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Synthesis of Modified Phenylalanine Bioisosteres: their Use for the Design of HIV Inhibitors.

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The search for new HIV inhibitors led us to study a novel series of compounds based on the bioisosteric replacement of the methylene group in a phenylalanine residue (Phe*) by various S, O or N heteroatoms. The synthesis of these modified Phe-Phe* templates (*Figure 1*) bearing various *N*-terminal and *C*-terminal groups need to develop specific synthetic routes because of the chemical unstability of the hemiaminal (Phe*) intermediates. Indeed, during the hydrolysis process, a chemical rearrangement can occured (*Scheme* 1). We report here the synthesis and the anti-HIV activities on HIV infected MT_4 culture cells of a series of new Phe-Phe* bioisosteres which structures differ from the structures of general HIV-antiproteases containing the hydroxyethylamine (HEA) motif.

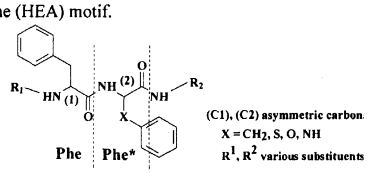
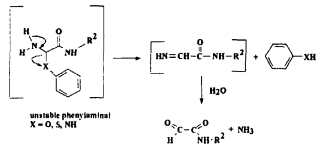


Figure 1. *Structure of new HIV protease inhibitors Phe-Phe*.*



HIGH THROUGHPUT SCREENING ASSAY FOR THE IDENTIFICATION OF PROTEASE SUBSTRATES

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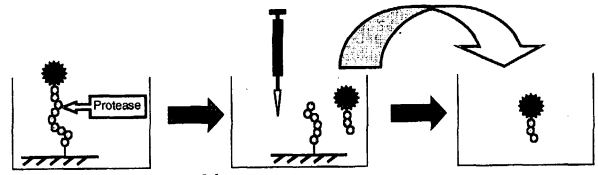
The characterization of protease specificities is important for the identification of protein substrates, development of peptidic substrates used in high troughput inhibitor screening for pharmaceutical purposes and the deduction of substrat sequence-based peptidic inhibitors. Therefore, large numbers of different peptides have to be synthesized and tested for proteolytic cleavage. This demand was achieved by the SPOT synthesis technology [1], which is a method for the rapid and highly parallel generation of large peptide arrays on cellulose membranes. 600 potential substrate peptides (spots of 1 cm in diameter) can be synthesized on a 20 x 30 cm sheet within a few days. The peptides are C-terminally coupled to the solid support and bear a 2-aminobenzoic acid residue as fluorescence marker at the N-terminus [2]. After synthesis the spots are punched out into 96-well microtiter plates and incubated with a protease solution. Thereby substrate peptides are cleaved and the N-terminal part with the fluoescence dye is released into the supernatant. After different times aliquots from the supernatant are pipetted into free wells and cleavage is quantified by fluorescent measurements. Results obtained with this assay are shown for the identification of trypsin and caspase-3 substrates using different types of peptide libraries as well as and substitutional analyses.



Schematic presentation of the protease assay

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Syntheses of New HIV Protease Inhibitors Containing a Novel (2-Phenylsulfanyl-1-Hydroxyethyl)amide Isostere

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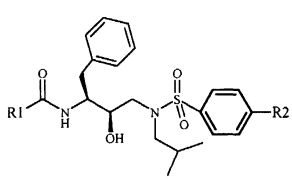
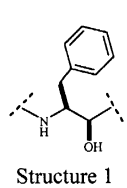
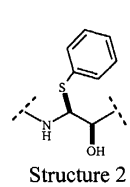
We report herein the synthesis, the chiral separation and the antiviral evaluation of Amprenavir bioisosteres (Agenerase®, a clinical used anti-HIV protease inhibitor) including a (2-Phenylsulfanyl-1-Hydroxyethyl)amide isostere. We presumed that a bioisosteric replacement of the methylene group of the phenylalanine derivative (structure **1**) by a sulfur atom (structure **2**) could improve the antiviral activity. Indeed, numerous examples of bioactive drugs based on such bioisosteric replacement have demonstrated remarkable pharmacological properties (ddC replaced by 3TC).
Synthesis and antiviral studies of such new chiral bioisosteres of Amprenavir represent interesting chemical challenges.



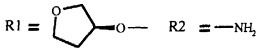


Structure 1



Structure 2

R1, R2 : various substituents



Amprenavir

TRYPTOPHAN-RICH INTEGRASE INHIBITORY PEPTIDES AND PEPTIDOMIMETICS

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Integrase enzyme (IN) plays a pivotal role in the infectious process of the HIV virus. It allows for the integration of the viral genome into its targeted host chromosome, and thus for the replication of the virus. Inhibition of IN is a promising approach towards the development of anti-HIV therapeutics. IN is already present in the infectious virions, and there is no known counterpart to this enzyme in mammalian cells. The complex mechanism of integrase function poses a challenging opportunity for efficacious enzyme inhibitory design. This enzyme removes two nucleotides from the 3'-end of the viral DNA, and it also functions in inserting the viral DNA through its 3'-end into the genomic DNA of the cell. In order to develop an improved peptide-based pharmacophore model for IN inhibitory action we have carried out detailed structure-activity studies on a lead peptide, H-C-K-F-W-W, 1, (IC_{50} = 40 uM, in our hands), identified earlier by synthetic peptide library methodology (Plasterk and coworkers, Proc. Nat. Acad. Sci, USA, 1995). Over 30 relevant analogs were prepared and evaluated. Our findings indicate that single D-a.a. substitutions decrease the inhibitory effectiveness of the parent peptide. On the other hand, the all-D analog showed improved activity (IC_{50} = 31 uM). Deletion of the two C-terminal Trp's abrogated the activity. Ala mutation studies indicate that His-1 was also essential. Backbone cyclization, and at the same time all D-a.a. substitution results in only 1.2 – 1.5 fold decrease in effectiveness, providing a conformationally better defined and proteolytically stable IN inhibitor. The C-terminally Trp extended all-D analog of 1 provided the most effective agent (IC_{50} = 15 uM). We also identified a known Trp-rich antimicrobial peptide as an effective IN inhibitor, that may serve both as an anti-infective agent in addition to its enzyme inhibitory function. Significantly, these Trp rich agents retain their activity in assays when the substrate DNA is pre-incubated with IN before the addition of inhibitors and the target DNA. Mechanistic studies are in progress on these agents.

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EFFECT ON HUMAN PLATELET AGGREGATION *in vitro* OF RGD ANALOGS INCORPORATING MOIETY OF SALICYLIC-DERIVATIVES

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Platelet aggregation is generally mediated by fibrinogen, an extracellular matrix protein, which is specifically bound to the platelet receptor GP IIb/IIIa. The RGD (Arg-Gly-Asp) sequence is an important component in the recognition of fibrinogen by the platelet receptor. Research studies have revealed the property of RGD-containing peptides to inhibit platelet aggregation and thrombus formation by interfering with fibrinogen-GP IIb/IIIa[1,2]. On the other hand, aspirin treated platelets failed to aggregate in response to arachidonic acid, owing to inhibition of platelet cyclooxygenase 1. Thus, the current knowledge recognizes that low doses of aspirin decrease platelet aggregation by causing an inhibitory effect on thromboxane A_2 production by platelets[3].

