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Statistical Molecular Design of an Embelin-Inspired Library of XIAP Inhibitors

A Rational Approach Towards the Identification of Ligands Directed to Single and Multiple Targets of Systems Biology Diseases

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The aim of having a large compound library is to cover chemical structure space well, but if we are smart we can do this more efficiently.
(Douglas B. Kell, *Tibitech* **2000**, *18*, 186-187)

Abstract

A re-evaluation of the modern principles of Fragment-Based Design is presented in this investigation, which opens new perspectives to the design of multitargeted ligands as biological tools and potential leads for Systems Biology-based diseases, with a special emphasis on cancer. The preliminary results of the methodology are here described, which integrates Chemometrics and Molecular Modeling with biostructural information available from naturally occurring, promiscuously active products, in an effort to anticipate the identification of multitargeted proapoptotic modulators in early and less expensive stages of the drug design. As a starting multiple-active chemotype, a dihydroxyquinone-based naturally occurring fragment was selected. This choice was driven by the intrinsic promiscuity of a wide range of natural quinone-based compounds and strictly related analogues thereof, as well as by the availability of detailed biostructural information pertaining their mechanism of action on phylogenetically related and unrelated proteins. In order

to expand the chemical opportunities, isofunctional but structurally diverse chemotypes were selected by similarity search methods against a database of fragments, basing the search on a combination of Principal Component Analysis, Hierarchical Cluster Analysis and Tanimoto coefficient methods. An 'ad-hoc' database of quinone-based analogues were designed biased against synthetic feasibility, drug-likeliness, and biostructural information concerning interactions of quinone-based Natural Products with XIAP and kinases. This database was merged to a pre-compiled database of 13,098 commercial fragments complying with the 'Rule of Three', and 328 molecular descriptors were calculated for each entry. Following the application of the similarity search methods, a discrete library of 66 diverse fragments were selected, which are under investigation by Surface Plasmon Resonance on Biacore against a panel of apoptosis modulator proteins including XIAP (X-Chromosome-linked Inhibitor of Apoptosis Protein), a Serine-Threonin Kinase (B-Raf), Receptor Tyrosine Kinases (VEGF-2, EGFR, HER2, IGF1R), and PI3 kinases (p110α-p110γ, mTOR).

A preliminary virtual screening of a small sub-collection of fragments was preformed on XIAP, and directed the choice of a few diverse fragments and their Structure-Based synthetic elaboration as potential non-quinone inhibitors of XIAP. In silico pre-validation of the library on the protein kinase set is in progress. From this investigation, a direct comparison of the different similarity methods selected in this study will be possible, which might disclose novel applications of Principal Component Analysis in Fragment-Based Design of single- and multitargeted ligands.

Introduction

Drug discovery is unrelentingly challenged by Nature. The more biologists unravel the complex regulatory pathways surrounding disease-relevant events, the more the 'one-target, one-disease' paradigm becomes a reductionist approach.[1] Biological pathways involved in diseases are multiple and complex, and still not well-understood. Most biological functions are the result of interactions among many 'modules' consisting of numerous interacting molecules. Expanding the drug discovery space, a new perspective is emerging from recent advances in Systems Biology, which rephrases the major drug discovery paradigm comprised in the concept of chemical interaction between drugs and biological targets, and unveils novel opportunities towards a 'systems'- or 'pathway-oriented' approach.[1] Instead of focusing on a compound's interaction with a molecular target, a pathway-based drug discovery suggests to seek for molecules that cause signature changes in the concentration of proteins and metabolites, or control the expression disease-mediating genes. Hits in pathways-based screens should emerge based on similarity of biochemical consequences rather than through specific chemical mechanisms. In this context, 'molecular' combinations of agents able to modulate multiple but selected targets are emerging as powerful tools for tackling Systems Biology and Medicinal Chemistry issues.[2]

