SHORT HISTORICAL REVIEW

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The practical utility of synthetic tumor suppressor peptides: a personal retrospective on 25 years

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Abstract

In this brief survey, I look back upon a quarter of a century of my personal experience with synthetic peptides. Thereby, I focus on major steps in the design and experimental exploration of peptides that I have derived from the retinoblastoma tumor suppressor protein (RB). Along this way, both Merrifield's solid phase peptide synthesis method and collaborations with established investigators in the peptide and cancer research fields have played an important role.

Keywords: synthetic peptide, tumor suppressor, retinoblastoma protein (RB), cancer therapy, review.

The preparatory years

It was in 1992 when I first came across Gregory Grant's book entitled "Synthetic Peptides" that had been published that year [1]. I had been interested in these compounds since the clinical part of my medical studies in the late 1980s, but this book certainly inspired me further. Even more importantly, it contributed to my decision to devote myself to the research and development of therapeutic peptides in the subsequent years.

There were two passages from that book that particularly stimulated me. On the one hand, it was Theodor Wieland's motto initially stated in a 1966 publication and preceding the overview chapter of the above-mentioned book: "...every synthesis of a complicated polypeptide represents the sum of prolonged intellectual and experimental exertions which, however, often find their reward in the interesting physical, chemical and biological properties of the final product". And, on the other hand, the reference by Grant within the same introductory part of his book to the work of the synthetic peptide research pioneers Vincent du Vigneaud (1901–1978) and Bruce Merrifield (1921–2006) for which he again cited Wieland with a remarkable metaphor, specifically "...the synthesis of glutathione opened the door to peptide synthesis a crack, and the synthesis of oxytocin pushed the door wide open...", which he then extended by remarking that "...the introduction of the solid-phase method blew the door off the hinges".

As I mentioned earlier, my interest for peptides had begun several years before. Specifically, in the course of my medical studies at the University of Münster/Westfalen in the second half of the 1980s, I was keen to learn more about immunology and especially neuroimmuno-endocrinology.

In the context of this area – dealing with the bidirectional communication between the nervous and immune systems through circulating peptide hormones and rapidly evolving at that time – it was in particular the endogenous opioid β-endorphin, which had caught my attention. The sequence of β-endorphin, a 31-amino acid neuroendocrine peptide hormone, had been reported by Choh Hao Li's group at UCSF in 1976 [2]. This molecule was initially shown to be involved in the mediation of endogenous analgesia [3]. Later on, it became clear that it has various effects on several important components of the immune system such as stimulating the activity of NK cells [4], which participate in the body's protection against the development of cancer.

Due to this interesting role, I had considered already in the 3rd year of medical school in 1988 to find a team of clinical investigators with whom I could organize a preliminary clinical trial building upon this observation, as a potential part of my doctoral thesis. Unfortunately, however, this plan did not pan out since we could not find a supplier for pure β-endorphin in quantities that would have been sufficient to conduct such a trial. Thus, I had to defer my therapeutic peptide ambitions for at least four years, *i.e.*, after I finished both my medical studies and my doctoral thesis in 1992.

Tumor suppressor peptide research and development

At that time, I came across Grant's above-mentioned book on synthetic peptides. This was serendipitous since it coincided with a period in which I had begun to thoroughly think about developing anti-cancer peptides whose structure I was planning to derive from my analysis of the crucial retinoblastoma tumor suppressor protein (RB) that I had chosen to focus on after becoming aware of its outstanding importance through the 1990 *Science* article by Bookstein *et al.* [5] and Robert Weinberg's review on tumor suppressors that appeared in the same journal one year later [6]. Specifically, I was then fortunate to identify such a potential peptide structure, more precisely the RB hexapeptide LFYKKV, which then led in 1992 to two publications in the prestigious *Journal of Molecular Recognition* [7, 8] that had among its distinguished editorial board members two Nobel laureates, Chris

Anfinsen (1916–1995) and Jean-Marie Lehn. Yet it took me another three years to conceive, based on this initial RB amino acid sequence, an expanded synthetic peptide and its more protease-resistant variants that could then be proven in the second half of the 1990s to exert significant antineoplastic activity both *in vitro* and *in vivo*.

Along this way, I initially teamed up with the Italian synthetic peptide expert Giorgio Fassina. During the course of this collaboration, we could experimentally demonstrate the recognition of the LXCXE RB-binding motif – present in insulin (as I had previously uncovered and published as part of one of the above-mentioned *Journal of Molecular Recognition* papers) and in the E7 oncoprotein of human papilloma virus (HPV) 16 – by my above-specified RB hexapeptide [9]. From Giorgio I have learned that, for the purpose of binding studies, it would be particularly useful to synthesize the RB peptide in a *branched* tetrameric form as a so-called multiple antigenic peptide (MAP).

One of these MAPs was revealed to have anti-cancer activity *in vitro*, as I could discover in 1996 [10–13], but, for practical purposes, its synthesis appeared rather complicated in view of potential clinical applications and, moreover, since RB is an intracellular tumor suppressor, I had to find a way to also ensure such intracellular localization for my RB hexapeptide. I therefore coupled it to a so-called nuclear localization sequence (NLS) such as that derived from the Antennapedia protein. Such obtained *linear* two-component polypeptide was then proven to inhibit cell cycle progression in a variety of human cancer cells *in vitro* [10–13]. In this context, I should note that I sequentially discovered that the RB hexapeptide by itself did not have antineoplastic activity (nor did the above-specified NLS alone have such effect) and, moreover, the (relatively protease-resistant) all-D two-component polypeptide was significantly more active than its (comparatively rather protease-sensitive) all-L variant. After having presented this work of mine at an international cell cycle meeting that took place at the Garda Lake in the spring of 1997, it was interesting to find out from David Lane, one of the discoverers of the p53 tumor suppressor, that there had previously been attempts to develop LXCXE motif antagonists, yet without fruition.