We have already reported that combination in the same molecule of dipeptide amides, containing amino acid(s) of RGD sequence, with salicylic-residue 2-RO-C_6H_4-CO~{where R=H or CH_3CO} at their N-terminal amino group have shown inhibitory activity on human platelet aggregation[4]. In this report, we present a series of RGD analogues incorporating salicylic-derivatives synthesized by conventional solution techniques and/or by solid phase. The synthesized RGD analogues were tested for inhibitory activity on human platelet aggregation in vitro, by adding the aggregation reagent (collagen) to citrated platelet rich plasma (PRP). The inhibitory activity of 2-HO-C_6H_4-CO-Arg-Gly-Asp-NH_2 was higher than the activity of HCl·RGD-NH_2, which shows that the combined molecule of salicylic acid and RGD may result in a synergetic action, giving more potent compounds against platelet aggregation. Comparing the side groups of Asp(OR), it was found that the 2-HO-C_6H_4-CO-Arg-Gly-Asp(OBzl)-NH_2 is more active than others, indicating that a lipophile character of the side chain of Asp(OR) is preferable by the receptor. The IC_{50} values of the synthesized and tested compounds, as well as their MS, IR and ^1H and ^{13}C NMR spectra will be discussed.

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TARGETING EXTRACELLULAR CATHEPSIN B BY EFFECTOR-FUNCTIONALIZED *ENDO*-EPOXYSUCCINYL PEPTIDES

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Extracellular cathepsin B has been implicated in several pathophysiological processes, most prominently tumor progression and metastasis. This enzyme therefore is an interesting target for the development of synthetic tools to study its pathogenetic role in more detail. In addition, the selective targeting of this enzyme by inhibitors is a promising approach for therapy. Our *endo*-epoxysuccinyl-peptide synthesized recently, i.e. MeO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH, was proven to be the most potent cathepsin B inhibitor among this class of inhibitors and excels for its high degree of selectivity (k_2/K_i = 1 520 000, specificity cathepsin B/cathepsin L: 1 262).[1] As shown by molecular modeling, the terminal glycine is exposed to the surface of the protein, thus allowing the chemical manipulation at this site. Via an additional C6 spacer the inhibitor was functionalized with rhodamine B, biotin, and mono-(6-deoxy-6-amino)-β-cyclodextrin. A comparison of the second order rate constants of enzyme inhibition by these constructs and the underivatized epoxysuccinyl peptide clearly shows that the inhibitory potency is not affected and that even an increased selectivity is obtained. The membrane permeability of the parent inhibitor molecule and the constructs was assessed using MCF-7 breast cancer cells. Both the inhibitor and the constructs are not cell-permeant even at concentrations 200-fold higher than those required for complete inhibition of cathepsin B in cell lysates. The cyclodextrin-functionalized inhibitor can form an inclusion complex with methotrexate, thus representing a potential site-selective drug carrier system.[2] Furthermore, affinity blot analysis using the biotinylated inhibitor allows a highly sensitive and selective detection of active cathepsin B.

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OPIOID ACTIVITY PROFILES OF TIPP-RELATED PEPTIDES CONTAINING 2'-HYDROXY,6'-METHYLTYROSINE (HMT) IN PLACE OF TYR[1].

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We previously reported that substitution of Tyr[1] with Hmt in the δ opioid antagonist TIPP (H-Tyr-Tic-Phe-Phe-OH) resulted in a compound with very high δ antagonist potency and extraordinary δ receptor selectivity (1). In an effort to develop compounds with further improved δ antagonist or δ agonist properties, a number of tetra- and dipeptide analogs containing the N-terminal dipeptide segment H-Hmt[1]-Tic[2]- were prepared. Compounds were tested in the guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassays and in μ-, δ- and κ-opioid receptor binding assays. Interestingly, the tetrapeptide amide H-Hmt-Tic-Phe-Phe-NH_2 turned out to be not only a potent δ antagonist (K_e = 0.504 nM) but also a moderately potent μ antagonist (K_e = 236 nM). To prevent chemical degradation due to diketopiperazine formation, analogs containing a reduced peptide bond between Hmt[1] and Tic[2] were prepared. The Hmt[1]-analog of the highly selective δ antagonist TIPP[Ψ] (H-Tyr-TicΨ[CH_2NH]Phe-Phe-OH) retained high δ antagonist potency but, unexpectedly, also displayed moderate μ agonist activity and, thus, was identified as a mixed μ agonist/δ antagonist. Surprisingly, replacement of Tyr[1] in the highly potent and selective δ antagonist TICP[Ψ] (H-Tyr-TicΨ[CH_2NH]Cha-Phe-OH; Cha = cyclohexylalanine) with Hmt led to a compound, H-Hmt-TicΨ[CH_2NH]Cha-Phe-OH, which retained high δ antagonist potency, but again was also a moderately potent μ antagonist. Substitution of Hmt for Tyr[1] in the potent and moderately selective dipeptide δ agonist H-Tyr-Tic-NH-CH_2-$CH(Ph)_2$ (Ph = phenyl) resulted in a compound with subnanomolar δ agonist potency in the MVD assay (IC50 = 0.630 nM) and with greatly improved δ receptor binding selectivity (K_i^{μ}/K_i^{δ} = 835). In a direct comparison, H-Hmt-Tic-NH-CH_2-$CH(Ph)_2$ was an 8 times more potent and 15 times more selective δ agonist than DPDPE. Interestingly, the Hmt[1]-analog of the δ agonist H-Tyr-Tic-NH-$(CH_2)_2$-Ph(*o*-Cl) was found to be a δ antagonist. These results indicate that Hmt[1]-analogs of TIPP-related peptides may have unexpected opioid activity profiles.

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NEW μ OPIOID RECEPTOR ANTAGONISTS RESULTING FROM TYR[1] MODIFICATION OF CYCLIC CASOMORPHIN ANALOGS

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Department of [a]Chemistry and [b]Pharmacology, AstraZeneca R&D Montreal; [c]Laboratory of Chemical Biology & Peptide Research, Clinical Research Institute Montreal, Canada.

Recently, the cyclic casomorphin analog H-Tyr-c[-D-Orn-2-Nal-D-Pro-Gly-] (**1**) was described as an opioid peptide with mixed μ agonist/δ antagonist properties. Substitution of Tyr[1] by 2',6'-dimethyltyrosine (Dmt, **2**) led to the first cyclic peptide with a *balanced* μ agonist/δ antagonist profile. Subsequently, Gly[5] replacement by D-Ala (**3**) produced a compound exhibiting subnanomolar affinity toward the μ and δ reeeptors that was a potent antagonist in the mouse vas deferens (MVD) comparable to the non-peptidic δ antagonist naltrindole. Furthermore, the 1-position of parent peptide **1** was modified by 7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**4**), C^{α}-methyltyrosine (**5**) and 2'-methyltyrosine (**6**). Compounds **4** and **5** possessed μ and δ binding affinities in the low μM range, while affinities displayed by analog **6** were comparable to those of compounds **2** and **3**. Analog **6** turned out to be a potent δ antagonist in the MVD bioassay, but surprisingly, was also an antagonist against the μ agonist H-Tyr-D-Ala-Phe-Phe-NH_2 in the guinea pig ileum (GPI) assay. Introduction of a [CH_2NH] Xaa[1]-D-Orn[2] peptide bond replacement in analogs **1**, **2** and **3** had no detrimental effect on μ and δ binding affinities and δ antagonism, however, this modification converted the μ agonists into potent μ antagonists. Since the Phe[3] analog of parent peptide **1** is a potent μ and δ agonist, the corresponding Dmt[1] analogs without (**7**) and with (**7a**) a reduced peptide bond were prepared. As expected, compound **7** showed subnanomolar μ and δ receptor binding and was a very potent agonist in the GPI and MVD assay. Analog **7a** displayed high μ and δ receptor affinity but turned out to be a partial agonist in both functional assays. This result indicates that the reduced peptide bond interferes with μ and δ opioid receptor activation. However, prevention of complete activation is only realized when both the reduced peptide bond and the bulky Nal[3] side chain are present. The conformational properties of compound **2** and its reduced peptide bond containing analog **2a** in a membrane mimetic environment were analyzed by NMR spectroscopy and computational methods, in an attempt to explain their different pharmacological profile.

