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What to Synthesize? From Emil Fischer to Peptidomics

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Abstract: The driving forces, incentives and strategic targets of peptide synthesis have undergone considerable evolution during the centenary following the pioneer work of Emil Fischer. In those days peptide synthesis was considered as a way of confirming the polypeptide theory of protein structure. The scientific community also expected (naively) that the synthesis would eventually lead to the creation of artificial living organisms. Only in the 1950s, when the first exact amino acid sequences were established did peptide chemistry obtain firmer ground and clearly defined targets. The total synthesis of peptide hormones and antibiotics became possible, providing valuable material for elucidating structure–functional relationships and the mechanisms of biological action. In the following years the number of peptides isolated from various biological sources grew with impressive speed and peptides became known as the most abundant, ubiquitous group of low molecular bioregulators. The design and synthesis of novel peptide based pharmaceuticals became an important area of peptide chemistry. At present we are facing the challenge of analysing the structures and bioactivities of total sets of peptides, i.e. peptidoms, present in concrete tissues or groups of cells. The results obtained along these lines at the IBCH RAS Institute of Bioorganic Chemistry are briefly considered in the review.

Keywords: peptide synthesis; biological activity; structure–function relationship; tissue specific peptide pool; peptidomics

For the first 50 years after Emil Fischer formulated his polypeptide theory of protein structure, peptide synthesis remained a purely academic exercise. The aim was to obtain protein-like material, thereby confirming the theory. By 1907, Fischer and his collaborators had synthesized about a hundred peptides of various lengths including a record 18-membered long peptide. Some properties of these preparations, in particular, their susceptibility to proteolytic enzymes indeed resembled those of genuine proteins [1,2]. Under the illusion of these results the more optimistic of Fischer's contemporaries began considering the chances of making artificial food and even artificial life. For example, as Fischer described in his autobiography, the famous naturalist Ernst Hekkel in private conversation expressed the hope that 'Whe Ihr, Chemiker das richtige Eiweiss macht, dann crabbelts!', which means: 'When you, chemists make the right protein it will swarm!' [3].

Leaving aside all these exaggerations, one should admit that for that time the result obtained was truly remarkable, reaching the limits of synthetic organic chemistry at the time. Some 9 years later E. Abderhalden, a pupil of Fischer extended the length of the synthetic peptide by one amino acid unit [4]. However, the ambitious goal set by the great chemist, i.e. the total synthesis of an authentic protein was still a long way ahead. Even the basic task could not be properly formulated, in the absence of reliable synthetic methods. For that to happen the chemical formulae of the target molecules had to be established.

It took several decades for analytical biochemistry to mature to the point of successfully tackling the problem. Initially, the progress was quite slow. For a long time there were no reliable methods

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for separating complex biological mixtures into individual components. There were only very limited approaches to the reconstruction of structural formula after chemical degradation of natural products. The isolated success stories of that time, such as the structure elucidation of glutathione (1930) [5–7] and gramicidin S (1947) [8–10] and even the synthesis of glutathione (1935) [11–12] only underlined the complexity of the task.

In 1953 V. du Vigneaud [13,14], Tuppy [15], Acher [16] and in 1956 Sanger and Smith [17,18] made their landmark contributions, elucidating the total structures of bovine oxytocin, vasopressins and insulin. Oxytocin and Lys-8 vasopressin were soon synthesized in the du Vigneaud laboratory [19,20]. After that starting point the structure elucidation of naturally occurring peptides continued at an increasing rate which in the 1970s reached the scale of an avalanche.

According to their biosynthetic origin, natural peptides are divided into two classes: (i) peptides formed in a variety of (in fact, in any) organisms from biologically inactive protein precursors by post-translational modification (primarily proteolysis but also amidation, *N*-terminal acylation, epimerization of L-amino acids, etc.) and (ii) produced in lower organisms (bacteria, actinomycetes, fungi) by direct synthesis from amino acid or hydroxy acid building blocks with the help of specialized synthetases. In this case unusual amino acids, not coded by the nucleotide triplet code, are often employed. The overall number of peptides of the first group in the modern data banks has passed the 4000 mark and continues a rapid growth. That multitude is made up of over 600 structural families ranging in size from one to several hundred peptides [21]. There are no exact data on the size of the second group, however, rough estimates clearly show that it is more numerous than the first one.