This situation is particularly emphasized in highly complex diseases (central nervous system, cardiovascular, metabolic, infective), and is especially pressing in anticancer drug discovery.[3] Following the recent advances on the study of the genetic determinants of cancer, a description of this pathology in molecular terms have improved the way of detecting, classifying, monitoring and treating human tumors. Particular mutations or variations in gene expression responsible for specific tumors account for the distinctive molecular features nowadays used to subdivide histopathologically equivalent cancers. In principle, each distinctive mutation and aberration constitutes the specific tumor Achille's heel to exploit with targeted therapies. Despite their

promise in the clinic, many of the therapies that have been developed to selectively hit cancer molecular targets have conferred only modest benefits on patient survival. Cancer is a pleiotropic phenomenon, and compensatory mechanisms are involved which limit the effectiveness of these cytostatic treatments. For these reasons, a significant tumor regression still relies on combined therapies with conventional cytotoxic agents or other targeted drugs.[4]

Despite this contradictory situation, a challenging opportunity is offered by an emerging paradigm that considers the development of agents that modulate multiple targets simultaneously and benefit from higher efficacy and safety compared to drugs addressing a single target.[5] Several drugs designed to target a single cancer protein have been discovered which actually bind other homologous targets (e.g. the multikinase inhibitors sorafenib[6] and sutinib[7]). Such 'promiscuous' drugs have offered the proof of concept that hitting the delicate balance between nondiscriminate and discriminate targeting by picking up the targets that arise at critical biological hubs might be an effective way to knock out multiple cancer defence systems at once, and by-pass cancer resistance mechanisms. Beside a growing number of targeted agents with 'serendipitously' assessed side-targeted activities, the development of targeted agents deliberately designed to hit two or more receptors at once is a more recent trend,[8] and opens significant challenges to the medicinal chemistry community.[9] This new age is marked by lapatinib, a specifically designed dual-targeted drug against EGFR and HER2.[10]

The general principles in the design of multifunctional agents have been recently reviewed by Morphy and Rankovich through two main strategies, the 'single ligand' and 'dual ligand' approaches.[11] The first strategy relies on expensive or time-consuming focused or random screenings (HTS or virtual screening), from which a single molecule is individuated that, in most cases, has a good activity for one target and at least some minimal activity on a second target(s). The multi-targeting property is attained by 'designing-in' and balancing the activity of the latter(s) by means of focused analogues. In the second strategy, two known individual compounds highly selective for their specific targets are combined in a single entity with a dual activity. The starting compounds are usually selected among known selective ligands such as old drugs or proprietary agents. The 'combining' process can be achieved through different ways, following a sequence where the molecular complexity increases. Individual functional frameworks from two original agents can be 'linked' through a spacer to form conjugates, but also 'fused' through the superimposition of functional motifs, or 'merged' in a highly integrated dually active entity. The main problem with this strategy is inherent with the design rationale, which tends to drive the potential leads to be not as drug-like as expected for a preclinical compound. For the same reason, chances are low to design ligands targeting proteins belonging to different phylogenetic families.[12]

To address these problems, a Fragment-Based Design[13] approach is proposed in this preliminary investigation, which is expected to offer several practical benefits.[14] Firstly, smaller compounds increase the probability of finding smaller and more drug-like ligands. Secondly, given that low-molecular weight molecules are often more promiscuous, the design of binders of structurally diverse targets from different protein families has greater likelihood to succeed. In addition, the molecular simplification into fragments permits a straightforward analysis of the chemical space using standard cheminformatics applications. To these objectives, an efficient identification of fragment-like multi-binders is of paramount importance. In this context, the inherent promiscuity of fragments underlying the structural complexity of Natural Products is an

intriguing option. Being selected by evolution for binding to structurally conserved but genetically mobile proteins, Natural Products are biologically and chemically pre-validated.[15] The chemical space covered by natural scaffolds possess the basic requirements for binding to multiple proteins and enzymes, and is an obvious choice in the search of protein binders. In the broad spectrum of bioactive natural space, a large number of small Natural Products are known which can be regarded as 'ad hoc' fragments with an innate, pre-defined promiscuous activity towards genetically dissimilar targets (e.g., flavonoids, coumarins, antraquinones, polyketides, etc.). A requisite promiscuity might also be achieved by chemical 'defragmentation' of complex products by Function-Oriented Synthesis,[16] especially when biostructural information are available. Such natural and natural-derived fragments are therefore privileged frameworks in the search for multiligand agents.