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STRUCTURE-ACTIVITY STUDIES WITH MELANIN-CONCENTRATING HORMONE (MCH), A REGULATOR OF FOOD-INTAKE BEHAVIOUR

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The receptor for melanin-concentrating hormone (MCH), a neuropeptide found in the brain of all vertebrates and regulator of various behavioural paradigms such as food intake behaviour, has recently been discovered to correspond with the orphan receptor SLC-1 (Nature 1999, 400: 261 and 265). Previously, we have reported the occurrence of receptors for MCH on mouse and human melanoma cells, based on binding experiments with the radioligand [^{125}I]-[Phe^{13},Tyr^{19}]-MCH and photocrosslinking experiments with [^{125}I]-[D-Bpa^{13},Tyr^{19}]-MCH (Drozdz et al., FEBS Lett. 1995, 359: 199 and J. Peptide Sci. 1999, 5, 234). In order to study the binding characteristics of the melanoma MCH receptor with the cloned SLC-1 receptor, we have developed a number of radioligands, such as [^{125}I]-MCH, [^{125}I]-[D-Phe^{13},Tyr^{19}]-MCH, [^{125}I]-[Met(O)^{4,8},Phe^{13},Tyr^{19}]-MCH, [^{125}I]-[Ser^{4,8},Phe^{13},Tyr^{19}]-MCH, [^{125}I]-[Hse^{4,8},Phe^{13}, Tyr^{19}]-MCH as well as linear analogues with and without cysteine. Using some of these radiotracers, we have undertaken a structure-activity study with a number of analogues and fragments of MCH in order to compare the ligand-binding characterstics of the SLC-1 receptor with the binding site on melanoma cells. We are reporting homologies but also considerable differences in the recognition pattern for individual peptides of the two types of receptor.

A FLUORESCENT AND PHOTOREACTIVE VASOPRESSIN LINEAR ANTAGONIST.

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For many years, we have been studying the binding sites of vasopressin and oxytocin receptors using site-directed mutagenesis, photolabeling and molecular modeling approaches. By the way of biochemical methods, such as polyacrylamide gel electrophoresis in sodium dodecyl sulphate, autoradiography and CNBr or enzymatic fragmentation, we have determined some essentials residues and regions of the vasopressin V_{1a} receptor subtype, interacting with two radiolabeled azido linear antagonists. These interactions were reported in a speculative tridimensional molecular model [1].
More recently, we have synthesized some fluorescent vasopressin V_{1a} antagonists and used them in the labeling of whole Chinese hamster ovary cells (CHO) or CHO membranes, expressing the human V_{1a} receptor [2].
Here, we report the synthesis and properties of a new probe:
4-N_3Ph$(CH_2)_2$CO-D Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(5-carboxytetramethylrhodamyl)-Tyr-NH_2, joining the rhodamine as fluorophore and the aryl azido as photoactivatable group. This compound has a K_i of 0.5 nM for the V_{1a} receptor.
Used in combination with receptor overexpression, purification and labeling as well as high performance liquid chromatography purification and mass spectrometry characterization of the fragments, this product will allow us to progress in the determination of receptor regions interacting with the antagonist ligands.
With this new kind of probe we hope to correlate photoaffinity and fluorescence techniques.

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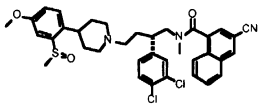
THE DISCOVERY OF NOVEL ORALLY ACTIVE DUAL NK1 NK2 ANTAGONIST ZD 2249

A. Shenvi, P. Bernstein, C. Ohnmacht J. Albert, D. Hill, R. Green, L. Shen, D. Stollman, B. Dembofsky, D. Andisik, R. Dedinas, S. Sherwood, G. Koether, B. Kosmider, T. Davenport, K. Kirkland, W. Potts, R. Bialecki, D. Aharony, W. Rumsey, and K. Russell. Discovery Research, AstraZeneca Pharmaceuticals LP, 1800 Concord Pike, PO Box 5437, Wilmington, DE 19850-15437

The discovery of ZD6021, a dual antagonist of NK1 and NK2 receptors prompted us to investigate the effect of substitution on the 4-aryl ring in the 4-arylpiperidine region of this class of compounds. The resulting SAR leading to the discovery of ZD2249 with improved pharmacological profile will be discussed in this presentation. ZD 2249 inhibited binding of [^{3}H]- SP



ZD2249
(3-cyano-N-((2S)-2-(3,4-dichlorophenyl)-4-[2-(methyl-(S)-sulphenyl)-4-methoxyphenylpiperidino]butyl)-N-methyl-1-naphthamide

and [^{3}H]-NKA to human NK-1 and NK-2 receptors (Ki = 0.09 and 0.34 nM, respectively), and demonstrated >100-fold reduced affinity for the human NK-3 receptor. In functional assays ZD2249, at 10-7 M, had : in rabbit pulmonary artery pA2 = 8.2 and 8.7 for NK-1 and NK-2, respectively, in human pulmonary artery pKB = 7.5 and in human bronchus pKB = 7.7, for NK-1. Oral administration of ZD2249 to guinea pigs dose dependently attenuated NK-1 induced extravasation of plasma proteins, ED50 = 1mg/kg, and NK-2 mediated bronchoconstriction, ED50 = 3 mg/kg.

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ISOLATION AND CHARACTERIZATION OF INSULIN FROM THE BROCKMANN BODY OF "DISSOSTICHUS MAWSONI", AN ANTARCTIC TELEOST.

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Osmoregulation in salt water fish is dependent upon the extrusion of NaCl from the blood. The Na/K-ATPase enzyme located in chloride cells of gill epithelium is responsible for catalyzing this extrusion process. A known modulator of the Na/K-ATPase activity in epithelia is insulin. To investigate the role of insulin in the osmoregulation of Antarctic fish, we isolated insulin from the anatomically well defined principal islets (Brockmann Bodies, Bb) of the Antarctic teleost Dissostichus mawsoni. The extract was partially purified on C18 SepPak cartridges prior to being purified to homogeneity by gel filtration and reversed-phase high performance liquid chromatography (RP-HPLC). Insulin containing fractions were identified by a radioimmunoassay using antisera raised against porcine insulin. The mass of the isolated product was 5725.57. Reduction and alkylation with vinylpyridine produced two products that were isolated by RP-HPLC. Edman degradation revealed these to be the A-chain of insulin with an amino acid sequence of G-I-V-E-Q-C-C-H-Q-P-C-N-I-F-D-L-Q-N-Y-C-N and the B-chain of insulin with an amino acid sequence of A-P-G-P-Q-H-L-C-G-S-H-L-V-D-A-L-Y-L-V-C-G-E-R-G-F-F-Y-N-P-K. This primary structure of insulin has a theoretical mass of 5725.52 in agreement with the measured mass and was further supported by amino acid analysis. The primary structure is similar to other teleosts with the generally accepted invariant regions important for biological activity being conserved. A novel substitution occurs at A9 (Q for usually an R, K or S) and there is an additional residue on the N-terminus of the B-chain which is common in teleost insulins. Supported by NSF-OPP 9613738

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BLOCKING THE N-TERMINUS OF CALCITONIN GENE-RELATED PEPTIDE (8-37) PRODUCES HIGH AFFINITY ANTAGONISTS.

