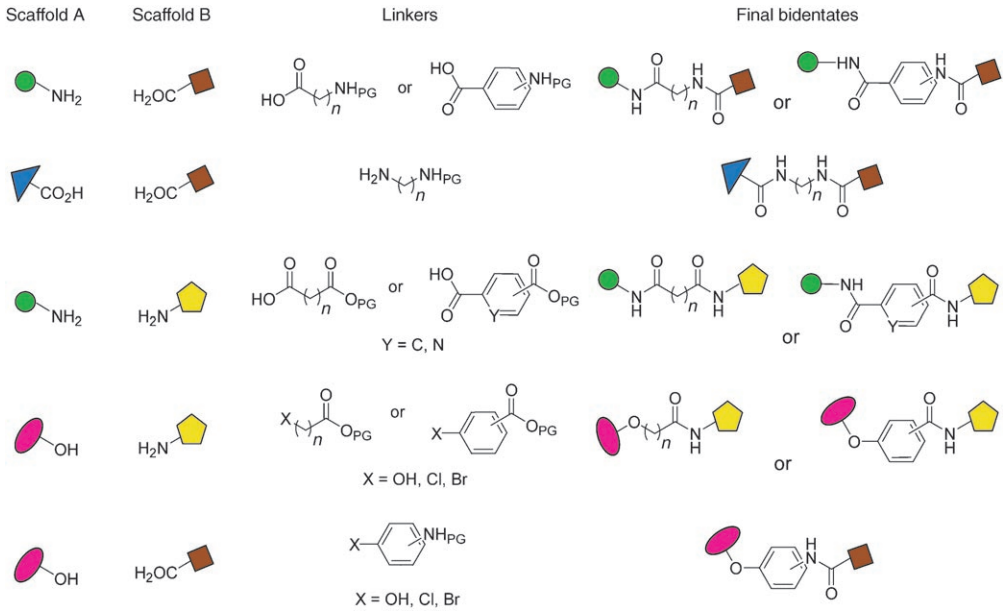

Figure 2. Linker chemistry and design.

for amide coupling when resin-bound reagents are used, and for the formation of amide bonds under neat conditions.[13] Chemical linkage to other functional groups can also be used, such as formation of ethers from phenols and alcohols, thio ethers, sulfonamides.

SAR by ILOEs-derived inhibitors of Bid: We have recently applied our approach to design compounds that are capable of binding on the surface of the protein Bid.[4] Bid is a key member of the Bcl-2 family protein involved in the regulation of tissue homeostasis and it represents a very attractive target for its crucial role in controlling the apoptotic process in cells.[14–16] Once activated by caspase-8, cleaved Bid translocates to the mitochondrial membrane where it triggers the apoptotic cascade.[17,18] However, the exact mechanism of this process is still unknown. Consequently, to date no inhibitors have been discovered for this protein.

Following the SAR by ILOEs approach, we were able to identify two fragments that bind on a deep hydrophobic crevice on the surface of the protein (BI-2A1 and BI-2A7, Figure 3a). Molecular docking analysis performed with FlexX[19] as implemented in Sybyl (TRIPOS, Inc.) was used to direct our synthetic efforts and covalently link the two fragments to obtain a tighter bidentate compound, namely BI-6C9. The synthesis of this 4-phenylsulfanylphenylamine derivative is shown in Figure 3b and it involves peptide bond formation using PS-CDI (Argonaut Technologies) in presence of the commercially available 4-amino-4′-nitrodiphenyl sulfide and *tert*-Boc-4-aminobutanoic acid. In vitro and in cell assays proved that the compound inhibits Bid activity in both isolated mitochondria and human cell lines.[4] When tested with isolated mitochondria our compound prevented Bid-induced release of the protein SMAC, a key promoter of cell-death. Moreover, an ultracentrifugation assay using isolated mitochondria and activated Bid shows that BI-6C9 prevents the association of Bid with the mitochondrial membrane (Figure 3c).

Therefore, by using our approach we could identify a functional region on the surface of Bid that seems to be important for its association with the mitochondrial membrane. Further cell-based and in vivo studies with BI-6C9 and its improved analogues are providing tremendous insight on the possible role of Bid in several human pathologies such as neurodegenerative diseases, liver and ischemic injuries.

