Advances in Methods for Therapeutic Peptide Discovery, Design and Development

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Abstract: Drug discovery and development are intense, lengthy and interdisciplinary processes. Traditionally, drugs were discovered by synthesizing compounds in time-consuming multi-step experimental investigations followed by *in vitro* and *in vivo* biological screening. Promising candidates were then further studied for their pharmacokinetic properties, metabolism and potential toxicity. Today, the process of drug discovery has been revolutionized due to the advances in genomics, proteomics, and bioinformatics. Efficient technologies such as combinatorial chemistry, high throughput screening (HTS), virtual screening, *de novo* design and structure-based drug design contribute greatly to drug discovery. Peptides are emerging as a novel class of drugs for cancer therapy, and many efforts have been made to develop peptide-based pharmacologically active compounds. This paper presents a review of current advances and novel approaches in experimental and computational drug discovery and design. We also present a novel bioactive peptide analogue, designed using the Resonant Recognition Model (RRM), and discuss its potential use for cancer therapeutics.

Keywords: Digital signal processing, drug discovery, cancer therapeutics, frequency, peptide *de novo* design, protein function.

DRUG DISCOVERY PROCESS

Drug discovery starts with target and lead discovery, followed by lead optimization and pre-clinical *in vitro* and *in vivo* studies to determine if such compounds satisfy a number of pre-set criteria for initiating clinical development. For the pharmaceutical industry, the number of years to bring a drug from discovery to market is approximately 12-14 years and costing up to $1.2 - $1.4 billion dollars. Traditionally, drugs were discovered by synthesizing compounds in time-consuming multi-step processes against a battery of *in vivo* biological screens and further investigating the promising candidates for their pharmacokinetic properties, metabolism and potential toxicity. This discovery process has resulted in high attrition rates with failures attributed to poor pharmacokinetics, lack of efficacy, animal toxicity, adverse effects in humans and various commercial and miscellaneous factors [1]. Nowadays, advances in genomics and proteomics, and development of new bioinformatics methods contribute greatly to the process of drug discovery. Combinatorial chemistry is a powerful tool used by medicinal chemistry for the design of new drug candidates [2]. Combinatorial methods provide a way to generate very large numbers of compounds in a relatively short period of time (compared to the traditional synthesis of a single compound). On the other hand, this aspect of combinatorial chemistry presents a problem as a balance is required between making everything possible and the constraints of economics, logistics, and time. In other words, there is a need to select the products to be synthesized from the vast pool of those possible to be produced [3].

In silico methods can help in identifying drug targets via bioinformatics tools. As the structures of more and more protein targets become available through crystallography and NMR analysis, computational methods can use the known structure of a protein target as a route to discover novel lead compounds. These *in silico* methods include *de novo* design, virtual screening and fragment based discovery.

Virtual screening and *de novo* design play important roles in lead discovery processes in the pharmaceutical industry. Virtual screening refers to computational screening of large libraries of chemicals for compounds that complement targets of known structure, which could be tested experimentally. Since virtual screening takes place in the three-dimensional (3-D) active site of a target, it is also called structure-based virtual screening. *De novo* design approaches attempt to use the unliganded structure of a protein to generate a novel chemical structure that can bind to the protein's active site. There are various algorithms, most of which depend on identifying initial putative sites of interaction that can be grown into complete ligands.

Fragment based discovery is based on the premise that most ligands that bind strongly to a protein active site, can be considered as a number of smaller fragments or functionalities. Fragments are identified by screening a relatively small library of molecules (400-20,000) by X-ray crystallography and NMR spectroscopy. The structures of the fragments binding to a protein can be used to design new ligands by adding functionality to the fragments or by incorporating features of the fragments onto existing ligands.

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The most powerful approach for designing drug-oriented peptides is hybridization of structure-based and combinatorial chemistry methodologies. This approach proposes to screen large quantities of drug candidates. The peptides are designed using a semi-rational process, the so-called "evolutionary computation" [3]. The main advantage of this approach is that novel structures, not contained in any database, can be designed, so the ligands can be built from scratch. However, to achieve this goal, the algorithms have to address two main issues. Firstly, a competent search method must be provided to explore this high-dimensional chemical space. Secondly, the search space (the set of all algorithmically treatable molecules) must be structured into regions of higher and lower quality to allow the prediction of desired properties. In order to perform the search task, researchers implemented and tested four different evolutionary algorithms [4]: Darwinist genetic algorithm (DGA), Lamarckian genetic algorithm (LGA), population-based incremental learning (PBIL) and Bayesian optimization algorithm (BOA).

Belda *et al*. [4] designed peptide drugs, which can serve as effective ligands to a target protein area defined by the user. This methodology is also known as surface patch. One application of such peptide drugs could be to act as inhibitors of some pathological functionalities of a target protein [5]. On the basis of the surface patch method the researchers developed a new methodology for specific cases: prolyl oligopetidase, p53, and DNA gyrase. They compared the proposed peptides with some other peptides that were designed using a purely chemical-knowledge based approach. In all the tested cases, the peptides designed *in silico* presented better docking energies than their counterparts designed chemically [4].

A genetic or evolutionary algorithm applies the principles of evolution found in nature to the problem of finding an optimal solution to a Solver problem. In a "genetic algorithm," the problem is encoded in a series of bit strings that are manipulated by the algorithm whereas in an "evolutionary algorithm," the decision variables and problem functions are used directly. Most commercial solver products are based on evolutionary algorithms. An evolutionary algorithm for optimization is different from "classical" optimization methods in several ways: (i) Random Versus Deterministic Operation, (ii) Population Versus Single Best Solution, (iii) Creating New Solutions Through Mutation, (iv) Combining Solutions through Crossover, and (v) Selecting Solutions via "Survival of the Fittest" [6-9].