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In membranes from pig coronary arteries, the binding affinity (IC_{50}) of the calcitonin gene-related peptide (CGRP) antagonist, CGRP (8-37) is 60-fold higher than its potency (K_B) in blocking relaxation of intact pig coronary arteries (see Table). This difference may be due to proteolytic breakdown of the antagonist in relaxation experiments. A protease inhibitor cocktail is present in binding experiments but damages intact arteries and cannot be used in relaxation experiments. A series of N-terminally blocked analogues of CGRP (8-37) were synthesized to prevent aminopeptidase degradation. Binding affinities and potencies in blocking relaxation of these analogues were measured and are listed below. All N- terminally blocked analogues have higher binding affinities and potencies in blocking relaxation compared to CGRP (8-37). Hydrophobic groups cause the largest increases. However, binding affinities versus potencies in blocking relaxation still differ significantly, suggesting aminopeptidase degradation is not a major cause of the lower relaxation potency. Supported by USPHS grant HL51131.

Table. Binding affinities (IC_{50}) and potencies (K_B) in blocking relaxation of CGRP (8-37) analogues on porcine coronary arteries.

Analogue	IC_{50} (nM)	K_B (nM)
h-α-CGRP (8-37)	14.38	970.1
acetyl-h-α-CGRP (8-37)	3.05	29.2
benzoyl-h-α-CGRP (8-37)	0.27	40.36
benzyl-h-α-CGRP (8-37)	1.58	118.7
dibenzyl-h-α-CGRP (8-37)	0.22	29.02

SERUM-STABLE NEUROTENSIN ANALOGS AS POTENTIAL IMAGING AND THERAPEUTIC AGENTS FOR PANCREATIC CANCER

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Neurotensin (NT) is a 13-amino acid regulatory peptide found in the brain and gut. Recent work [*Gut*, **42**, 546 (1998)] has indicated that 75% of human exocrine pancreatic tumors express neurotensin receptors in high density. This information suggests that a radiolabeled neurotensin derivative has potential for the detection and therapy of exocrine pancreatic cancer. Structure-activity relationships have demonstrated that the *C*-terminal sequence (Arg-Arg-Pro-Tyr-Ile-Leu-OH, NT (8-13)) is sufficient in preserving high affinity receptor binding. This truncated peptide, however, has poor serum stability. One site of enzymatic instability is the Arg^8-Arg^9 bond. By replacing one or both of the arginines with a suitable arginine mimic, we have been able to prepare neurotensin analogs with increased serum stability while preserving the receptor binding. The *C*-terminal portion of the peptide also appears to undergo degradation, so additional derivatives have been designed and synthesized to address this problem. Both these modifications led to highly serum-stable derivatives of neurotensin. All of the analogs contain diethylenetriaminepentaacetic acid (DTPA) at the *N*-terminus for incorporation of radioactive isotopes for γ-ray scintigraphy. These receptor-specific peptides can also be coupled to chelates capable of carrying β-emitting isotopes for therapeutic purposes. *In vitro* binding studies and biodistribution studies in HT-29 tumor-bearing mice using ^{111}In-labeled derivatives will be presented. In conclusion, these serum-stable neurotensin analogs should be ideal agents for the imaging and therapy of exocrine pancreatic cancer.

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SYNTHESIS AND *IN VITRO* EVALUATION FOR ANTI-HIV EFFECT AND TOXICITY OF A NEW PEPTIDES ANALOGUES OF 3'-AZIDO-2',3'-DIDEOXYTHYMIDINE

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To surmount the side effect of longterm antiretroviral therapy a study based on 3'-azido-2',3'-dideoxythymidine(azidithymidine, AZT, zidovudine) peptides analogues was undertaken. New prodrugs of AZT were synthesized by peptides in 5' position in sugar moiety. A high stability at pH 1 and 7.4 was established. *In vitro* studies for toxicity and anti-HIV effect were performed on NT4 cells infected with HIV-1 (H9/HTLV-IIIB). One of the prodrugs tested showed the same anti-HIV effect and five times low toxicity comparing to the parent drug. This prodrug with established anti-HIV effect, low toxicity and high stability is a perspective candidate for a new anti-retroviral agent.

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STRUCTURE-ANTIBACTERIAL ACTIVITY RELATIONSHIPS OF 15-RESIDUE MURINE LACTOFERRICIN DERIVATIVES

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LFM W8 is a synthetic 15-residue lactoferricin derivative (NH_2-EKCLRWQWEM-RKVGG-COOH), corresponding to residues 16 to 30 of the mature murine lactoferrin protein [1] except that glutamine in position 8 of the peptide is replaced by tryptophan. It is previously reported that the two tryptophan residues in position 6 and 8 are of crucial importance for the antibacterial activity of many lactoferricin derivatives, but that LFM W8 is inactive against *Escherichia coli* and *Staphylococcus aureus* despite having both tryptophan residues [2].
In the present study, the structure-antibacterial activity relationships of synthetic LFM W8 derivatives was investigated by replacing the glutamate residues in positions 1 and 9 with arginine or alanine, and by replacing the valine residue in position 13 with tyrosine. In general, the arginine substitutions increased the activity more than the alanine substitutions, and the replacement of the N-terminal glutamate was more important than the replacement of the interior glutamate. Thus, both the net charge and the positions of the positively charged residues were important parameters affecting the antibacterial activity of these murine lactoferricin derivatives. Furthermore, the positive effect of the arginine-modification was additive. Thus, the doubly substituted peptide LFM R1,9 W8 was one of the most active derivatives, displaying a minimal inhibitory concentration of 10 μM against *Escherichia coli* and 37 μM against *Staphylococcus aureus*.
A special effect of the amino acid tyrosine was also revealed. Replacing the valine residue with tyrosine resulted in further improved antibacterial activity, especially against *Staphylococcus aureus*. The most active derivative of this series, LFM R1,9 W8 Y13, displayed a minimal inhibitory concentration of 10 to 12 μM against both *Escherichia coli* and *Staphylococcus aureus*. This represented a 40- to 50-fold increase in antibacterial activity as compared with LFM W8. We suspect that the tyrosine-modification increased the affinity of the peptides for the bacterial cell membrane, leading to increased antibacterial activity of the derivatives.

1)Pentecost, B. T.; Teng, C. T. *J. Biol. Chem.* **1987**, *262*, 10134-10139.
2)Strøm, M. B.; Rekdal, O.; Svendsen, J. S. *J. Pept. Res.* **2000**, *submitted*.

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF DOUBLE CYCLIC RGD CONTAINING PEPTIDES

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The double cyclic disulfide bonded analogue of ACDCRGDCFCG undecapeptide, which was found in a phage display peptide library, is one of the most potent ligands for integrin receptors. The linear peptide containing four cysteine residues has been synthesised on solid phase and cyclised in solution, however the exact location of the disulfide bonds was not determined (Koivunen E., Wang B. and Ruoslahi E. Biotechnology, **1995,** 13, 265-270).
To find out the arrangement of the disulfide bonds in the above double cyclic peptide, we have synthesised the theoretically possible three model structures of the double cycle :

A[CD(CRGDC)FC]G **I** A[CD(CRGDC]FC)G **II** A[CDC]RGD(CFC)G **III,**

and performed spontaneous cyclisation of ACDCRGDCFCG, too.
Synthesis and cyclisation of **I, II** and **III** were carried out on solid phase with selective protection of the cysteine SH pairs to be oxidised, with Acm and Fm, respectively. For the spontaneous cyclisation the linear peptide with Meb SH-protection was prepared on solid phase and oxidised in solution as described in the literature. Using the models **I, II** and **III** for comparison, HPLC analysis revealed that the structure formed in solution cyclisation corresponds to **II**, and the previously supposed structure **I** has only been formed as a side product. For structure-activity relationship investigations the inhibitory effect of the cyclopeptides and of control peptides, c(CRGDC)G and SRGDP on melamoma cell binding to fibronectin was investigated.