In the second half of 1997, I was then able to demonstrate for the first time *in vivo* activity for my RB peptides in a syngeneic mouse tumor model [14]. When I presented this work in Amsterdam, at an NCI/EORTC meeting the next year, this drew the attention, among several established investigators, of Judah Folkman (1933–2008), who was a tumor angiogenesis pioneer and, moreover, had appreciated my work published in the *Journal of Endocrinology* in 1993 on the importance of (intracellular) hormone and growth factor subunits for cellular signal transduction (cf. *Addendum* to this review) ever since this commentary had been published [15].

In retrospect, I realize that the steady encouragement by both Judah Folkman (Figure 1a) and Irwin Chaiken, the *Journal of Molecular Recognition* Editor-in-Chief (Figure 1b), were crucial for me in these early years of my research career. Although I did not have the privilege to personally meet Vincent du Vigneaud and Bruce Merrifield, I think that, based on what I have read of them, they had the same kind of supportive and generous attitude towards their younger colleagues once they had recognized their talent.

(a)

(b)

Figure 1 – ***(a) Judah Folkman and me in Amsterdam, 1998. (b) Myself and Irwin Chaiken in Munich, 2002.***

In the second half of 1998, I then teamed up with Gabriele Jaques from the University of Marburg who was an expert on the experimental study of human lung cancer cells. By that time, we were already looking back upon a successful two-year collaboration in the period 1995–1997 on the demonstration of the nuclear localization of the IGFBP-3 protein [16] that I had predicted based on my structural analysis of this molecule in 1994 [17].

In late October 1998, we obtained the first results that showed significant antineoplastic effects of my peptides against various human lung cancer cells *in vitro*. In the summer of the next year, I wrote up the initial manuscript version on these data that, further to some modifications, was published a few months later [18]. In this context, a truly defining moment was on October 9, 1999 when we could ascertain that my fluorescently labeled MCR-4 peptide enters both the cytosols and nuclei of human lung cancer cells, as I had envisaged by its design for this NLS-carrying synthetic peptide. Subsequently, MCR-4 could also be shown to be active *in vivo* against both NSCLC [19] and SCLC [20] cells.

Together with Kai Kehe – who had been my colleague at the Walther-Straub-Institute of Pharmacology and Toxicology of the Ludwig-Maximilians-University in Munich during the time period 1999–2002, which encompassed our proof (along with Harald Mückter) of the physical interaction between insulin and RB and its disruption by the MCR peptide MCR-10 in human hepatoma cells [21] – I could then elucidate several mechanisms of action for MCR-4, mainly its activation of the pro-apoptotic p21 cyclin-dependent kinase (cdk) inhibitor protein [22] and its inhibition of the complex formation between insulin and RB [23] in human lung cancer cells.

More recently, I have underscored the potential of these particularly useful peptides both in my *PNAS* Letter to the Editor [24] and in a joint article [25] with Robin Fåhraeus who had worked with David Lane in the mid-1990s on antiproliferative synthetic peptides derived from cdk inhibitors such as p16 [26].

In conclusion, I remain confident that a golden era of peptides [27] will return soon. As Günter Blobel, who has received the Nobel Prize for his discovery of the signal peptide sequence, once told me, the time for peptides should come (again) in the foreseeable future (after other directions such as gene therapy and stem cells will have been tried out) and therefore one ought to be patient. Yet, the considerable potential of my RB-derived MCR peptides resulting from their successfully completed proof-of-concept stage both *in vitro* and *in vivo* as well as the global responsibility for improving the treatment of cancer patients warrants their accelerated exploration as part of clinical trials.

Acknowledgments

I dedicate this work to my parents, and thereby especially to my mother who has supported and encouraged me in an invaluable way all along during my research.

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Addendum

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To Whom it May Concern:

I am happy to write on behalf of Dr. Razvan Radulescu and to support his concepts on subunits and nucleocrine interactions between growth factors and tumor suppression.

I have read his paper in the Journal of Endocrinology entitled, "Hormone and Growth Factor Subunits: A Novel Perception of Cell Growth Regulation." The paper proposes a novel hypothesis and provides a considerable amount of supporting evidence.

I heard Dr. Radulescu's presentation at the recent NCI-EORTC Symposium in Amsterdam on antineoplastic peptides. I discussed with him the need that he pursue the development of his unique ideas. The autocrine intracellular mechanism of action of growth factors and their fragments may be particularly applicable to neoplastic transformation. For example, Michael Klagsbrun in my laboratory showed that in normal cells transfected with basic fibroblast growth factor (bFGF) fused to a signal peptide that neoplastic transformation occurred. However, while the experiment was designed to cause the secretion of bFGF, most of the bFGF was not secreted but remained intracellular and led to the malignant transformation (Rogelj S, Weinberg RA, Fanning P, Klagsbrun M. *Nature* 331:173-175, 1988). There has not been a satisfactory explanation for this phenomenon until Dr. Radulescu's 1993 paper in the *Journal of Endocrinology*.

I believe that Dr. Radulescu's hypothesis provides an important new direction to our understanding of the role of growth factors as mediators of oncogenesis. These ideas have potential for the development of anti-cancer drugs and certainly deserve support so that this fundamental work can be continued.

Sincerely yours,

Judah Folkman

Judah Folkman, M.D.

1998 Judah Folkman evaluation of my research work.

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