A drawback of any evolutionary algorithm is that a solution is "better" only in comparison to other presently known solutions. Such an algorithm actually has no concept of an "optimal solution," or of any way to test whether a solution is optimal. For this reason, evolutionary algorithms are best employed on problems where it is difficult or impossible to test for optimality. This also means that an evolutionary algorithm never knows for certain when to stop, aside from the length of time, or the number of iterations or candidate solutions that can be allowed to be explored [9]. Despite this limitation, evolutionary algorithms are perfect candidates for applications were deterministic or analytical methods fail. For instance, cases where the underlying mathematical model is ill-defined or the search space is too big [10].

Peptide aptamers are short peptides (up to 20 amino acids in length) that can be selected from a random peptide library and specifically bind to a given target protein under intracellular conditions [11]. The principle behind the approach is that each oligopeptide of the library is displayed in a unique formation and the library is large enough to contain a particular peptide, which is able to recognise and bind to any given protein target structure. Selection occurs through screening of a high-complexity peptide library, which is usually performed *in vivo*. To select an aptamer with a specific binding affinity, screening of millions of different peptides can be performed in 1-2 weeks. Aptamers with high binding affinities might have useful biological properties, for example, preventing the interaction of the target protein with functionally important cellular partners. High-affinity binding also limits the peptide concentrations necessary for an inhibitory effect. If the binding affinity is too low, affinity maturation by randomised mutagemers into more efficient inhibitors can occur [12]. The important advantage of this approach over conventional drug design is that the structure of the target protein can remain unknown. Aptamers are able to block only one specific function of a target protein, for example, the interaction site of a specific network member or the substrate [13].

Structure-based drug design plays a key role in the process of developing a new compound/drug. It exploits the recognition and discrimination capabilities of a target protein to create favourable interactions in three dimensions with a particular molecule.

Fragment-Based Drug Discovery

At Evotec (http://www.evotec.com/), researchers use the combination of a high-quality fragment library with sensitive biochemical screening methods, for the identification of weakly active fragment molecules as novel starting points for medicinal chemistry optimization. The application of this technology to enzyme targets such as renin and various kinases is extremely powerful and can dramatically reduce the time for moving from target to preclinical development as well as generating novel chemical start points. The traditional approach to screening for candidate compounds is high-throughput screening. The hit compound identified via this approach is of a similar molecular weight and size to that expected of the final drug, so the optimization of the hit compound entails the sequential removal and addition of appropriate functional groups aimed at increasing the potency and improving the pharmacokinetics of the compound. The advantages of fragments are that they are about one-half the molecular weight and so additional functionality can be quickly added to rapidly improve the potency and pharmacokinetic (PK) properties. The fragment-based approach ensures early access to crystal structures of the fragments interacting with a biological target. Knowing the crystal structures provides valuable information on how ligands interact with the target, the interactions of the molecular bonds, and what functionalities are most important. This allows researchers to quickly build improved molecules and

bypass the usual lengthy process of trial and error in molecule design.

Multiple Kinase Inhibitors

Prior to the completion of the Human Genome, the mapping of all the kinases in the human genome and drug discovery around kinases was problematic. It is known that cancer is heterogeneous. Each kind is involved with multiple pathways, angiogenesis, tumor growth, and metastasis, so hitting one pathway isn't going to be enough. Computational techniques have provided a start for designing inhibitors against individual targets. However, the design of compounds that are effective against multiple kinases has remained a challenge. By starting with validated targets and known kinase inhibitors, it is possible to eliminate the hit-to-lead stage of drug development by two to three years.

Protein and Ligand Structure-based Design for GPCRs

Protocols for structure-based drug design with soluble proteins are now used fairly routinely. However, for *G protein-coupled receptor* (GCPR) targets, structure-based approaches are likely to be limited to homology models for a few more years. These approaches are: examining traditional ligand-based-drug design; target design using structural and computational analysis—or taking advantage of what is already known about the system; and ligand re-design using structural information about proteins to tune out unwanted activity. The methods used include studying structure-activity relationships and high-throughput docking.

Lead Optimization

Lead optimization is the complex, non-linear process of refining the chemical structure of a confirmed hit to improve its drug characteristics with the goal of producing a preclinical drug candidate. This stage frequently represents the bottleneck of a drug discovery program. Lead optimization employs a combination of empirical, combinatorial, and rational approaches that optimizes leads through a continuous, multi-step process based on knowledge gained at each stage. Typically, one or more confirmed hits are evaluated in secondary assays, and a set of related compounds, called analogues, are synthesized and screened.

Renin Inhibitors

In this method a homology model of an enzyme is used to characterize the binding mode of a peptide compound, CGP38560, in complex with a model of renin. The medical model used in the development of the compound is the renin-angiotensin cascade leading to hypertension, which occurs in three levels. Most drugs in this class can block the cascade at the second and third levels, but the goal is to successfully block the cascade at the first level.

Role of Peptides in Pharmacology

The importance and broad functional role of peptides in life processes became apparent only in the 1950s and early 1960s, when the continuous development of increasingly sensitive analytical methods and techniques for isolation and purification started a new era in pharmacology. Size-exclusion chromatography [14], chromatography on cellulose-based ion-exchangers [15], countercurrent distribution [16] and other methods developed in various areas of biochemistry complemented the techniques for peptide isolation that had been developed previously [17]. Native peptides can be directly applied as pharmacologically active compounds only to a very limited extent. The major disadvantages of the application of a peptide in a biological system – for example, rapid degradation by proteases, hepatic clearance, undesired side effects by interaction of conformationally flexible peptides with different receptors, and low membrane permeability due to their hydrophilic character – prohibit the use of oral application in most cases because of their detrimental effects [17]. However, peptide chemistry can contribute considerably to drug development. The interaction of a peptide or a protein epitope with a receptor or an enzyme is the initial event based on molecular recognition, and generally elicits a biological response. Many efforts have been made to develop pharmacologically active peptide-based compounds, including peptide modification and the design of peptidomimetics [17]. Whilst modified peptides contain non-proteino-genic or modified amino acid building blocks, peptidomimetics are non-peptidic compounds that imitate the structure of a peptide in its receptor-bound conformation and – in the case of agonists – the biological mode of action on the receptor level. According to the definition by Ripka and Rich [18], three different types of peptidomimetics may be distinguished:

Type I

These are peptides modified by amide bond isosteres and secondary structure mimetics. These derivatives are usually designed to closely match the peptide backbone.