IMMUNOSUPPRESSORY ACTIVITY OF UBIQUITIN FRAGMENTS CONTAINING RETRO-RGD SEQUENCES

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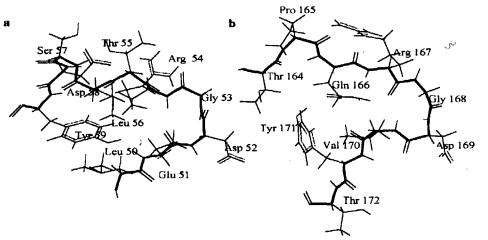
Our previous studies revealed that the nonapeptide fragment of HLA-DQ located in the ß164-172 loop with the TPQRGDVYT sequence suppresses the immune responses. As can be seen in the picture below, there exists topological correspondence between the 48-59-loop of ubiquitin (a) and the ß164-172 loop of HLA-DQ (b), suggesting that their biological roles are similar.



Ubiquitin is a small protein (8.5 kDa) present in all eukaryotic cells which plays an important role in tagging proteins for destruction. The biological role of the 48-59 ubiquitin fragment still remains unknown. The sequence is located in the loop of the molecule exposed toward the solvent and therefore may be involved in interactions with other proteins.
We synthesized ubiquitin(48-59) and a series of its shorter fragments. The peptides were characterized by CD spectroscopy and investigated for their activity in humoral and cellular immune response. We found that the synthetic peptides possess unusually high immunosuppressory potency, comparable with that of cyclosporine. The analogues with restricted peptide backbone were also synthesized and examined for their immunological properties. Our results suggest that ubiquitin may possess its own immunomodulatory domain located in the 48-59-loop.

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DEVELOPMENT OF BIFUNCTIONAL OPIOID LIGANDS CONTAINING DIMETHYLTYROSINE

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The opioid system consists of three main receptor subtypes (δ, μ and κ) and their corresponding ligands (enkephalin, endorphin, dynorphin) which govern diverse physiological functions in peripheral tissue and in the central nervous system. Recently, endomorphin-1 (H-Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH_2), which exhibited high affinity and selectivity for μ-opioid receptor, were isolated from bovine brain, indicating the importance of the μ-opioid receptor system.[1] This presentation deals with design and synthesis of opioid mimetics derived from endomorphin sequence.
The studies on the structure-activity relationship of endomorphin revealed that H-Tyr-Pro-Phe-NH_2 is the smallest sequence to exhibit μ-opioid receptor binding activity. Various substituents were employed for X of H-Tyr-Pro-Phe-NH-X. The resulting peptide derivatives exhibited various μ-opioid receptor binding activity profiles. H-Tyr-Pro-Phe-NH-1-naphthyl and 5-isoquinolyl exhibited K_iμ values of 2.41 and 61.1 nM, respectively. The use of 2,6-dimethyl-L-tyrosine (Dmt) instead of Tyr increased binding activity significantly. Dmt-Pro-Phe-NH-1-naphthyl and Dmt-Pro-Phe-NH-5-isoquinolyl exhibited K_iμ values of 0.30 and 0.19 nM respectively. Although both compounds exhibited antinociceptive activity in tail pressure test in mice, Dmt-Pro-Phe-NH-5-isoquinolyl exhibited an IC_{50} value of 0.94 nM with GPI and >10,000 nM with MVD. Interestingly, this compound acts as agonist toward μ-opiod receptor and antagonist against δ-opioid receptors. We designated this compound as a bifunctional opioid receptor ligand.

1) Zadina, J.E., Hackerler, L., Ge, L., Kastin,A.J. (1997) *Nature,* **386/13**, 499-502.

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BIOACTIVE PEPTIDES FROM TRYPTIC HYDROLYSATE OF BOVINE α_{S2}-CASEIN

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Since the end of the 70's, milk proteins are not simply recognised as amino acid providers but also as delivering agents of biologically active peptides. These functional peptides can stimulate the immune system, reduce blood pressure, provoke analgesia, enhance calcium absorption *etc...* Bovine α_{S2}-casein represents 10% of the total caseins. Its gene has a common ancestor with α_{S1} and β-caseins ones. These last two proteins are known to release opiate-like and antihypertensive peptides after proteolysis. Furthermore, bovine α_{S2}-casein contains sequences corresponding to active parts found in many opiate peptides. Consequently, this protein could potentially release these two kinds of peptides. Pure α_{S2}-casein was obtained through a two-step semipreparative chromatography : an anion exchange chromatography was pursued by an hydrophobic interaction one. Trypsin was chosen for its high specificity and also because it is a leading proteolytic enzyme in neonate which is by nature the target of bioactive milk peptides. A kinetic study of the liberation of the peptides was purchased and their identification was obtained by amino acid analysis and mass spectrometry. Opiate-like activities upon μ and δ receptor subtypes were established by competitive binding of some peptides contained in total hydrolysates against specific opiate radioligands. A new RP-HPLC methodology was achieved to investigate antihypertensive activity and to obtain inhibition constants of the angiotensin converting enzyme (ACE), a dipeptidyl carboxypeptidase which is one of the great actors in vasoconstriction phenomena. The formation of hippuric acid from hippuryl-His-Leu-OH substrate was followed at 228 nm after its separation on a C_{18} column. At least four peptidic fractions significantly inhibited ACE activity with IC_{50} values less than 80 μM. Identification of the peptides responsible of the opiate-like and antihypertensive activities is in progress.

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MAPPING OF THE INTEGRIN GPIIb-IIIa REGIONS PARTICIPATING IN PLATELETS AGGREGATION

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The integrin family of adhesive receptors are heterodimeric proteins which mediate a variety of cell-cell and cell-matrix interactions via their recognition of specific ligands. GPIIb-IIIa is the most prominent integrin of platelets and can interact with a variety of adhesive proteins including fibrinogen, fibronectin and Von Willebrand factor. Up to date, several attempts have been made in order to determine the specific ligand binding sites of GPIIb-IIIa. However, due to conformational changes that take place upon activation of GPIIb-IIIa, the determined ligand binding sites, using immobilized receptor or in its non-activated state, are strongly contradicted and extended in a very large area of the receptor.
In this work we present the mapping of the GPIIb-IIIa receptor in the activated state. Using soluble synthetic peptides of twenty amino acids length, overlapping by eight residues, we were able to define distinct regions of the receptor that are involved in the aggregation process. These regions are located in both proteins GPIIb and GPIIIa and inhibit the platelet aggregation, induced by ADP, in micromolar concentration range. The synthesis of the overlapping peptides was carried out using either the Geysen's multipin peptide synthesis or the multiblock method. Experiments are now in progress in order to evaluate the specificity of each determined region.
(Grant from GGSRT).

NEW ENDOMORPHIN ANALOGUES: DESIGN, SYNTHESIS AND BIOLOGICAL PROPERTIES.