Description of the investigation

The quinone nucleous is well represented in Nature. Several classes of quinone- and quinone-related-based natural products are known as biological modulators, antielminthic, analgesic, antifertility, antitumor and antioxidant agents.[17] Mitomycin C, doxorubicin and daunorubicin are known examples of quinonoid anticancer agents, and certain cannabinoid quinones have been recently disclosed as potent antineoplastic agents (Figure 1).[18] The naijquinone family is a group of marine sesquiterpene quinones which have been identified as inhibitors of Receptor Tyrosine Kinase (RTK) Her-2/Neu (also called erbB-2, EGFR-2), an oncogene vastly over-expressed in about 30% of primary breast, ovary, and gastric carcinoma (Figure 1).[19] The structural requirements for binding this kinase have been elucidated by detailed bioinformatic studies, and have led to the identification of simplified analogues with selective activity towards certain RTKs, such as Vascular Endothelial Growth-Factor Receptor 2 and 3 (VEGFR-2 and VEGF-3), Insulin Growth-Factor-1 Receptor (IGF1R), and Tie-2. Further examples of quinone-based synthetic leads and their binding mode on VEGFR-2 have been reported.[20] Strictly related to najiquinones are certain natural 1,4-benzoquinones such as embelin and rapanone, ilimaquinone, which are known for their antielminthic, analgesic, antifertility, antitumor and antioxidant activity (Figure 1).[21] Embelin has recently been reported to bind X-chromosome-linked Inhibitor of Apoptosis Protein (XIAP),[22] an important modulator of natural apoptosis executioners, the serine proteinase caspases.[23,24]

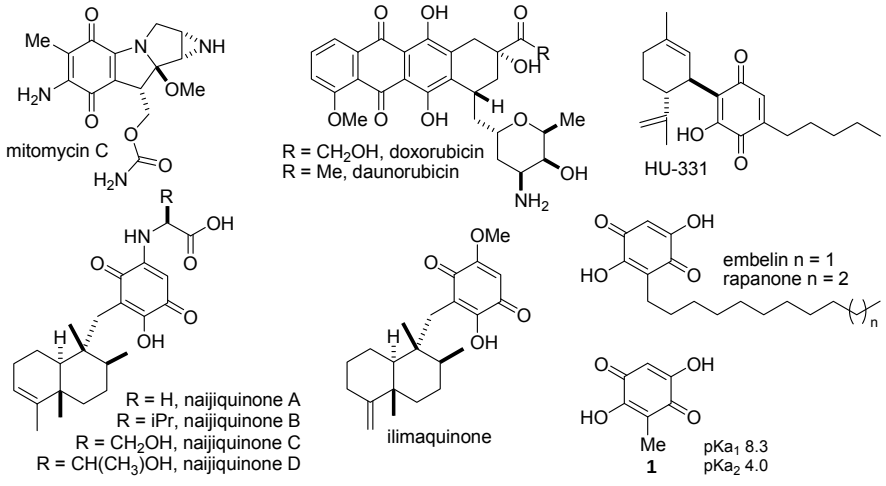

Figure 1. Structures of biologically relevant quinone-based Natural Products, and dihydroxyquinone fragment **1** (pka calculated with Marvin, ChemAxon, www.chemaxon.com/marvin/sketch/index.jsp).

Based on these premises, the simple dihydroxyquinone fragment **1** (Figure 1) was envisaged in this study as a suitable 'consensus motif' that brings together structurally related proteins (i.e. kinases) with unrelated ones (i.e. XIAP). Despite its biochemical benefits, the dihydroxyquinone **1** suffers from several drawbacks related to its inherent toxicity as a Michael acceptor and redox-active molecule,[25] as well as to the presence of several ionization and resonance forms at physiological pH, which complicates the understanding of the interactions with target proteins. The availability of analogues of **1** is thereby a precondition in the search of multiple ligands of apoptosis modulator proteins, and call for the use of appropriate databases of fragments as a source of isofunctional yet structurally diverse chemotypes.

Database preparation and similarity search

Alternative bioisosteres of **1** were designed accounting for all the ionization forms expected at pH 7.4 for **1**, whilst keeping the resonance possibilities as limited in number as possible. Quinone-based analogues, but also non-quinone equivalents (e.g. lactons, lactams, and *N*-containing heterocycles) were considered. A small collection of 45 drug-like fragments were designed based on biostructural information available from known natural quinones with target proteins (kinases, XIAP). These fragments are accessible by parallel and divergent synthesis by readily available starting materials.