Type II

These are small non-peptide molecules that bind to a receptor or enzyme (functional mimetics). However, despite being often presumed to serve as structural analogues of native peptide ligands, these non-peptide antagonists often bind to a different receptor sub-site and, hence, do not necessarily mimic the parent peptide.

Type III

These may be regarded as ideal mimetics, because they are non-peptide compounds and contain the functional groups necessary for the interaction of a native peptide with the corresponding protein (pharmacophoric groups) grafted onto a rigid scaffold.

The design of all three types of peptidomimetics may be assisted by X-ray crystallographic or nuclear magnetic resonance (NMR) data, computational *de novo* design ("*in silico* screening"), and combinatorial chemistry.

In peptide-based drug design it is most important to determine a specific peptide sequence with a high affinity for binding to a particular protein surface. Solving a peptide binding problem involves finding a region on the protein surface suitable for peptide binding, finding the appropriate peptide for this region and peptide refinement to enable stable binding which is required for inhibition. When the bind-

ing surface is known, a peptide can be designed *de novo*. In other cases, for a given peptide the region on the protein's surface with the optimum binding conditions should be searched. Petsalaki *et al.* [19] introduced a method based on a bioinformatics approach that could successfully find the binding sites for the peptides. A similar approach, the *de novo* molecular design computational tool ProLigand, was adopted by Frenkel et al [20]. Known peptides were docked to unknown locations on given proteins by Hetenyi and Spoel [21] using AutoDock. There have been successful attempts for computational peptide design that use knowledge-based search strategies and diverse sets of statistical descriptors, different training databases, hydrophobicity scales, and motif regularities, etc. [22]. Automated peptide binding search techniques from known epitopes or protein libraries have been successfully used as bioinformatics tools [23-25]. There are different computational binding tools such as the sequence moment concept, artificial neural networks, fuzzy neural networks and Hidden Markov Model for checking the suitability of inhibitory peptides for binding on MHC class II proteins [26]. The suitability of a ligand as a drug was tested using Bayesian neural network analysis [27]. The application of genetic algorithms for the design of peptides has been an important line of research, examples of which are: *in silico* peptide screening and the application of a genetic algorithm to determine an inhibitory peptide against the Parkinson's disease-related protein a-Synuclein [28]; peptides as thrombin inhibitors [29,30]; integer linear programming [31]; design of hexapeptides against stromelysin protein [32], and a peptide build up approach in combination with a genetic algorithm [33-35].

Therapeutic Approaches to Cancer Treatment

To date, the morbidity and mortality associated with cancer is second only to heart disease [36]. Although some types of cancers are clinically managed quite effectively with conventional therapy, the most common and life-threatening cancers such as lung, breast and prostate require development of more effective and curative treatments by clinicians and scientists. Surgery, where appropriate, remains a leading treatment for many cancers. Less invasive therapies have historically been divided into chemical and radiological treatments. However, the sometimes complicated and severe side-effects, associated with chemo- and radiation therapies, have prompted oncologists and cancer researchers to look for new approaches to cancer therapy. The genetic events associated with tumorigenesis involve the gain and loss of entire chromosomes, specific chromosomal translocations, gene amplifications, and deletions or point mutations [37-40]. These events can lead to increased activity of oncogenes and/or the loss of function of tumour suppressor genes and thus, contributing to tumor cells' growth and development [41]. For therapeutic purposes, it is most important to know which of these genetic alterations are rate-limiting and possibly reversible. The answer to these questions will determine which genes or gene products will become the most promising drug targets in the future [42].

Currently immunotherapy is at the forefront of experimental cancer therapies. This methodology utilises the power of the immune system and its focused ability to destroy cancer cells [36]. Cancer vaccine development is becoming more complex and challenging with each advance in the field. It ranges from molecular characterization of candidate vaccine antigens or peptides, to formulation of an optimal vaccine, and to administration and monitoring of such a vaccine in appropriately designed clinical trials [43]. Although many approaches based on the employment of immune cells or immune molecules to treat cancer have been and are being studied, active immunization stands out as the most promising methodology [44]. The idea of vaccinating to treat cancer, i.e. the administration of a therapeutic vaccine, is not new. For decades researchers and clinicians have studied and debated the possibility of vaccinating against cancer [44]. Only recently the focus of such debates has changed from pre-clinical proof-of-principle methodologies to what can define a tumor antigen and to ways of optimal vaccine delivery to tumor sites to induce anti-cancer immunity. Vaccines consisting of peptides derived from the protein sequence of a candidate tumor-associated or specific antigens represent the tip of the anti-cancer vaccine spear [36, 45]. To date, the vast majority of published pre-clinical studies have demonstrated the requirement of T-lymphocytes for the eradication of solid tumors. Cytotoxic T-lymphocytes (CTLs) or CD8+ T cells represent the primary cells involved in tumor-specific immune-mediated destruction of cancer cells. CTLs recognize, engage and destroy target cells through the tri-molecular interaction of the antigen-specific receptor (TCR) on the CTL and peptides that are presented by the target cell to the CTL in the context of class I major histocompatibility antigens (referred to in people as human leukocyte antigens or HLA) [36].

Insights into the genetic defects of cancer cells lead to new technologies being developed to extend and advance the application of current therapeutic approaches to cancer treatment [46]. These include the replacement of defective versions of tumour suppressor genes and the specific inhibition of inappropriately activated oncogenes. The spectrum of genetic tools used to interfere with the function of a given gene product includes antisense and siRNA, intracellular antibodies, dominant-negative proteins and RNA aptamers. Selected inhibitory peptides can also contribute to this spectrum. In a few model systems, peptides have already been used to manipulate crucial regulatory networks in cancer cells [47-53]. They can target specific intracellular proteins required by cancer cells for proliferation and invasion. Additional essential signalling components in cancer cells are being discovered and it has been shown that individual peptides can be derived to inhibit their function in a targeted fashion [54-56]. These peptides can be used for monotherapy or in combination with conventional chemotherapeutic agents. Since multiple pathways become dysfunctional when a tumor develops, and cancer cells accumulate oncogenic mutations as they progress, the greatest advancement can be achieved by combining therapeutic agents, which address different hallmarks of cancer. This concept, called "multi-focal signal modulation therapy" (MSMT), is a very promising approach and researchers have demonstrated that combinations of signal modulators achieve dramatic suppression of tumor growth [54-56].