Generally, peptides are the most abundant, ubiquitous class of low molecular weight bioregulators. They participate in a vast number of biological processes. In fact, there is hardly a biochemical process that is not, or could not be, influenced by a peptide. As a result, the immense biological potential of peptides attracted the unremitting attention of academic researchers and industry to the chemistry and biology of peptides.

Starting with the work of du Vigneaud and Sanger, a new level of target setting for peptide synthesis became possible compared with the time of E. Fischer. The present view of principal objectives and strategic targets of peptide synthesis is summarized in Figure 1.

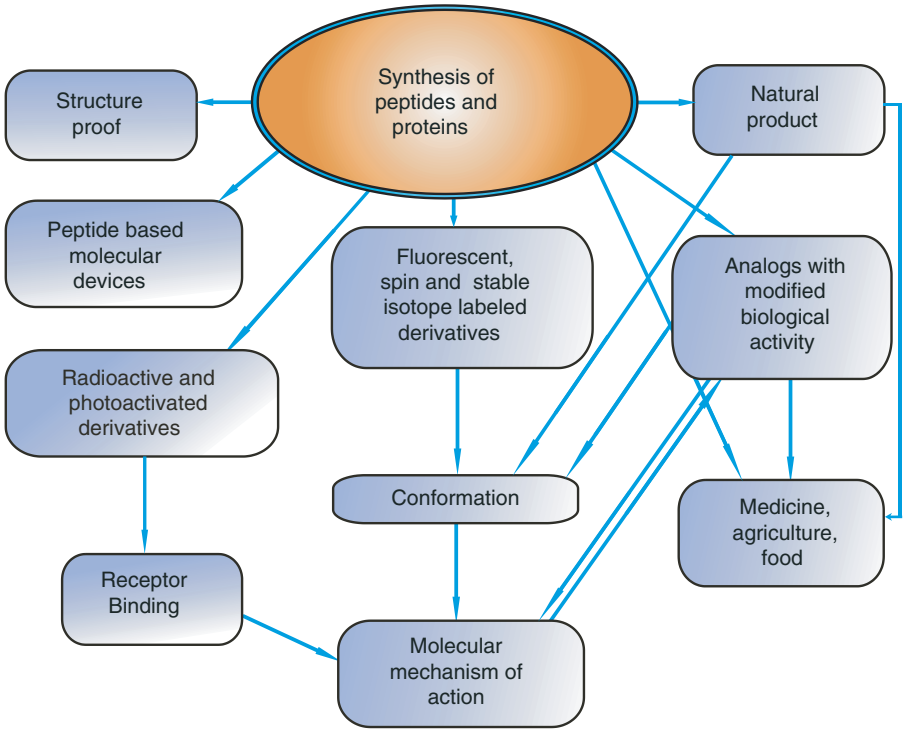

Figure 1 The world of peptide synthesis.

Initially, when the structural analysis of peptides still remained a formidable challenge, total synthesis was considered as an essential step for the final acceptance of the proposed chemical formula. If synthesis resulted in material not identical to the natural sample, the reason for the discrepancy had to be elucidated, and in several cases it was the inaccuracy of the initial formula.

The story of the cyclic depsipeptide antibiotics valinomycin and enniatins A and B serves as a typical example (see [22] and references therein). The respective compounds with structures taken from earlier literature were synthesized in the 1960s in the laboratories of M. Shemyakin and Yu. Ovchinnikov of this Institute which now carries their names. The products obtained were totally inactive and their physico-chemical constants differed considerably from the expected values. The following study which culminated in the total synthesis of all three antibiotics showed that the correct structures differed from the initial ones in having 50% larger molecular masses. At present, with highly developed mass-spectrometry, NMR-spectrometry, x-ray analysis and other spectroscopic techniques total synthesis is no longer considered a necessity for structural proof.