In addition, a pre-compiled database of 13,098 commercial fragments was used. This database is freely accessible on the web,[26] and contains fragments complying with the 'Rule of Three'.[27] Both databases were merged and elaborated using MOE (Chemical Computing Group). Manual filtering was performed to exclude heavy metals. Molecules were washed in order to deprotonate strong acids, protonate strong bases, add explicit hydrogens, and scale bonds to reasonable length. Partial charges were calculated using MMFF94, and adjusting hydrogens and lone pairs as required. Molecules were minimized under RMS gradient 0.1, calculating forcefield charges and preserving the existing chirality if necessary. Finally, 1D, 2D and 3D molecular descriptors were calculated (328 variables).

Similarity search was performed comparing the database of fragments with both the ionized and non-ionized forms of **1** as the queries, and basing the search on a combination of Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA) and Tanimoto coefficient (Tc).

PCA is a popular multivariate statistical procedure in modern data analysis, useful to find patterns in data of high dimension and express them under linear combination of their vectors ('principal components').[28] By PCA most the variability present within an original system of observations and variables is captured, and the pattern of similarities and differences among observations and variables is simply represented by points in a bidimensional plot.

A PCA analysis was performed on the previously prepared database using Simca-P 12.0 (Umetrics). A model based on 38 components explaining 98% of the variability of the whole set (>13,000 observations, 213 variables) was obtained. The first three components accounted for 30%, 50% and 62% of the variability, and were considered for the similarity analysis. The PCA scatter plot of the resulting model is depicted in Figure 2, where the query entries – ionized and non-ionized **1** – are shown as green and red spots, respectively.

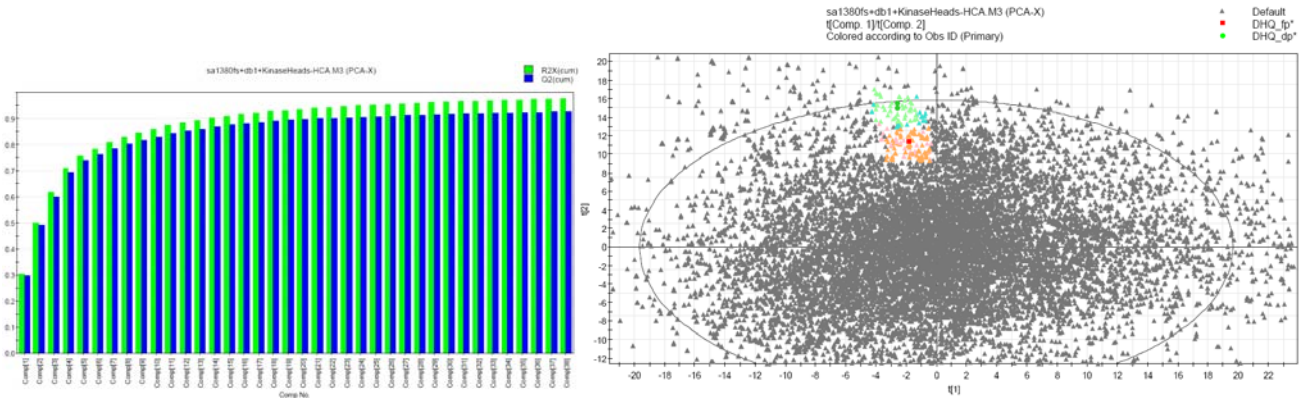

Figure 2. PCA analysis: model (left) and scatter plot (right). Query fragments (ionized and non-ionized **1**) are shown as green and red spots, respectively. Green light spots: fragments similar to ionized **1** across the first and second component. Turquoise spots: fragments similar to ionized **1** across the first three components. Orange spots: fragments similar to non-ionized **1** across the first and second component. Pink spots: fragments similar to non-ionized **1** across the first, second, and third component.

An arbitrary 'similarity space' surrounding the queries was defined by fixing a maximal area of ±1.5 from the normalized score distance (t value) defining the location of each query molecule in the plot relative to the first and second component (light green and orange spots), and of ±2 from the location of each query molecule in the plots relative to the first three components (pink and turquoise spots).