ANTICANCER PEPTIDE THERAPY

Anticancer peptide therapy is an emerging field that uses bioactive therapeutic peptides (TPs) to kill cancer cells. In

the past 15 - 20 years, much effort has been directed to developing peptides capable of eliciting therapeutic responses in cells. Early work was pursued with the goal of using peptides as tools to probe the mechanisms and functional consequences of various protein–protein interactions, but it soon became apparent that peptides capable of mimicking or interfering with important intraprotein contacts could be useful as therapeutic molecules [1].

Peptide therapy has many promising characteristics:

- First, as opposed to small molecule drugs, peptides are easily designed to target almost any protein of interest using 'rational' methods. As the sequence, structure and interaction partners of many oncogenic proteins are known, peptides can be designed to inhibit these interactions by using a sequence from the interaction domain.
- Second, peptides are easily produced, and their sequence easily modified using chemical synthesis or molecular biology techniques. However, the utilisation of peptides for cancer therapy is limited at present by poor pharmacokinetic (PK) parameters and tumor deposition [57, 58]. When applied *in vivo*, peptides are rapidly degraded in circulation, and their relatively large size and often charged nature make them unable to penetrate cancer cell membranes. These limitations can be overcome through the use of non-natural amino acids or macromolecular carriers to enhance peptide stability and through the use of cell penetrating peptides (CPPs) to increase membrane permeability [1].

TPs can be grouped into three classes: (i) peptides that interfere with proliferative signal transduction cascades, (ii) peptides that arrest the cell cycle by modulating cyclin-dependent kinase activity and (iii) peptides that can directly induce apoptosis by modulating proteins that control apoptotic response [1]. There are several types of peptide therapeutics that are being currently investigated for cancer treatment: (i) peptide inhibitors of Ras activation; (ii) peptide inhibitors of MAP kinases; (iii) peptide inhibitors of NF-κB activation; (iv) peptides that affect p53 function; and (v) peptide inhibitors of c-Myc activation.

APPLICATION OF DIGITAL SIGNAL PROCESSING FOR COMPUTATIONAL ANALYSIS OF PROTEINS

Resonant Recognition Model

Small molecular weight peptides have been recently applied in developing cancer therapeutics, mostly for their ability to easily penetrate cellular membranes and to interfere with enzymatic functions or protein-protein interactions within cells [59]. In the development of such therapies the focus is on small peptides with strong tumoricidal activity and low toxicity. This therapy aims at obtaining high therapeutic indices on cancer cells and to minimize undesirable side effects on normal cells [60]. Computational approaches have grown in their effectiveness due to improved understanding of the basic science, biological events and molecular interactions that define a target for therapeutic intervention, and advances in mathematical algorithms [61]. It is generally recognized that the relationship between the structure and biological function of a protein and its ability to bind to a specific ligand, can be enunciated in terms of a multistage process which involves specific biorecognition, chemical binding and energy transfer. The Resonant Recognition Model (RRM) [62, 63] is one attempt to identify the selectivity of protein interactions within an amino acid sequence.

The RRM allows investigation of the periodicity of structural motifs with defined physicochemical characteristics, which determine biological properties of protein and DNA sequences. The RRM presents a physico-mathematical approach to the analysis of protein-protein and protein-DNA interactions. The RRM interprets a protein sequence's linear information using digital signal analysis [62-65]. It is assumed that proteins with the same biological function or interactive activity have the same periodic components in the distribution of delocalized electron energies along the protein molecule. This postulate is supported by the fact that electrons delocalized in a particular amino acid have the strongest impact on the electronic distribution of the whole protein sequence. The RRM is based on the findings that there is a significant correlation between spectra of the numerical presentation of amino acids and their biological activity [62, 63]. It was found that the RRM frequencies represent the characteristic features of different proteins' biological functions or interactions [62, 63]. It is proposed that these characteristic frequencies (RRM frequencies) are relevant parameters for mutual recognition between bio-molecules, and are significant in describing the selectivity of interactions between proteins and their substrates or targets but are not chemically binding [64, 65].

Bioactive Peptide Design Using the RRM

It is possible to determine the RRM characteristic frequency from analysis of the power spectra of proteins. In addition, from the analysis of their phase spectra we can identify the corresponding phase for a particular frequency. On the basis of determined RRM characteristic frequencies and phases for a particular group of protein sequences, we can design amino acid sequences (short peptides) having those specific characteristics related to a protein's biological function. It is expected that the designed peptide will exhibit the desired biological activity.

The strategy for design of such defined peptides is presented below:

1) The RRM characteristic frequency is determined from the multiple cross-spectral function for a group of protein sequences that share a common biological function (interaction).
2) The phases are calculated for the characteristic frequency or frequencies of a particular protein, which is selected as a parent for an agonist/antagonist.
3) The minimal length of the designed peptide is defined by the appropriate frequency resolution. An Inverse Fourier Transformation (IFT) is used to calculate a numerical sequence of different lengths, which exhibits the same prominent characteristic frequency as a parent protein.
4) To determine the amino acids that correspond to each element of the new numerical sequence defined above,

the tabulated Electron Ion Interaction Potential (EIIP) parameter values are used. The resulting new amino acid sequence represents the anticipated designed peptide [62, 66].