The main effort of synthetic chemists has been and is being spent on the preparation of naturally occurring peptides and their analogues which are employed for a number of purposes: elucidation of structure–functional relationship, analysis of the molecular mechanism of action and, of course, practical applications in human and veterinary medicine. Each newly discovered peptide with interesting biological properties becomes an object of synthesis which is often carried out by standard methods and is not considered as a difficult hurdle. Despite notable exceptions, such as insulin and oxytocin, the natural peptides themselves (especially those derived from protein precursors) often have a number of drawbacks as pharmaceuticals. They are rapidly degraded by proteases and therefore usually cannot be taken orally and are too short-lived in the bloodstream. They trigger a broad range of biological effects (giving rise to undesired side effects), often do not permeate biological barriers (such as the haematoencephalic barrier), in some cases are insufficiently active or too expensive.

An obvious approach to improving the practical value of the original molecule is its modification with the help of the practically unlimited arsenal of synthetic peptide chemistry. The trial and error approach is routinely used for the empiric evaluation of the structural features essential for biological activity. For that purpose analogues are prepared shortened at either or both ends as well as analogues with amino acid substitutions at one or several positions.

Considering the importance of the spatial relationships in biological interactions, considerable effort is spent on conformational studies of peptides, both in the free state and within the receptor complex. Properly labelled derivatives are frequently used as convenient conformational probes. Based on the information obtained, analogues are made with limited conformational mobility in the required parts of the molecule. For that purpose a variety of peptidomimetics have been engineered with preformed secondary structure elements (α-helices, β- and γ- turns, etc.) or the peptide bonds replaced by stereochemically similar moieties, such as esters, or carba- and aza- amides, etc.

An interesting method for modifying active peptides, called topochemical was introduced in the late 1960s by Shemyakin, Ovchinnikov and one of the authors of this review [23]. As shown in Figure 2, reversal of the amino acid sequence of a cyclic peptide (retro-transformation) with simultaneous inversion of all *C*-alpha configurations (enantio-transformation) results in a retro-enantio isomer which can potentially imitate the parent molecule with respect to the side chain arrangement, thereby reproducing its topochemical and biological properties. The concept is also applicable to linear peptides if care is taken to block the charged *N*- and *C*- termini (Figure 3). The concept was further developed by Goodman and Chorev who suggested partial, instead of the total, retro-inverso, as they named the transformation [24,25]. Whether or not the potential topochemical mimicry actually takes place depends on the individual conformational properties of the peptide and requires separate study. Van Regenmortel, Guichard, Briand and their collaborators demonstrated in a series of elegant papers (e.g. [26–28]) that all L- and respective retro-inverso peptides can cross-react in immunological test systems, the latter being as good as, or even superior to, antigens or immunogens. In particular, the transformed fragment of the VP1 foot-and-mouth disease viral protein was able to elicit long lasting protective antibodies after a single injection [29,30].

The combined efforts of many peptide laboratories around the world resulted in the synthesis of a huge number of peptide derivatives, by far exceeding the number of naturally occurring prototypes. Analogues have been obtained with considerably