A Hierarchical Cluster Analysis (HCA)[29] was then performed by calculating the Euclidean distances between observation in the same dataset. This classification method is commonly used to arrange sets of observations into clusters. The similarity depends on the method used, and is a measure of the distance between observations when Euclidean metric is used. In the hierarchical ('tree-like') CA, clusters are joined at increasing levels of dissimilarity providing a dendrogram. In this study, Ward algorithm was used for the calculation, which is implemented in Simpca-P 12.0. Most of the fragments detected by PCA showed to closely cluster together, substantiating the results previously obtained (Figure 3). However, a few extra fragments were detected by HCA which were excluded by PCA.

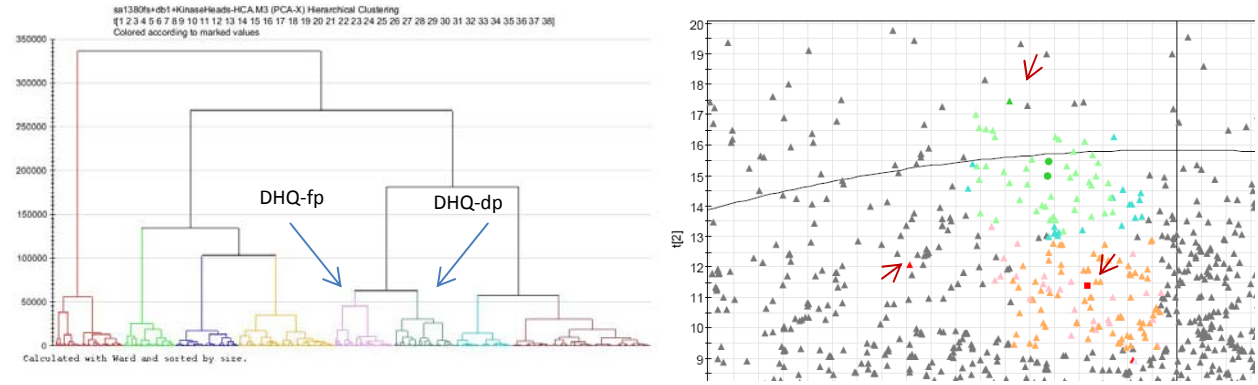

Figure 3. HCA dendrogram (left), and selected fragments in the PCA space (right).

The fragments selected by the combination of these methods (about 150) were then filtered applying a bias against drug-likeliness and synthetic feasibility, to obtain 49 fragments (Library 1). Of these, 4 derived from the originally designed library of 'ad hoc' fragments, while the remaining were commercially available.

A second PCA was performed on the 49-fragment library, which was further diminished to a discrete sub-collection of 20 fragments (Sub-library 1a) in order to cover as much as possible of the PCA chemical space and keep the number of entries sensibly limited in number. A depiction of the fragment distribution in the PCA space is presented in Figure 4. The fragments in this sub-library contained structural indications relative to the location of the C11 aliphatic chain of embelin, and were selected to be elaborated into embelin analogues.

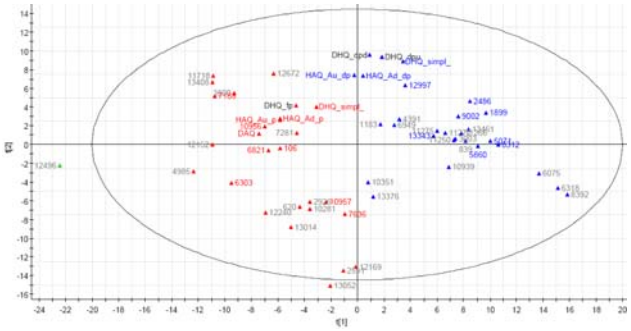

Figure 4. PCA distribution of fragments selected by PCA and HCA (Library 1). Red entries are close analogues of the hydroxyquinone nucleus in the non-ionized form; blue entries are close analogues of the hydroxyquinone nucleus in the ionized form; the green outlier is a positively charged analogue. Fragments selected for the Sub-library 1a are labelled in blue, red and green.

To expand the scope of the study, a similarity search was performed scoring the queries against the whole database according to Tanimoto similarity coefficient, basing the search on MACCS 166 Structural Keys (bit packed, Bit_MACCS) as the molecular fingerprints, and using MOE (Chemical Computing Group). Different fragments emerged compared to the PCA method, and 17 were selected which showed similarity scores higher than 50-60% (Library 2). All of these fragments were commercially available. Their PCA distribution is shown in Figure 5.