In previous studies the RRM approach was applied to structure-function analysis of basic fibroblast growth factor (bFGF) [67]. Property-pattern characteristics for biological activity and receptor recognition for a group of FGF-related proteins were defined and then used to aid the design of a set of peptides which can act as bFGF antagonists. Molecular modelling techniques were then employed to identify the peptide within this set with the greatest conformational similarity to the putative receptor domain of bFGF. The 16 mer peptide, which exhibits no sequence homology to bFGF, antagonised the stimulatory effect of bFGF on fibroblast thymidine incorporation and cell proliferation, but exerted no effect itself in these *in vitro* bioassays [67].

The RRM was also successfully applied for the analysis of HIV envelope proteins. The interaction between HIV virus envelope proteins and CD4 cell surface antigen has a central role in the process of virus entry into the host cell. Thus, blocking the interaction between the envelope glycoproteins and CD4 surface antigen, known to be the HIV receptor, should inhibit infection [68]. For this purpose, six peptides, each of 20 amino acids in length, were designed using the RRM methodology. To validate the RRM computational predictions, the activities of the designed peptides were evaluated experimentally. These investigations were performed initially by evaluating the reactivity and cross-reactivity of all designed peptides with their corresponding antibodies [68]. The results obtained showed significant cross-reactivity to the polyclonal antibodies raised against peptides that share at least one characteristic frequency and phase at this frequency. The results provided an experimental confirmation of the concept that RRM frequency characteristics present important parameters associated with biomolecular recognition and in particular, the antibody-antigen recognition.

Design of Peptide Analogue with Anti Cancer Activity

Interleukin-12 (IL12) is a key regulator of cell-mediated immunity that has therapeutic potential in cancer and infectious diseases [69]. It has an essential role in the interaction between the innate and adaptive arms of immunity by regulating inflammatory responses, innate resistance to infection, and adaptive immunity. IL12 can utilize the effects or mechanisms of both innate resistance and adaptive immunity to mediate anti-tumor resistance [70]. The potent *in vivo* antitumor and antimetastatic effects of IL12 against murine tumors were reported in the 1990s [71]. These findings were followed by other studies on experimental tumor models, which concluded that recombinant IL12 treatment has a dramatic anti-tumor effect on transplantable and chemically induced tumors and in tumors arising spontaneously in genetically modified mice [72].

IL12 has been shown to have potent antitumor effects in murine models of melanoma, sarcoma, kidney cancer, lung cancer, colon cancer and ovarian cancer [73-63]. Systemic or peritumoral injection of IL12 can induce complete regression of established tumors, inhibit the formation of distant metastases and substantially prolong the survival of tumor-bearing mice. These studies have identified doses of IL12 that can induce impressive tumor responses without causing overt toxicity. In some tumor models, mice that had experienced complete responses after IL12 therapy were subsequently able to reject implants of the same tumor, but not of a different tumor, suggesting that specific antitumor immunity had been established [73-76]. In models of colon cancer, ovarian cancer, lung cancer, renal cell cancer and melanoma, IL12 was found to be more effective and/or less toxic than IL2 [73-76]. Moreover, a combination of IL2 and IL12 was more effective than either cytokine alone in models of primary and metastatic renal cell cancer [73]. IL12 can also augment the graft versus- tumor effect of bone marrow transplantation without promoting graft-versus-host disease [75, 76]. The mechanisms underlying the antitumor activity of IL12 are likely to be complex and have not been fully elucidated [70].

Computational Analysis and Peptide Design Using the RRM

Here the RRM approach was employed for structure-function analysis of IL12 proteins and the computational design of a short therapeutic peptide having IL12-like activity. Its toxic anti-tumor effect was then validated experimentally. Thirteen IL12 proteins from different origins were analysed using the RRM. The characteristic RRM frequency (most prominent) was identified at f_{RRM}=0.4531 Fig. (**1**). According to the RRM concepts this prominent peak characterises the common biological activity of analysed MV proteins. Less prominent peaks observed in Fig. (**1**) confirm that these selected IL12 proteins can be involved in different biological processes (interact with other proteins). Mouse IL12 sequence (NP_032377, 215 aa, Entrez Protein Database) was selected as a parent protein to design a short bioactive peptide having IL12-like activity. The RRM-IL12 (sequence AREDLDERAQQKREDLDP for an 18 aa linear peptide. The length of the designed peptide is defined using the ration $L=1/f_{RRM}$) was designed with the frequency f_{RRM}=0.4531 and phase φ=3.069. The synthetic peptide sequence has the following characteristics: a molecular weight of 2.184 kDa' theoretical pI of 4.39 and an estimated half-life of 4.4hr in mammalian reticulocytes.

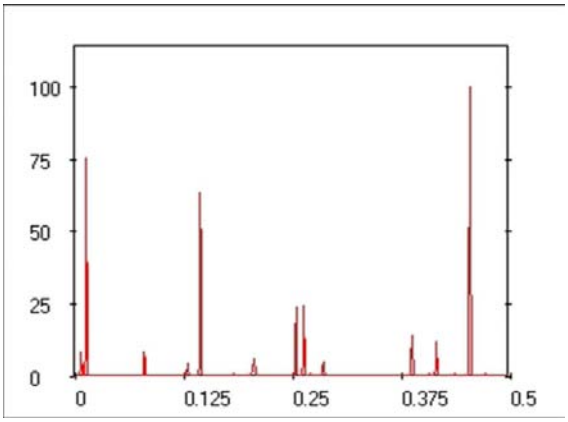

Fig. (1). Multiple cross-spectral function of 13 mammalian IL12 protein sequences. The prominent peak(s) denote common frequency components. The abscissa represents the RRM frequencies, and the ordinate is the normalized intensity.

A similar procedure was used to design the negative control peptide analogue (RRM-C), which has a different "inactive" frequency and phase (f_C=0.2, φ_C=1.5) and does not express IL12 activity. The 22 amino acids linear peptide CVLQDCVLQDCVIQDCVLQDCV was designed as a negative control for biological cytotoxicity assays (molecular weight of 2.454kDa, theoretical pI of 3.32, and estimated half-life of 1.2hr).