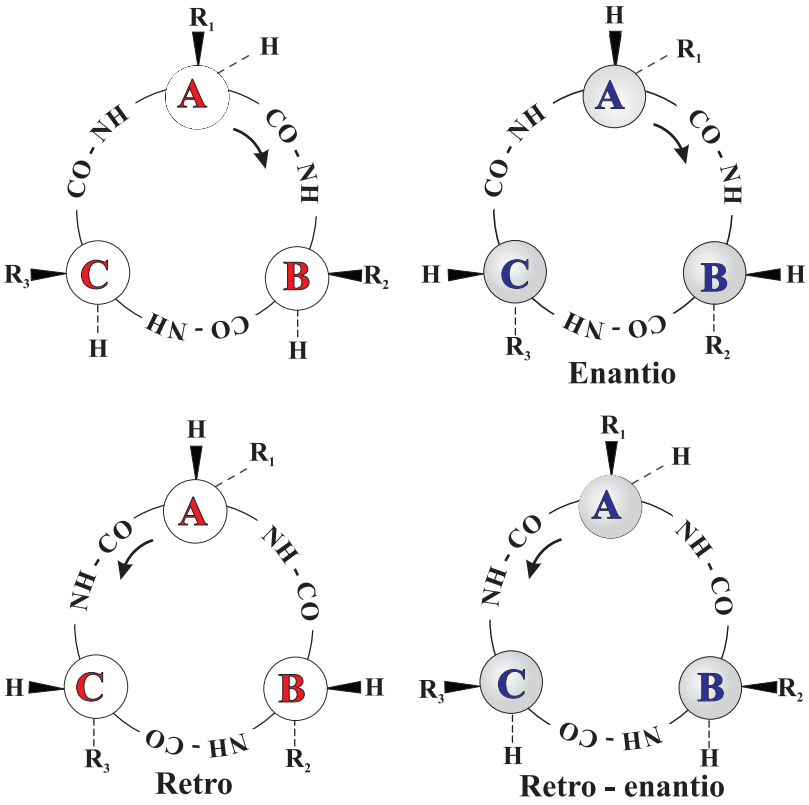

Figure 2 Topochemical similarity of a cyclic peptide to its retro-enantio isomer. A, B, and C designate C^{α} atoms of amino acid residues. Empty circles belong to L-residue and shaded — to D residues.

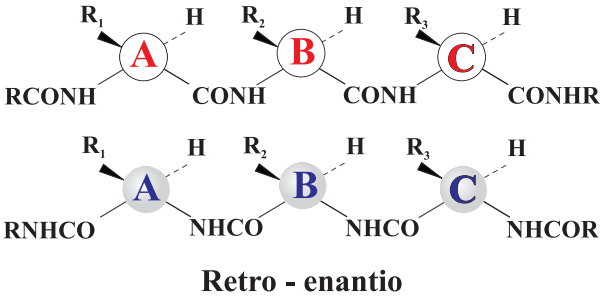

Figure 3 Topochemical similarity of a protected linear peptide to its retro-enantio isomer. Designations are the same as in Figure 2.

enhanced, reversed, or modified in a desirable way, parameters, such as the spectrum of biological actions, distribution in tissues and cells, etc. Among the peptides commonly developed or marketed as pharmaceuticals, the artificially designed and accordingly prepared analogues are at least as well represented as the natural hormones and antibiotics.

It would be not true to state that structures produced by Nature are the only source of inspiration for synthetic peptide chemists. In several cases the inquisitive mind of the researcher chooses an independent path, creating totally artificial molecules. The world of peptides made of beta amino acids or numerous peptide combinatorial libraries serve as examples. The well known sweetener, the dipeptide aspartame produced and consumed in multi-ton amounts is also a product of pure organic chemistry with no roots in the chemistry of natural products.

A growing, although still modest, part of the synthetic peptide repertoire is occupied by the so-called peptide based molecular devices [31]. These are synthetic enzyme models [32,33], peptide transmembrane nanostructures [34], helix bundles covalently attached to various prosthetic groups [35–38], peptide dendrimers [39], etc.