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Endomorphin 1 (Tyr-Pro-Trp-Phe-NH_2) and endomorophin 2 (Tyr-Pro-Phe-Phe-NH_2) isolated from the bovine and human brain have been suggested to be endogenous ligands for μ opioid receptor (1). New analogues of these endomorphins were synthesized using conformational constrained amino acids (βMePhe, 2',6'-dimethyl-Tyr (Dmt)) with L and D configurations.The affinity and selectivity of the peptides for the different opioid receptor types were evaluated by radioreceptor binding assays using tritiated opioid ligands and *in vitro* assays in isolated organs (GPI and MVD). D-amino substitutions at Tyr or Phe of the endomorphins decrease their biological activities. Some of these analogs with L-aromatic amino acids were more active (5-20 times) and/or more selective μ agonists compared to the parent peptides in all assays. The most promising analogues (Tyr-Pro-Trp-(2S,3S)βMePhe-NH_2, Tyr-Pro-Phe-(2S,3S)βMePhe-NH_2, Dmt-Pro-Trp-Phe-NH_2, Dmt-Pro-Phe-Phe-NH_2) were increased the tail flick and paw pressure latencies in rats. The most important finding was the strong antiallodynic effect of endomorphins in rats subjected to sciatic nerve crushing.
(Supported by OTKA T 030086, T 002104, T 029460)
[1] Zadina,J.E., Hackler,L., Ge,L.J. and Kastin,A.J., *Nature* 386, 499-502 (1997)

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DESIGN AND SYNTHESIS OF POTENT LINEAR AND CYCLIC ANALOGUES OF THE MYELIN BASIC PROTEIN EPITOPE MBP_{87-99}

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Experimental Autoimmune Encephalomyelitis (EAE) is a demyelinating disease of the central nervous system and an animal model of multiple sclerosis (MS). The immunodominant Myelin Basic Protein peptide 87-99 has been implicated as a candidate antigen in Multiple Sclerosis (MS) by several lines of evidence. In the present report, a linear and a cyclic analogue were designed and synthesized based on the human myelin basic protein (MBP_{87-99}) epitope (Val[87]-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro[99]). These analogues were designed looking for suppressors of EAE induced by guinea pig MBP_{72-85} epitope (Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val) in Lewis rats. The linear analogue [Arg[91], Ala[96]] MBP_{87-99} (Val[87]-His-Phe-Phe-Arg[91]-Asn-Ile-Val-Thr-Ala[96]-Arg-Thr-Pro[99]) in which Arg substitutes Lys[91] and Ala substitutes Pro[96] was found to be a strong inhibitor which when administered to Lewis rats together with the encephalitogenic agonist MBP_{72-85} completely prevented the induction of EAE. Similarly the N,C- termini amide-linked cyclic analogue, cyclic [Arg[91], Ala[96]] MBP_{87-99} was found to be as well a strong inhibitor of EAE. These analogues could be used as drug leads in the design and synthesis of more potent substances for yse in the treatment of MS. The analogues have been synthesized using the Fmoc/tBu methodology and the cyclization was achieved using O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronioum tetrafluoroborate (TBTU), 1-Hydroxy-7-azabenzotriazol and 2,4,6-collidine as cyclization reagents.

SEARCHING FOR LINEAR ANTIBODY EPITOPES OF HSP 60/65 HEAT SHOCK PROTEINS SPECIFIC FOR HIV INFECTION

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Our previous work indicated that autoantibodies could contribute to the immunopathogenesis of HIV infection [1]. Levels of various autoantibodies, including anti-heat-shock protein antibodies, were measured in the sera of HIV seropositive patients, and found to be in correlation with the stage of disease. Assuming that anti-hsp60 antibodies are mainly directed to linear epitopes on the protein we found it interesting to test, whether a distinct set of epitopes can be characteristic for HIV seropositive sera. To localise the potentially antigenic sites we have performed Chou-Fasman secondary structure prediction calculation for β-turns. Hhydrophilic regions of human HSP60 and *Mycobacterium bovis* HSP65 proteins were also detected. Peptides corresponding to the selected regions of these proteins were synthesised in duplicates on Fmoc-β-alanyl-glycine ester functionalised polyethylene pins with Fmoc/tBu chemistry. Side chain deprotected peptides attached to pins were used for testing sera of HIV infected, coronary heart diseased and healthy individuals in modified ELISA experiments [2]. According to our results there are differences between the patterns of reactive peptides detected by antibodies in sera of HIV infected and of healthy individuals.
These studies were supported by grants from the Ministry of Education (FKFP 0101/97) and from National AIDS Committe (NABKP-4/98).
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[2] Prohászka, Z., Daha, M.R., Süsal, C., Daniel, V., Szlávik, J., Bánhegyi, D., Nagy, K., Várkonyi, V., Horváth, A., Ujhelyi, E., Tóth, F.D., Uray, K., Hudecz, F. and Füst, Gy. C1q Autoantibodies in HIV infection: Correlation to elevated levels of autoantibodies against 60-kDa heat-shock proteins. 1999 J. Clin. Immunol., 90: 247-255.

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CYANOBACTERIUM *ANABAENA* 90: BIOACTIVE PEPTIDES AND GENES RESPONSIBLE FOR THEIR PRODUCTION

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In Finland cyanobacteria belonging to the genus *Anabaena* frequently form toxic blooms. One hepatotoxic strain, *Anabaena* 90, isolated from a Finnish lake, was selected as a model strain to study the bioactive peptides and their production. Three classes of cyclic peptides have been isolated from *Anabaena* 90: two types of heptapeptides, microcystins and anabaenopeptilides, and one type of hexapeptides, anabaenopeptins. Microcystins are hepatotoxic and their general structure is cyclo(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), where X and Z are variable L-amino acids, and Adda is (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimetyl-10-phenyldeca-4*E*,6*E*-dienoic acid. Anabaenopeptilides are cyclic depsipeptides and they contain a unique amino acid unit, 3-amino-6-hydroxy-2-piperidone (Ahp). The structural characteristics for anabaenopeptins are the ureido group and one _-amido linkage between two amino acids. Microcystins and other cyclic, cyclic branched, or linear bioactive peptides of bacteria and lower eukaryotes are produced non-ribosomally by multidomain peptide synthetases, employing so called thiotemplate mechanism. Different domains act as independent enzymes whose function is to join one amino acid to the growing polypeptide chain and make possible modifications. The specific order of the domains forms the protein template that defines the sequence of the incorporated amino acids. Possible analogy to the known peptide synthetases was the basis for an approach to clone the peptide synthetase genes from *Anabaena* 90. The lenght of the first characterized peptide synthetase operon is 29 kb. The translation of the DNA sequence shows the modular structure typical to all known peptide synthetases. This gene region from *Anabaena* 90 contains seven amino acid activating domains that belong to the highly conserved family of peptide synthetases. Based on the sequence analysis, it seemed probable that it codes for the synthesis of anabaenopeptilides found in *Anabaena* 90. This was also confirmed by the knock-out experiment. The characterization of the second and the third peptide synthetase operons in *Anabaena* 90 is in progress.

New $ACTH_{4-10}$ peptide analogue "SEMAKS" stimulates regeneration of mouse motor nerve in vivo.

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Several fragments of ACTH have recently been found to exert neurotrophic influence on sensory-motor neuronal and axonal regeneration in vitro and in vivo. In the present study a new synthetic analogue of $ACTH_{4-10}$ i.e decapeptide $ACTH_{4-10}$-Pro-Gly-Pro, has been tested as a possible promotor of axonal regeneration and limb muscle reinnervation after the nerve damage in vivo.