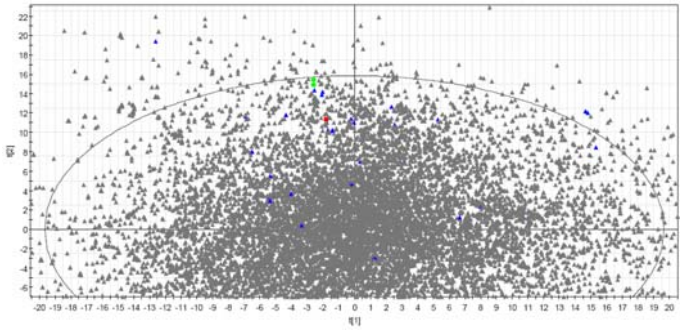

Figure 5. PCA distribution of fragments selected by Tanimoto similarity criterion (blue entries) (Library 2). Query entries are shown as green and red spots.

Overall, 66 fragments were selected by PCA, HCA and Tc (Library 1 and 2, 62 commercially available fragments) to test by Surface Plasmon Resonance (SPR) on a panel of selected modulator of apoptosis proteins (Figure 6). Given that small promiscuous fragments might have weaker binding affinity than a traditional selective lead, routine direct binding assays using SPR have been chosen for measuring their affinity.[30] All fragments are currently under evaluation in SPR assays and, whenever possible, their binding mode will be confirmed by high resolution NMR experiments and/or crystallographic analysis of their complexes with individual proteins. Further molecular modelling analysis of these fragments will drive the optimization and refinement of their potential single- and dual binding modes by means of fragment

extension and linking operations. Novel building blocks can emerge by extension of the central fragment in appropriate directions (fragment evolution), or by joining different fragments (fragment linking) if several fragments are identified with different locations within the binding site. The application of techniques for scaffold hopping and statistical molecular design will hence direct the lead optimization and the design of focused libraries.

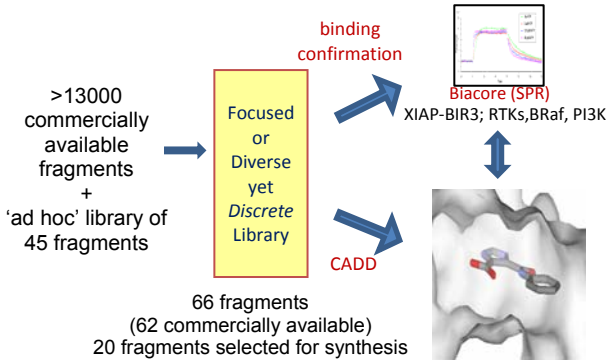

Figure 6. Library design and validation process.

Systems Biology rationale and Structure-Based Design tools

Inspired by the known activities of embelin and najiquinones, four known and medicinally validated protein groups were selected as a crucial biological checkpoint: proteins of the Receptor Tyrosine Kinases (RTK) family (e.g. EGFR; HER2, also called EGFR-2; VEGFR-2, and IGF1R), a Serine-Threonine Kinase (B-Raf), Lipid Kinases of the Phosphatidylinositol-3-Phosphate (PI3K) family (e.g. p110α-p110γ, mTOR), and an Inhibitor of Apoptosis Protein (X-Chromosome-linked Inhibitor of Apoptosis Protein, XIAP). These protein families are known validated targets and oncogenes involved in key signalling pathways controlling crucial cellular functions such as endothelial cell angiogenesis, tumor cell growth, proliferation and survival, and apoptosis. Recently, benefits from the so-called 'antiangiogenic chemotherapy' have been validated by a number of cancer therapy protocols. Among them, novel combinations of agents directed at the apoptotic pathways (e.g. IAPs) with drugs targeting 'indirect' apoptosis regulators (e.g. kinases) are emerging.[31]

A Systems Biology rationale underlines the choice of the target proteins, which permit the investigation of several potential strategies for achieving the maximization of inhibition phenomena upon targets and pathways in a logical way. Depending on the biological response displayed by the selected fragments, three systematic strategies might turn out, involving the (1) maximization of specific pathways inhibition: this will be possible if signalling components within the same pathway will be inhibited, for instance by simultaneously blocking the activity of an RTK and a Ras/Raf protein, and/or a PI3K; (2) inhibition of related pathways: this will be possible if a simultaneous block of two growth factor receptors or two downstream components in parallel pathways will occur, for instance if a simultaneous inhibition of two RTKs will be observed; (3) inhibition of unrelated pathways: this will be put in practice if a combined inhibition of a kinase and XIAP will be detected.