In spite of the above mentioned developments, the participation of peptides in biochemical processes remains the core around which the main activity of peptide science revolves. The remaining part of the review will be devoted to that area. As already mentioned, the protein precursors of endogenous peptides as a rule have no other specific activity except for serving as precursors. Until recently it

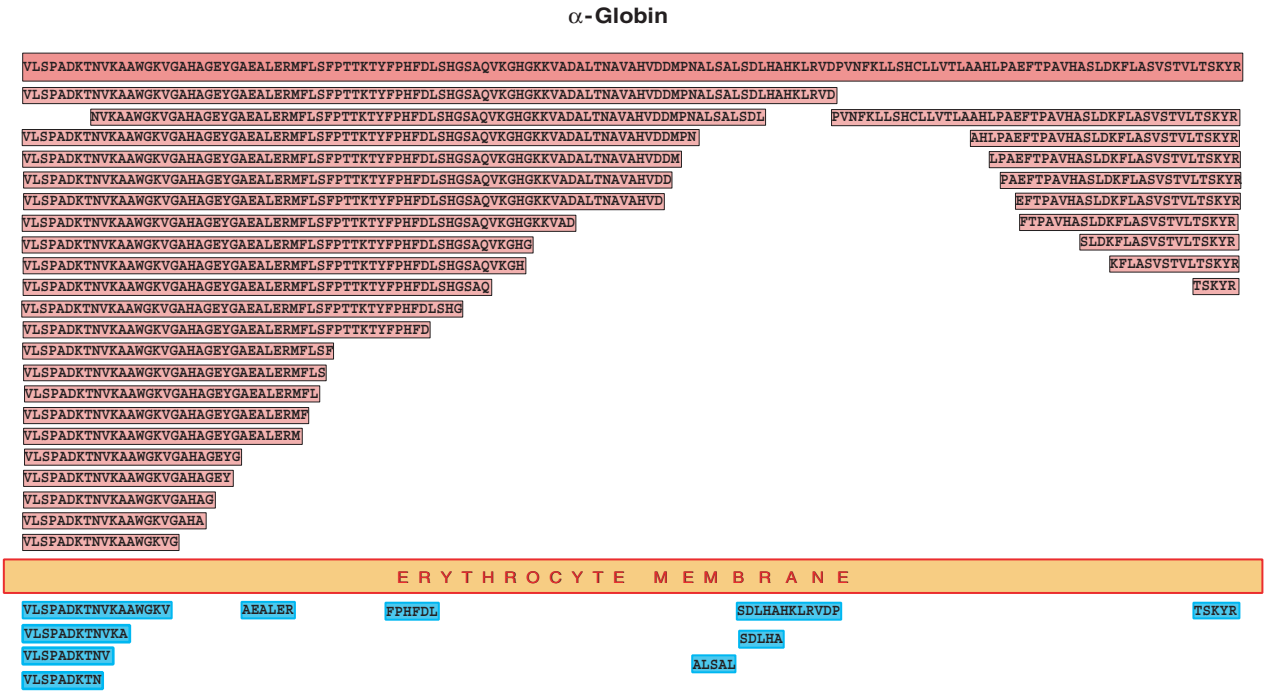

Figure 4 Endogenous fragmentation of alpha-globin in human erythrocytes.