Biological Examination of the RRM-IL12 Peptide Analogue

The cytotoxic effects of RRM-IL12 on mouse cancer and normal cells were evaluated on the following adherent and semi adherent cell lines: (a) a mouse melanoma (B16F0) cancer cell line; (b) a non-transformed, normal mouse skin fibroblast primary cell culture; (c) Chinese Hamester Ovary (CHO), a normal transformed cell line, and (d) the semi-adherant mouse macrophage cell line (J774). The cell cultures were maintained in complete Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat-inactivated foetal bovine serum at 37°C in 10% CO_2. The evaluation of cytotoxic effects was performed on 95% confluent cell cultures by incubation with various concentrations of RRM-IL12 or RRM-C (100ng/ml-1600ng/ml) with the bioactive peptide RRM-IL12 or the negative control RRM-C as explained below.

a) Phase Contrast Microscopy

One of the parameters used to evaluate the cytotoxicity of RRM-IL12 and RRM-C on mouse cancer and normal cell lines is the detachment of a large number of cells from the adherent confluent layer of treated cell cultures versus the untreated cultures. This was performed by phase contrast microscopy Fig. (**2**). Similar concentrations of the total protein ova albumin (OA) were also used as a second negative control. The cultures were checked every three hours by phase contrast microscopy to detect the initiation of cellular morphological changes (cell shape, membrane blebs etc.) and detachment of the 95% confluent layer. Cellular detachment of the adherent cell culture was detected in B16F0 melanoma cell culture after three hours of incubation. These had the highest concentrations of RRM-IL12 peptide analogue (1600ng/ml) Fig. (**2A**) when compared with the non-treated culture Fig. (**2D**). Interestingly, even after sixteen hours of treatment of normal cell cultures (mouse skin fibroblasts, mouse macrophages J774 and CHO cell line), with up to 1600ng/ml of the bioactive peptide RRM-IL12, no morphological changes or detachment were detected (data not shown). Furthermore, there was no detachment in normal and cancer cultures treated with the negative control peptide RRM-C or with the OA protein at any concentration within sixteen hours of incubation, Fig. (**2B** and **2C**).

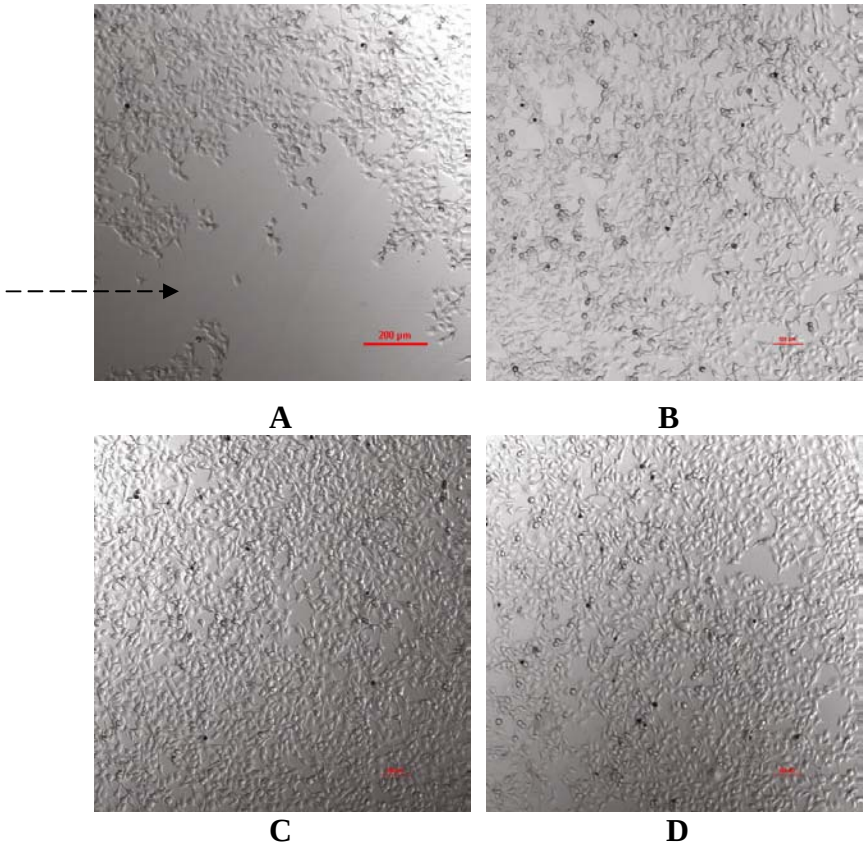

Fig. (2). Phase contrast micrographs with 40X magnification for B16F0 cell cultures treated with 800ng/ml of RRM-IL12 (A); RRM-C (B), or OA (C) for three hours. The dashed black arrow in A indicates the detachment of the cellular layer and loss of adherent cells in a culture treated with RRM-IL, while there was no detachments and loss of cells in cultures treated with similar concentrations of RRM-C or OA (B&C) as compared with the non treated cell cultures in (D).

b) Detection of Cytotoxicity by Confocal Immunofluorescence Microscopy

Cellular cytotoxicity in normal and cancer cells was assessed by detection of apoptosis and necrosis using a Vybrant Apoptosis Assay kit which contains annexin V-Alexa Fluor 488 conjugate (AF) and propidium iodide (PI). Cells in DMEM only were treated with selected concentrations of the peptide analogues at 37°C for 3h. After treatment, cells were washed once with ice-cold 1× PBS and labelled with annexin V-AF 488 and PI according to the manufacturer's instructions. Confocal laser scanning microscopy (CLSM) imaging of cells was carried out with a Nikon Eclipse Ti-E A1 laser-scanning confocal system (Nikon Instruments Inc, USA), using the 10×, 20× and 40× objectives. In order to compare the extent of apoptosis between treatments, the pinhole aperture and other settings were fixed. Cell images captured were analysed with the NIS-Element imaging software.