Having diverged late in evolution, kinases are closely related to each other. While from one side the high level of homology in the catalytic site of kinases helps the individuation of promiscuous fragments, on the other side this might hamper the search for specifically bifunctional inhibitors. However, the catalytic site of kinases is not static, and new opportunities are emerging to design selective kinase inhibitors.[9b,32] The

recent individuation of structural differences in their catalytic pocket as well as of non-conserved extra back- and front-pockets nearby the ATP binding site, help to set the structural requirements for addressing kinases with small molecules inhibitors in a selective way.[32]

Although the inhibition of multiple tumor processes by targeting genetically unrelated proteins, such as XIAP and kinases, might be envisaged as a challenging situation, the availability of a promiscuous fragment for these proteins at the outset sets up the conditions for the Structure-Based Design of dually selective ligands. Interaction models of XIAP with its natural ligand (Smac) or unnatural probes (eg, AVPI peptide and peptidomimetics thereof) are available,[24] and further biostructural information have been recently disclosed pertaining the inhibition of XIAP-related proteins (e.g. cIAPs, ML-IAP).[33]

A preliminary virtual screening on XIAP was preformed for the small sub-collection of 20 fragments previously selected by PCA (Sub-library 1a), with the aim to guide their Structure-Based elaboration into potential non-quinone small molecules inhibitors of XIAP. The protein was prepared using MOE (Chemical Computing Group) starting from the complex with AVPI peptide (pdb code: 2OPZ). Omega2 (OpenEye Scientific Software Inc.) was used to generate 3D conformational ensembles of the selected fragments. MMFF94 force field was applied to generate up to 20,000 conformers (rms 0.3). A rigid docking assessment was performed by examining exhaustively all possible poses within XIAP active pocket using Fred (OpenEye Scientific Software Inc.), filtering for shape complementarity before selecting each single pose based upon a consensus of scoring functions (ShapeGauss, PLP, ChemGauss3, ChemScore). Ligands were then scored and ranked with these scoring functions. Top-binding poses were detected for several fragments displaying interactions with specific residues in the binding pocket of XIAP (E314, W323, Y324), which are critical in stabilizing AVPI peptide, peptidomimetics thereof, and embelin in both ionized and non-ionized form. These diverse fragments were selected to be elaborated into embelin analogues by connecting a C11 aliphatic tail on the fragment nucleus in the proper location. Their structures, syntheses and biological evaluation will be disclosed in due course. In silico pre-validation of the library on the protein kinase set is in progress.

Summary and Perspectives

In this preliminary investigation, a method has been described for the simplification of natural-like architectures into molecular ‘passe-partout’ and the individuation of promiscuous binding determinants from Natural Products. Fragments targeting single and multiple proteins are expected to be identified which will be elaborated in selective single- and multitargeted binders by means of conventional Structure-Based Design on individual apoptosis modulator proteins. A direct comparison of the different similarity methods used in this study will be performed, which might disclose novel applications of Principal Component Analysis in Fragment-Based Drug Design of ligands targeting single and multiple proteins.

In pursuing this investigation, the ability of Organic Synthesis to reorient its creative processes and strategies depending on targets, diversity and functions, will be the driving force to interface Systems Biology and Medicine. Novel biological tools might emerge which will help to understand multiple inhibition phenomena on a mechanism, phenotypic and genetic basis, and to individuate novel potential leads for human cancer cell lines (e.g. melanoma, neuroblastoma, glioma, ovarian, breast, lung, prostate cancer, neuroendocrine cancers such as pancreas, thyroid and parathyroid carcinomas). It will be possible to develop novel resistance models to assess possible compensatory processes on targeted oncogene-resistant cell lines.

Models might also be developed to understand the toxicity and tissue selectivity in in vitro, ex vivo and in vivo models. Finally, it will be possible to elaborate, integrate and decode 'local' and systemic activity data underlying certain types of cancer.

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