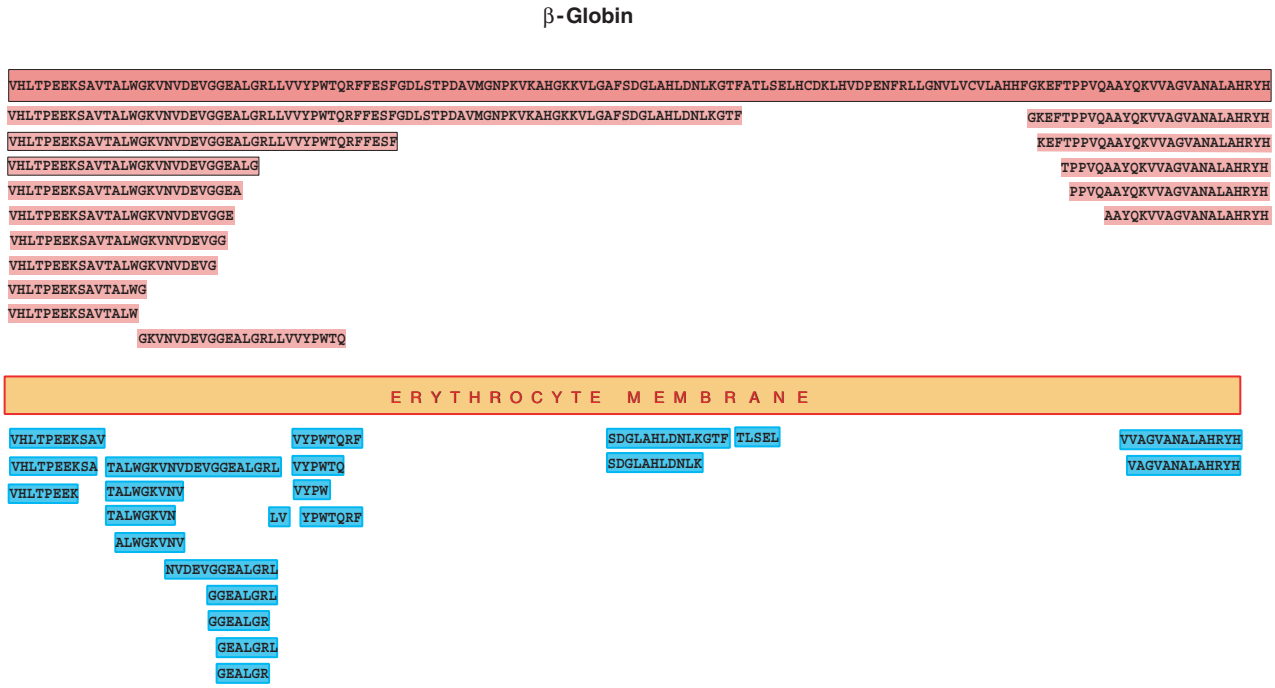

Figure 5 Endogenous fragmentation of beta-globin in human erythrocytes.

remained an open question whether thousands of other proteins participating in standard metabolic processes gave rise to functionally active peptides. The fact that according to genomic data several hundred of these thousands are proteases with various specificities adds ground to the question. Total screening of biological material (tissues, cells, biological fluids) for peptide components is the only way to answer it and a few years ago we were surprised to learn that there is no such information in the literature. For a number of sometimes accidental reasons we became involved in such work and soon found that, indeed, extracts of several animal tissues (bovine brain and bone marrow, rat brain, spleen, lung and heart) contain fragments of haemoglobin, actin, several intracellular enzymes, neurospecific proteins, ubiquitin and other proteins with well defined metabolic function [40–45].

As a result of these studies, the concept was formulated [42, 46–48] according to the following points.

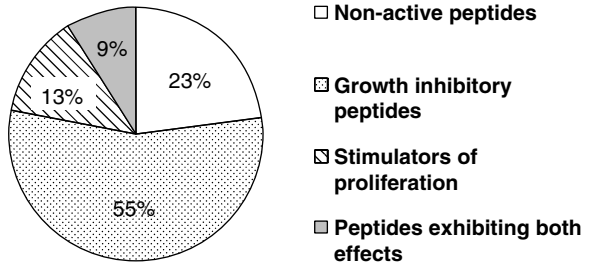

Figure 6 Fractions of pool components exhibiting different types of activity.

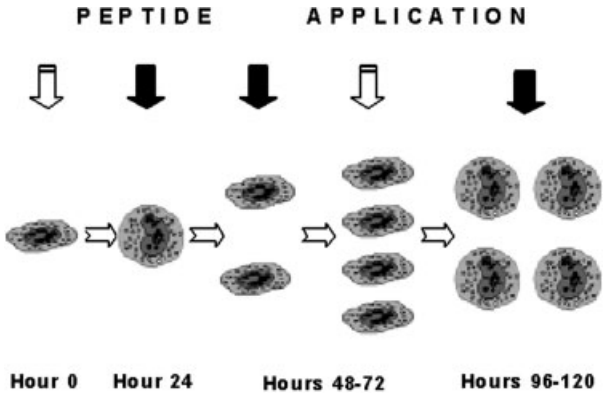

Figure 7 Pool components induce reversible cell cycle arrest and the acquired temporary resistance to the following treatment [71]. White arrows — the cells are sensitive to peptides action, dark arrows — cells are resistant to peptides action. The time intervals when cell proliferation is arrested are indicated by larger cells.