Evaluation of results for the *in vitro* assays via CLSM was done through the assessment of the effects of the different treatments on normal and cancer cells. The assessment included the determination of the difference in the number of apoptotic cells (green fluorescence) and necrotic cells (red fluorescence) between treated and non treated cultures within the same cell line and between treated cultures from different cell lines (normal *vs* cancer cells). Our results revealed that the treatment of the B16F0 mouse melanoma cells with RRM-IL12 peptide analogue induced both cellular apoptosis and necrosis (cell death) in the cancer cells as compared with the non treated B16F0 cell culture and also with the B16F0 cells treated with the negative control peptide RRM-C Fig. (**3A**).

The effect of the RRM-IL and RRM-C on normal cell lines was also assessed Fig. (**3B; 3C**; and **3D**). The cytotoxic effects (apoptosis and necrosis) of the RRM-IL on the normal cell lines were minimal when treated and non-treated cultures from the same cell line were compared. It was clearly shown that the bioactive peptide analogue RRM-IL12 has selectively induced apoptosis and necrosis on cancer cells while it has a negligible effect on normal cells Fig. (**3C, 3D**).

c) Quantitative LDH Assay

The cell cytotoxicity of the RRM-IL12 and RRM-C treated cultures was quantitatively assessed by measuring the release of cytoplasmic lactate dehydrogenase (LDH) into cell culture supernatants. Cell cultures were seeded and grown as previously indicated and then incubated with specific concentrations of the peptide analogues (800ng/ml and 1600ng/ml) at 37°C for 3h. LDH activity was evaluated using the Cytotoxicity Detection Kit (Roche Diagnostics). Experiments were performed in triplicates with three repeats within each experiment. According to the manufacturer's instructions, the percentage of cell cytotoxicity was calculated using the following formula: 100×[(experimental LDH release - spontaneous LDH release)/(maximum LDH release - spontaneous LDH release). The statistical analysis on the cytotoxicity data was conducted with one-way ANOVA and Dunnett's test, which compares the means of all treatments with a designated control (negative control peptide or untreated cells).

The values of the LDH assay indicated that the bioactive peptide RRM-IL12 has a significant cytotoxic effect on the mouse melanoma cell line (B16-F0), inducing high LDH release and cytotoxicity, when compared to both untreated (blank) and RRM-C -treated cells Fig. (**4**). RRM-IL12 treatment produced no cytotoxic effect on both the mouse macrophage J744 and CHO cell lines when compared with the non-treated cultures Fig. (**4**). On the other hand, no cytotoxic effects were detected in cancer or in normal cell cultures treated with the non bioactive peptide, RRM-C. Interestingly, the percentages of cellular cytotoxicity measured for this assay in all three cancer and normal cell lines incubated with RRM-C, were less than the percentages of cytotoxicity in non-treated cultures. This needs further investigation as it is possible that RRM-C may affect the cellular proliferation rate in these cell cultures, leading to cell survival. The LDH cytotoxicity data Fig. (**4**) substantiates the observations seen in the CLSM micrographs Fig. (**3**), where levels of LDH release from RRM-IL12-treated mouse melanoma cells corresponded to the intensity of cellular apoptosis and necrosis detected by CLSM in these cells. Our experimental results clearly indicate that significant cytotoxic effects due to the RRM-IL12 peptide analogue only occurred for cancer cells, while cytotoxicity effects could not be detected in normal cell lines treated with RRM-IL12.

CONCLUSION

This paper presents a review of current advances and novel approaches in experimental and computational drug discovery, design and development. The reviewed literature demonstrates that therapeutic peptides can be developed for the inhibition or reactivation of a huge variety of important signaling molecules. Furthermore, these peptides can be very specific with regard to their target proteins and in some cases can also be specific with regard to the cancerous cell types. As knowledge grows about the proteins involved in tumor cell development, peptides will be the first available inhibitors for therapy of the newly discovered target proteins. Therefore, owing to their ease of design and production and wide spectrum of potential targets, therapeutic peptides have a promising future in cancer therapy.

Therapeutic peptides have many attributes that make them attractive as drugs for cancer therapy. First, because so much is known about the sequence and structure of interacting proteins, the rational design of therapeutic peptides to inhibit interactions of interest is relatively easy, and certainly much easier than designing small molecules to inhibit the same interactions. This gives drug developers access to inhibitors of many important protein–protein interactions to which small molecule inhibitors are not available. Furthermore, because therapeutic peptides can be very specific, this can reduce the likelihood of "off-target" effects. However, so far, the use of peptides for cancer treatment has been limited due to their poor performance pharmacologically. Limitations of stability in plasma, bioavailability and tumor cell penetration have prevented the advance of peptides beyond preclinical testing. Therefore, the key issue for the development of this new class of drugs is not finding new therapeutic peptides, but finding new ways for their delivery to tumor cells (tumor sites for *in vivo* validation) [31].