Conclusion

In the crowded milieu of the cell, protein's function and activity are highly regulated by a complex network of specific interactions. Therefore, every protein is expected to be engaged in different interactions with other proteins, membranes, nucleic acids or cofactors during the lifespan of a cell, resulting in the activation or attenuation of different signaling pathways. In this context, the application of the SAR by ILOEs approach to functional studies represents a valid complement to traditional molecular and cell biology to unravel the intricate series of events at the basis of cellular functions. Moreover, defects or alterations of the regulation of molecular pathways are often at the basis of the onset and progression of most human diseases. Therefore, the ligands that can be obtained by means of the SAR by ILOEs approach could also serve as pharmacological tools for target validation in disease models and may even translate in potential lead compounds for the development of novel therapies.

We anticipate that the method will find wide application in tackling protein targets with unknown or less characterize function and/or to target macromolecules involved in complex intermolecular interactions.

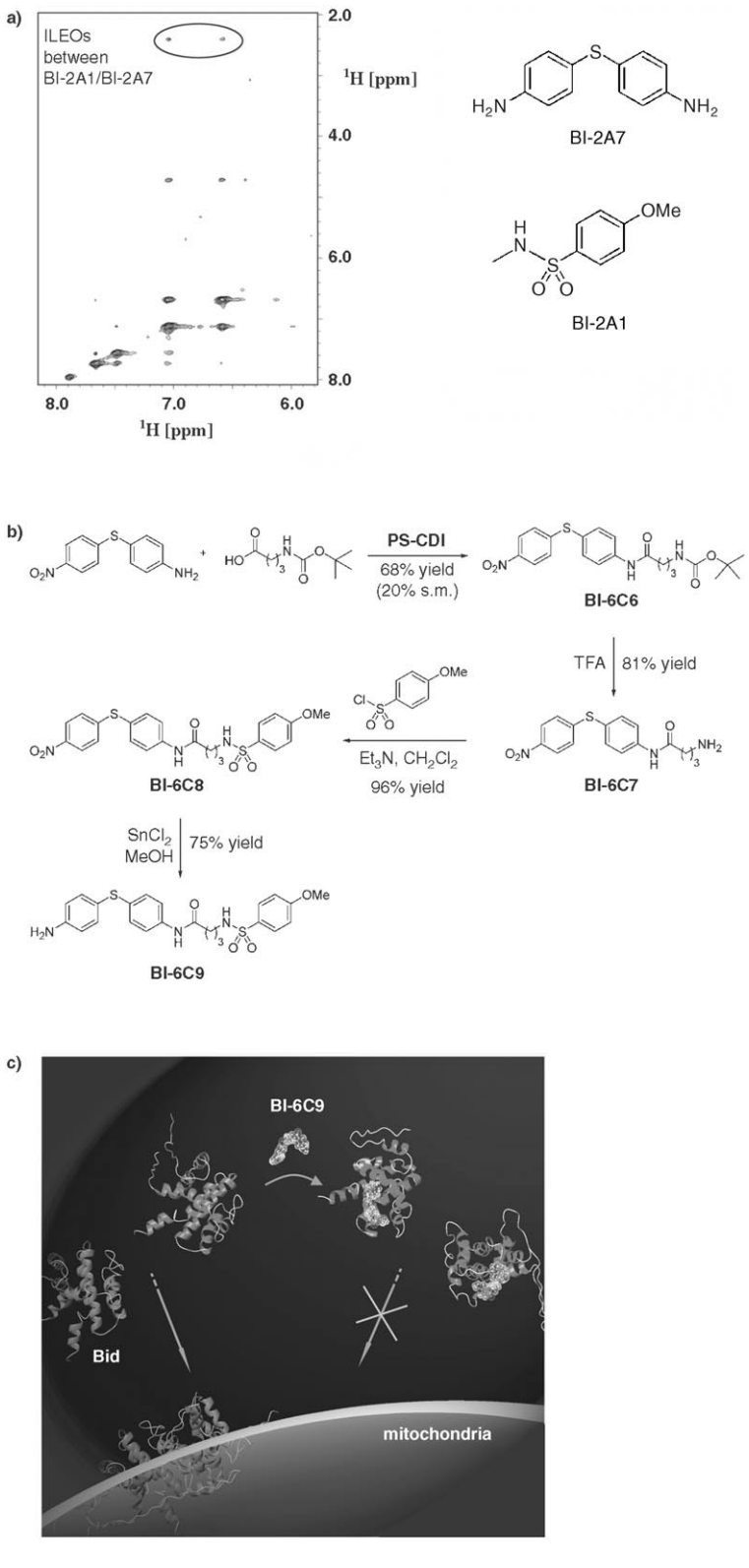

Figure 3. Example of the SAR by ILOEs approach: NMR, Chemical synthesis, and proposed mechanism of BI-6C9. a) NOESY spectrum measured in presence of a sub-stoichiometric amount of Bid (10 μM) for the identified pair of binders BI-2A1 and BI-2A7. b) Synthesis of BI-6C9: the peptidic bond was formed by using *N*-cyclohexylcarbodiimide-*N*′-propylmethyl polystyrene (PS-CDI) (Argonaut Technologies) in presence of the commercially available 4-amino-4′-nitrodiphenyl sulfide and *tert*-Boc-4-aminobutanoic acid. Stirring the reaction mixture at room temperature resulted in the corresponding Boc-protected amine BI-6C6. Deprotection with trifluoroacetic acid (TFA) gave the free amine BI-6C7 in good yield. Following reaction with 4-methoxybenzenesulfonyl chloride afforded the corresponding sulfonamides BI-6C8 in very high yield. The synthesis was completed by reducing the aromatic nitro group to the amines BI-6C9 in presence of tin dichloride ($SnCl_2$). c) Proposed mechanism for BI-6C9 biological activity: in isolated mitochondria, BI-6C9 prevents the association of activated Bid with the mitochondrial membrane.