- Each tissue under normal conditions contains a conservative set of peptides present in considerable amounts.
- The content and the composition of these peptides is tissue specific [49,50]. The sets of such peptides are defined as 'tissue-specific peptide pools'.
- These peptides are generated by endogenous proteolytic enzymes from proteins with well established function (haemoglobin, actin, cellular enzymes).
- The content and the composition of pool components correlate with the pathologies related to alterations of tissue metabolism [51–55].
- The *in vivo* function of the components of the peptide pools was suggested as the maintenance of tissue homeostasis, i.e. of the appropriate ratio of dividing, differentiating and functional cells.

A large portion of pool components is represented by haemoglobin-derived peptides. Therefore, the proteolytic degradation of haemoglobin in a primary culture of human red blood cells was studied in detail. After a series of preliminary attempts [42,56–58], it was eventually found that erythrolysate prepared in the presence of the inhibitors of the main classes of proteases contains an impressive set of long α- and β-globin derivatives [52,59]. About 50 peptides were isolated and sequenced which can be considered as products of *N*- and *C*- terminal degradation of the peptides released after splitting at the (94–95) site of the α-globin molecule and at the (70–118) segment of β-globin. These peptides are 15–94 amino acid residues long, except for α-globin (137–141), i.e. neokyotorphin. The content of the individual haemoglobin fragments comprises 0.01–2.5 nmol/ml blood, the overall fraction of haemoglobin fragments being about 0.1% of the total haemoglobin content (Figures 4 and 5).

The erythrocyte peptides are subjected to further degradation probably by membrane associated protease(s) and the resultant peptides are released into the surrounding medium [42–48,60]. These peptides are also shown in the Schemes. In addition to these peptides, tissue extracts contain sets of haemoglobin fragments differing from those produced by erythrocytes, as well as peptides derived from other functional proteins [40–44].

Over 500 peptides derived from functional proteins have been identified in extracts of mammalian tissues and sequenced. Some of these peptides were found earlier and showed pronounced activity in a variety of biological test systems [61–68]. These data are reviewed in [42,47]. At present study of about