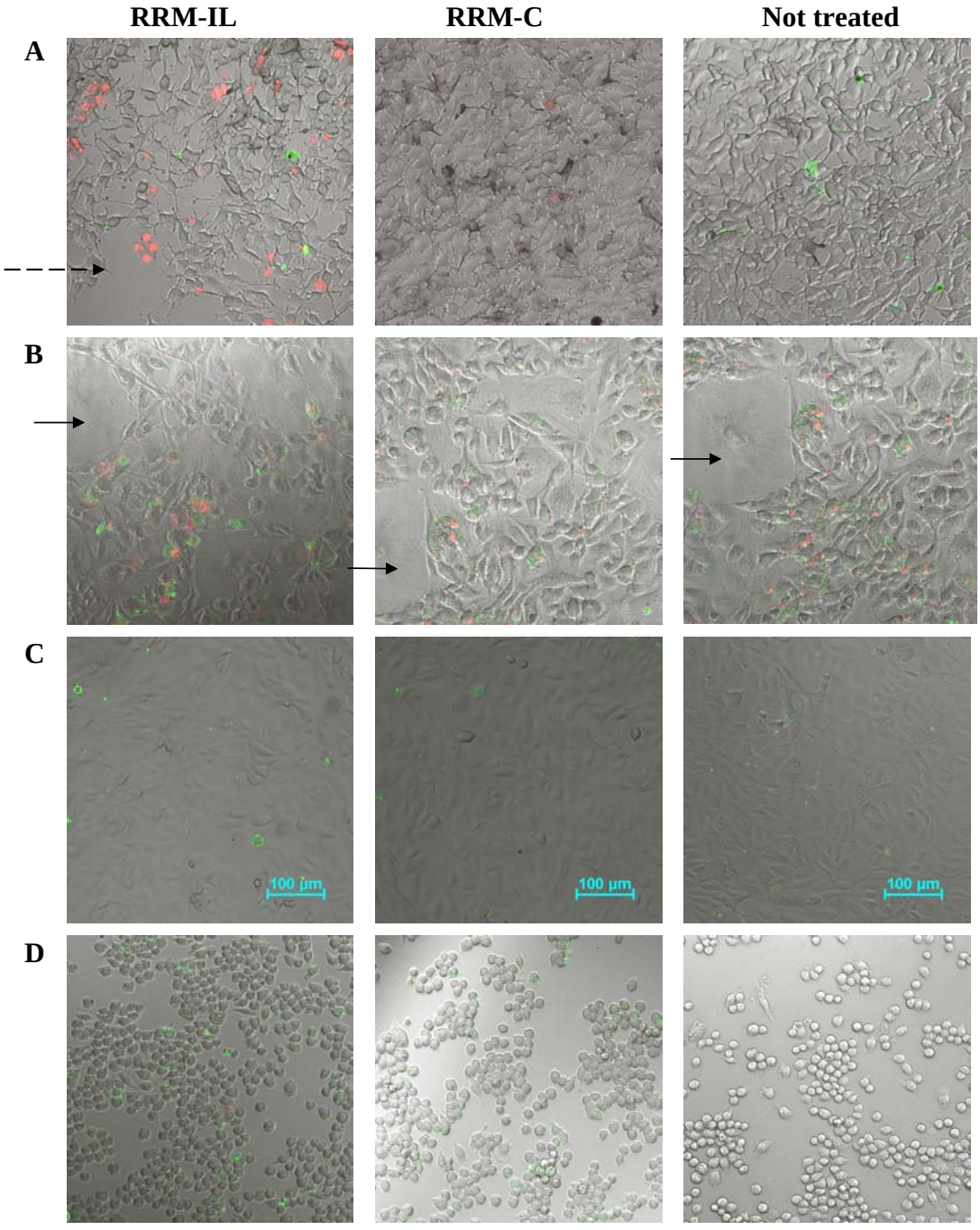

Fig. (3). Effects of RRM-IL12 and RRM-C treatment on mouse melanoma B16F0 cell line in A, normal mouse skin primary cell culture in B, CHO cell line in C, and mouse macrophages J774 cell line in D. The cell lines were incubated with 800ng/ml of the peptide analogues for three hours. The non- treated cell cultures were similarly processed. Cellular apoptosis (green cells) and necrosis (red cells) were detected by Vybrant Apoptosis Assay kit (invitrogen), which contains annexin V-Alexa Fluor 488 (AF488) conjugate and propidium iodide (PI) and viewed by Confocal Laser Scanning Microscopy (CLSM), with 100x magnification. The abundance of apoptotic and necrotic cells is clear in the mouse melanoma cell line B16F0 (in A) treated with RRM-IL12 as compared with the negative control RRM-C and the non treated cell culture. The dashed black arrow in A indicates the detachment of the confluent layer due to cell death, while the solid black arrows in B indicate another form of cells in this primary cell culture. Cellular changes or detachment were minimal in the three normal cell lines (B, C & D) as the number of apoptotic and necrotic cells in the treated and non treated cultures were not significantly different.

In this paper we also discussed the use of de novo designed peptides for cancer therapy using the example of the IL-12-like short peptide, which was computationally designed using the RRM approach. Experimental evaluation of the designed peptide's efficacy for cancer treatment has been undertaken on B16F0 melanoma cancer and normal mouse

cell lines. The results obtained indicated that the cytotoxic effects of the computationally designed RRM-IL12 peptide analogue were directed against cancer cells leading to cellular necrosis, yet it has negligible effects on normal cells. Furthermore, the findings revealed that the effects of the non-bioactive control peptide RRM-C were minimal on both normal and cancer cells. The outcomes of our experimental validation are encouraging and lead to the conclusion that bioactive RRM designed peptide analogues should be further investigated as potential cancer therapeutics.

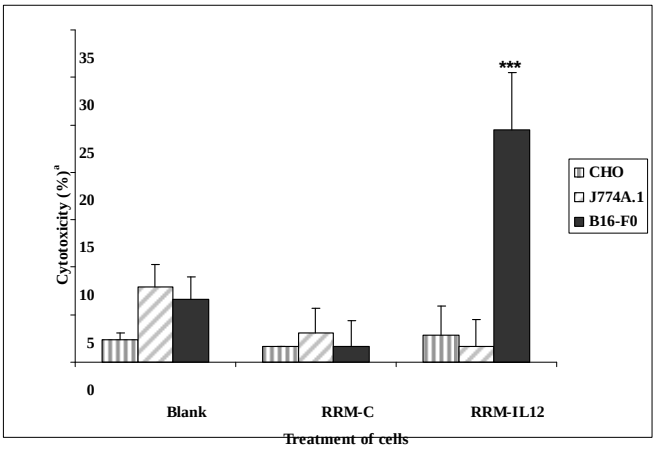

Fig. (4). Effect of RRM designed peptide analogues on cells by LDH cytotoxicity assay. Cells were incubated without treatment (blank), with negative control peptide (RRM-C) and IL-12 bioactive peptide (RRM-IL12) for 3h at 1.6μg/mL. Each bar represents the mean ± standard errors for three separate experiments done in triplicate. Data values that are significantly altered (***) when compared to the negative control (RRM-C) and when compared to untreated cells (Blank) at a significant level of $p < 0.05$ (ANOVA and Dunnett's post-hoc analysis)

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