Acknowledgements

Financial support for this work was provided in part by NIH grants CA107875, CA102583, AI061139, AI055789, AI058123.

[1] M. Pellecchia, B. Becattini, K. J. Crowell, R. Fattorusso, M. Forino, M. Fragai, D. Jung, T. Mustelin, L. Tautz, *Expert Opin. Ther. Targets* **2004**, *8*, 597–611.
[2] M. Pellecchia, D. S. Sem, K. Wüthrich, *Nat. Rev. Drug Discovery* **2002**, *1*, 211–219.
[3] M. Schade, H. Oschkinat, *Curr. Opin Drug Discovery Dev.* **2005**, *8*, 365–373.
[4] B. Becattini, S. Sareth, D. Zhai, K. J. Crowell, M. Leone, J. C. Reed, M. Pellecchia, *Chem. Biol.* **2004**, *11*, 1107–1117.
[5] B. R. Stockwell, *Neuron* **2002**, *36*, 559–562.
[6] D. Li, E. F. DeRose, R. E. London, *J. Biomol. NMR* **1999**, *15*, 71–76.
[7] R. E. London, *J. Magn. Reson.* **1999**, *141*, 301–311.
[8] J. Fejzo, C. A. Lepre, J. W. Peng, G. W. Bemis, Ajay, M. A. Murcko, J. M. Moore, *Chem. Biol.* **1999**, *6*, 755–769.
[9] M. Pellecchia, D. Meininger, Q. Dong, E. Chang, R. Jack, D. S. Sem, *J. Biomol. NMR* **2002**, *22*, 165–173.
[10] S. J. Vincent, C. Zwahlen, C. B. Post, J. W. Burgner, G. Bodenhausen, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 4383–4388.
[11] K. C. Nicolaou, Y. L. Zhong, P. S. Baran, J. Jung, H. S. Choi, W. H. Yoon, *J. Am. Chem. Soc.* **2002**, *124*, 2202–2211.
[12] A. Lew, P. O. Krutzik, M. E. Hart, A. R. Chamberlin, *J. Comb. Chem.* **2002**, *4*, 95–105.
[13] A. Loupy, *Microwaves in organic synthesis*, Wiley-VCH, Weinheim, Germany, **2003**.
[14] J. M. Adams, S. Cory, *Science* **1998**, *281*, 1322–1326.
[15] A. Gross, J. M. McDonnell, S. J. Korsmeyer, *Genes Dev.* **1999**, *13*, 1899–1911.
[16] J. C. Reed, *Oncogene* **1998**, *17*, 3225–3236.
[17] N. Plesnila, S. Zinkel, S. Amin-Hanjani, J. Qiu, S. J. Korsmeyer, M. A. Moskowitz, *Eur. Surg. Res.* **2002**, *34*, 37–41.
[18] N. Plesnila, S. Zinkel, D. A. Le, S. Amin-Hanjani, Y. Wu, J. Qiu, A. Chiarugi, S. S. Thomas, D. S. Kohane, S. J. Korsmeyer, M. A. Moskowitz, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 15318–15323.
[19] B. Kramer, M. Rarey, T. Lengauer, *Protein J.* **1999**, *37*, 228–241.

Published online: August 25, 2005