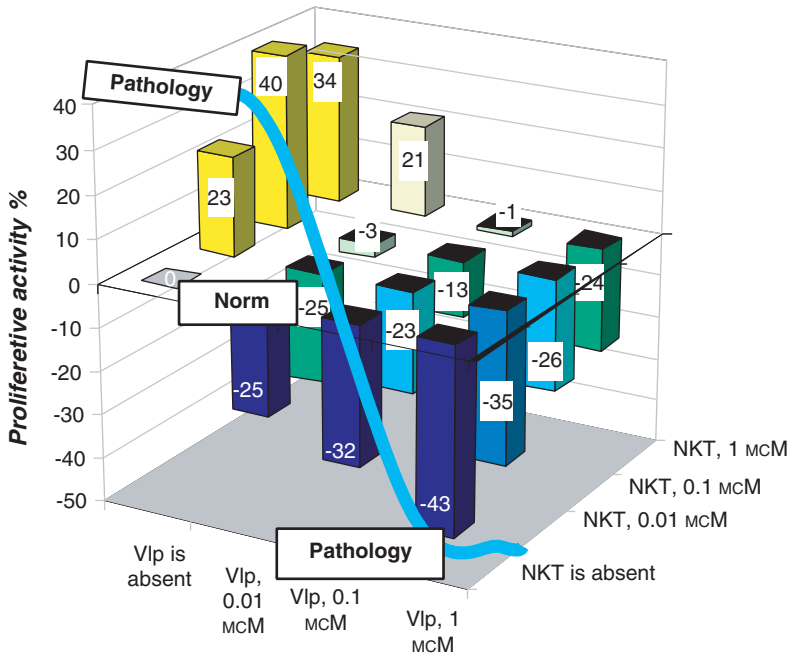

Figure 8 Inhibition of neokyotorphin (NKT) activity by valorphin (VLP) in L929 cells. The peptides were co-incubated with L929 cells for 24 h. The effect was evaluated by visual cell count [70–72].

150 peptides in normal and tumour cell cultures has been completed [69].

The majority (77%) of them affect the proliferation of tumour cells. Screening allowed the separation of these compounds into three major functional groups (Figure 6): (1) inhibitors of cell proliferation, (2) stimulators of cell proliferation, (3) peptides exhibiting both growth inhibitory and growth stimulatory effects depending on the concentration.

In general, these results indicate that peptide pools predominantly contribute to the negative regulation of cell number, rather than to growth stimulation.

The majority (>80%) of the inhibitors belong to the structural families of β-globin (32–41) fragments (haemorphins), to β-actin segments (75–90) and (68–77) and to 3-4-membered peptides enriched with acidic amino acid residues. The peptides corresponding to the same group exhibit similar effects in cell cultures, concentration range and the maximal activities depending upon the concrete amino acid sequences [70]. These peptides exhibit a considerably higher antiproliferative effect in tumour cells than in normal cells. The representatives of all groups were shown to induce a reversible arrest of cell division accompanied by a reversible resistance to the next treatment [71] (Figure 7). The ability to reduce tumour growth *in vivo* could indicate the involvement of pool components in the antitumour defence of an organism.

Growth stimulatory peptides are represented by long alpha-globin (106–141) fragments and by the neokyotorphin group, the peptides corresponding to the 134–141 segment of alpha globin. In contrast to growth-inhibitory pool components, the effects of proliferative peptides are similar or even higher in normal cells, compared with tumour cells. The effects of growth stimulatory peptides strongly depend on the presence of growth factors and cell density. Typically, growth stimulatory effects are more pronounced in unfavourable conditions, such as deficiency of growth factors, low cell density and the presence of cytostatic chemopreparations [60,72].

The third group is represented by two families of peptides corresponding to (1–32) and (12–25) α-globin segments. These substances exhibit both growth inhibitory or growth stimulatory activities, depending on the concentration [57].

As a model of the total *in vivo* pool effects, the effects were studied of a combined action of the most abundant inhibitory and stimulatory pool components, valorphin (VV-hemorphin–5) and neokyotorphin (α-globin-(137–141)). The proliferative effect of neokyotorphin was detected only when the neokyotorphin concentration was 100-fold higher than

that of valorphin. At an equimolar concentration corresponding to that present in several tissues normally [49–50], the former peptide suppressed by 50% the growth inhibitory effect of valorphin, i.e. the rate of cell proliferation can be regulated by changing the ratio of these peptides (Figure 3) [69].

We believe that the patterns of action of the most abundant peptide families reflect the overall effect of pool components in tissue cells. Bearing in mind the domination of growth inhibitors among the pool components (Figure 1), we consider that under normal conditions these peptides are directly involved in the control of excessive cell proliferation. In the case of deviation from the norm (tissue damage, lack of growth factors, etc.) proliferative peptides could contribute to tissue regeneration.

We believe that the results presented here add a new dimension to the complexity of the regulatory peptide network. The newly emerging field which implies the total screening of biological tissues, cells and fluids is appropriately called peptidomics and should be considered as a logical sequel to genomics and proteomics. These are all grounds for expecting that the peptides discovered in the course of such studies will provide a variety of interesting targets for synthetic chemists. Pool components can be considered as a source of derivatives applicable both as potential drugs and as tools for the investigation of cell proliferation processes.

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