The motor nerve(*n.peroneus*) of mouse hind limb was gently crushed which is followed by full degeneration of distal nerve and regrowth of proximal axonal endings to their original target - i.e. limb skeletal muscles. The procedure of nerve regrowth and muscle reinnervation was shown to take about 7-8 days after the nerve crush. The mice were distributed in 3 groupes: 1st - mice with nerve crushing treated with intraperitoneal SEMAKS injections (50mg/kg); 2nd - mice with nerve crushing treated with intranasal "SEMAKS" injections(50mg/kg); 3-d - sham-operated mice treated with saline(0,9% NaCl) injections. Repeated daily SEMAKS or saline injections commenced at day of surgery and lasted 10 days. At 11-th day after nerve crush the following parameters of regenerated nerve and reinnervated muscle by control and experimental animals vere tested *in vivo*: a) threshold of nerve electrical excitability (TEE); b) angular coefficient (AC) of muscle contraction increment in response to gradual nerve electric stimulation; c) latent period(LP) of integral muscle action potential evoked by nerve stiimulation; d) Light microscopical morphological analysis of nerve cross sections(axons number and their diameter distribution were tested). At the 11-th day after nerve crushing statistically significant run down of mean value of TEE for 23-26%($P<0,05$) in both (1 and 2) experimental groupes($n_1=10$; $n_2=12$) was revealed. Independent on the manner of SEMAKS injections, positive but statistically nonsignificant shifts of AC and LP parameters were also found in both experimental groupes: the mean values of AC increased for 11-12%; the LP shortenes for 10 -12 % ($P>0,05$). Morphological analysis of nerve sections showed, that the mean value of axons number per nerve does not change significantly in experimental groupes, but the the axonal diameter distribution does change evidently: pool of large-diameter motor axones (D> 20μkm) of regenerated nerves increases in statistically significant manner for 18% ($P<0,05$) after chronic SEMAKS injections.

The result show, that chronic SEMAKS administration improved the functional status of regenerating motor nerves. The possible mechnisms related to the beneficial effect of SEMAKS on nerve regeneration and neuro-muscular transmission in regenerated muscle are discussed.

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IN VITRO AND IN VIVO POTENCY OF A NOVEL PHOSPHINIC PEPTIDE INHIBITOR OF MATRIX METALLOPROTEINASES.

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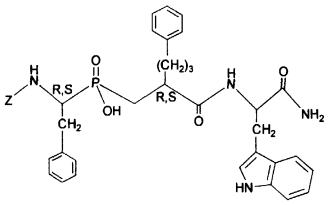
Matrix metalloproteinases (MMPs), also known as matrixins, have been recognized since the early 1980s as therapeutic targets of particular interest. In a previous work[1],the identification of highly potent phosphinic peptides inhibitors for MMPs was described. Among these inhibitors, a phosphinic peptide called RXP03 (Z-$_{(R,S)}$PheΨ(PO_2-CH_2)$_{(R,S)}$pPhe-Trp-NH_2 was selected for further in vivo evaluation.



The four diastereoisomers of RXP03 were prepared and their abilities to inhibit various MMPs were determined. The efficacy of RXP03 F_1 pure diastereoisomer to slow the growth of primary tumors will be reported.

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NEUROTENSIN : SYNTHESIS AND PHARMACOLOGICAL PROPERTIES OF NEW CYCLOPEPTIDIC ANALOGUES

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Neurotensin (NT) is a tridecapeptide (pGlu[1]-Leu[2]-Tyr[3]-Glu[4]-Asn[5]-Lys[6]-Pro[7]-Arg[8]-Arg[9]-Pro[10]-Tyr[11]-Ile[12]-Leu[13]) distributed throughout the central nervous system and the small intestine of the mammals. Structure-activity studies have demonstrated that the minimal sequence needed for full biological activity is the C-terminal hexapeptide (Arg[8]-Arg[9]-Pro[10]-Tyr[11]-Ile[12]-Leu[13]), NT(8-13). This peptide elicits a potent hypothermic and naloxone-insensitive analgesic effects after intracerebroventricular injection but it has been demonstrated that this peptide does not cross the blood-brain barrier. Analgesia induced by neurotensin may present a great interest in the relief of pain. Contrary to morphine and its others opioïds, which are up to date the most efficient compounds to relieve pain, neurotensin doesn't lead to side effects such as physical dependence and tolerance. For this reason, it could be of great importance for therapeutic interest to find active compounds of this family able to develop analgesic activity after intraveineous administration. Cyclisation of peptide is suitable for this purpose. Indeed cyclic structures increase the bioavailability and the resistance towards exopeptidases degradation compared to the linear peptide parent. The synthesis and the biological activities of cyclopeptidic analogues of NT(8-13) will be discussed here.

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ANTIBIOTIC ACTIVITIES OF TEMPORIN A ANALOGS

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Temporin A is a small, basic, highly hydrophobic, antibacterial peptide amide (FLPLIGRVLSGIL-NH2) found in the skin of the European red frog, *Rana temporaria*. It has variable antibiotic activities against a broad spectrum of microorganisms, including clinically important methicillin-sensitive and -resisitant *Staphylococcus aureus* as well as vancomycin-resistant *Enterococcus faecieum* strains. Studies with synthetic analogs of temporin A showed that the following molecular features were important or necessary for antibacterial activity: some characteristic of the N-terminal amino acid, its side chain hydrophobicity and/or its single positive charge; a positive charge at amino acid position 7; bulky hydrophobic side chains at positions 5 and 12. Replacing isoleucine with leucine at amino acid positions 5 and 12 resulted in the greatest enhancement of antibacterial activity. Also, there was little difference between the activities of temporin A and its all-D enantiomer, indicating that the peptide probably exerts its effect on bacteria via nonchiral interactions with membrane lipids.

INTERACTION OF THE VASOACTIVE INTESTINAL PEPTIDE Asp^3 WITH THE HUMAN $VPAC_1$ RECEPTOR Arg^{188}.

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Vasoactive Intestinal Peptide (VIP) receptors belong to a G protein coupled receptor family with no sequence homology to rhodopsin. An arginine or lysine residue is highly conserved in the second putative transmembrane domain, except in the calcitonin and Calcitonin Gene Related Peptide (CGRP) receptors where it is replaced by an asparagine. We tested in this work the hypothesis that it participates to the recognition of the ligand or to signal transduction. [Arg188Leu] and [Arg188Gln] mutant $VPAC_1$ receptors were constructed and stably expressed in transfected CHO cells. These mutant receptors had a reduced affinity for VIP with an Asp residue in position 3 (from 1nM to >50 nM) and an increased affinity for [Asn^3] and [Gln^3] VIP analogues. They also had a significantly greater affinity for the VIP fragments VIP (4-28) than for VIP fragments (2-28) and (3-28), that retained the aspartate residue. These results supported the hypothesis that the positive charge of Arginine 188 is necessary to facilitate the introduction of the VIP aspartate^3 anion in a hydrophobic environment.
VIP behaved as a partial agonist compared to [Asn^3]-VIP adenylate cyclase stimulation. This result, together with the observation that VIP modifications in position 1 had a reduced impact on VIP recognition by the mutant receptor suggests, that Asp^3 participates in the anchoring of the whole N-terminal VIP region, necessary for receptor activation.

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STUDY ON STRUCTURE ACTIVITY RELATIONSHIP OF A NOVEL SPIDER TOXIN NPTX-594 FROM *NEPHILA MADAGASCARIENCIS*

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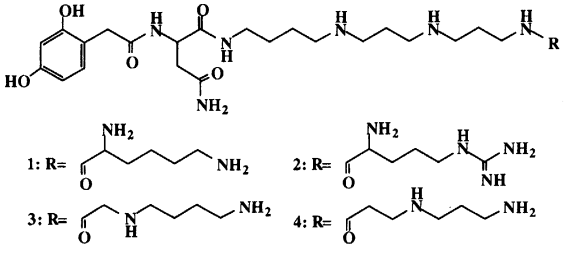
Spider toxins seem to be a good tool for elucidating the mechanism of glutamatergic neuromuscular transmission in the brain. We recently synthesized a novel acyl-polyamine toxin termed NPTX-594 (**1**) from the venom of *Nephila madagascariencis*, a Madagascarian Joro spider. NPTX-594 is comprised of four residues, i.e., 2,4-dihydroxyphenylacetic acid (Dhpa), asparagine, 4,8-diaza-1,12-dodecanediamine (Dada), and lysine. So far SAR study of NPTX containing Dada as a polyamine has not been carried out yet. In the present paper we will present the synthesis of several NPTX-594 analogs **2** – **4** and their biological activity.



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SYNTHESIS, STRUCTURE-ACTIVITY RELATIONSHIP OF NOCICEPTIN AND ITS FRAGMENTS

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Nociceptin is a new kind of endogenous opioid peptide and was discovered in 1995. It shares some structural homology with the known opioid peptides in N terminal sequence (nociceptin: PheGlyGlyPheThrGlyAlaArgLysSerAlaArgLysLeuAlaAsnGln-OH) and differs from them in some physiological activities such as inhibition to the electrically evoked contractions in mouse vas deferens (MVD) with a naloxone insensitive mechanism and the hyperalgesia activity. We designed and synthesized its four fragments and an analog, which were abbreviated as $NC(1\text{-}15)NH_2$, $NC(1\text{-}13)NH_2$, $NC(1\text{-}11)NH_2$, $NC(1\text{-}5)NH_2$ and $[Tyr^1]NC(1\text{-}13)NH_2$. The opioid activity assay showed that $NC(1\text{-}13)NH_2$ is the smallest fragment which shares whole activity of nociceptin. $[Tyr^1]NC(1\text{-}13)NH_2$ had affinity with both ORL1 receptor and μ receptor, and showed hyperalgesia activity and could antagonize the analgesia activity caused by morphin in mouse tail flick test (i.c.v) but it also could inhibit the electrically evoked contractions in guinea pig ileum (GPI) assay *in vitro*. The results of vasodilatation test showed that $[Tyr^1]NC(1\text{-}13)NH_2$ had the hypotensive activity *in vivo* and small artery expansion activity *in vitro* with a naloxone insensitive mechanism. All the results showed that the last four residues (LeuAlaAsnGln) in C terminal of nociceptin were not necessary for all physiological activity. The residue Phe^1 in N terminal acted an important role in the receptor selectivity.

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LONG ACTING, SELECTIVE, PERIPHERAL KAPPA AGONISTS

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Selective κ-opioid agonists offer great promise for the treatment of pain without the side effects commonly associated with μ-opioid agonists. However, non-peptidic κ agonists developed to date exhibit CNS penetration, which limits their clinical application. By contrast, selective peptidic κ agonists could result in peripheral compounds devoid of CNS side effects.

Selective peptide κ-agonists have been recently identified by screening positional scanning format mixtures of a tetrapeptide combinatorial library. The all D-amino acid tetrapeptide H-D-Phe-D-Phe-D-Nle-D-Arg-NH_2 (FE 200041) exhibited high affinity, selectivity, and agonist activity for the human κ-opioid receptor (KOR) *vs.* μ (MOR) and δ opioid receptors *in vitro*. In order to develop potent and selective peripheral κ-agonists with long duration of action *in vivo*, we prepared a series of secondary and tertiary tetrapeptide amides based on the FE 200041 lead and tested them for antinociceptive potency in the mouse acetic acid writhing test (mWT) and the rat formalin flinch assay, and for sedation in the mouse rotarod test (mRT).

Over a hundred N-alkylamides were synthesized by solid phase/solution phase peptide chemistry. Fully protected tetrapeptide acids were prepared using 2-chlorotrityl chloride resin and then coupled to a series of diverse amines by the mixed anhydride method. The protective groups were removed, and the peptides were purified by preparative HPLC. Derivatization of the C-terminus resulted in analogs that retained excellent affinity, selectivity, and agonist activity at KOR with significantly improved duration of action *in vivo*. The SAR for the series will be presented. Two long acting compounds: H-D-Phe-D-Phe-D-Nle-D-Arg-NH-4-Pic (FE 200665) and H-D-Phe-D-Phe-D-Leu-D-Orn-morpholine amide (FE 200666) had K_i at KOR 0.24 and 0.08 nM, with a selectivity index for KOR vs MOR of 16,000 and 88,000, respectively. A_{50} in the mWT was 7 and 12 μg/kg, i.v. for FE 200665 and FE 200666, respectively. The periphery selectivity index (ratio of A_{50} values in the mRT and mWT) was 643 and 84, being 322- and 42-fold higher than the one of the non-peptidic KOR agonist enadoline. In conclusion, both FE 200665 and FE 200666 exhibit unprecedented affinity and selectivity at KOR with high antinociceptive potency and excellent peripheral selectivity.

TRYPSIN HYDROLYSIS OF GH-RH ANALOGUES

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Growth hormone-releasing hormone(1-29)-NH_2 stimulates the release of growth hormone (GH) from the pituitary gland and accelerates growth velocity in GH-deficient children. However, this peptide, and many analogues which are potent *in vitro*, are rapidly cleaved in plasma, mainly by dipeptidylpeptidase IV and trypsin-like enzymes, to form inactive products. It has been demonstrated that trypsin-like degradation in human plasma was prevented by trypsin inhibitor, Trasylol [1]. In order to obtain information about hydrolysis at 4 basic amino acid residues (2Arg and 2Lys) we studied digestion of [Nle^{27}]-GH-RH(1-29)-NH_2 (TyrAlaAspAlaIlePheThrAsnSerTyrArg^{11}Lys^{12}ValLeuGlyGlnLeuSerAlaArg^{20}-Lys^{21}LeuLeuGlnAspIleNleSerArg-NH_2) (**I**) and its $Orn^{12,21}$ analogue (**II**) by trypsin. The course of degradation was studied using RP-HPLC and ESI-MS of separated samples. Several intermediates and final degradation products were identified and conclusions concerning the rate of cleavages at different positions in **I** was drawn. It was observed that replacement of Lys in position 12 and 21 by Orn resulted in the elimination of 12-13 and 21-22 cleavages in **II**. This finding explains the extremely high potency *in vivo* of several Orn analogues of GH-RH(1-29)-NH_2 obtained earlier [2] and justifies the incorporation of this replacement to receive new potent and long acting analogues.

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DESIGN, SYNTHESIS, CONFORMATION, DNA-BINDING CHARACTERISTICS, AND ANTITUMOR AND TOXICITY ACTIVITY OF ACTINOMYCIN D ANALOGS

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Actinomycin D (ActD), containing a planar phenoxazone ring and two cyclic pentapeptides, is one of the most intensely studied anticancer drugs and is currently used to treat highly malignant tumors such as Wilms'tumor and gestational choriocarcinoma. It has been well known that the antitumor activity of ActD is due to its ability to bind to double-stranded DNA, resulting in the inhibition of DNA-directed RNA synthesis. Although ActD possesses high antitumor activities, its clinical usefulness is limited by its extreme cytotoxicity. Thus, if the structure of ActD can be modified to reduce its cytotoxicity while retaining its activity, such an analog would be a better antitumor drug. Based on the results of X-ray crystal structures of ActD-DNA complexes, we designed and totally synthesized a series of ActD analogs in which both of *N*-methylvaline residues of cyclic pentapeptides lactones were replaced with *N*-methyl-L(D)-phenylalanines, *N*-methyl-L(D)-alanines, *N*-methyl-L-tyrosine, and *N*-methyl-L-serine. The circular dichroism (CD) spectra of the analogs suggested that they had the similar conformation with ActD. Difference spectra of the drugs with Calf thymus DNA showed that all of the analogs intercalated strongly to the DNA as ActD did, which was exactly supported by the association constants (K's) with the intercalation mode, calculated from the UV absorption spectra of ActD and its analogs titrated with Calf-thymus DNA. The antitumor activities and toxicity of the analogs also were examined. [This work was supported by National Natural Science Foundation of China and Natural Science Foundation of Gansu province, China.]

N-CAPROYL-L-PROLYL-L-TYROSINE METHYL ESTER - POTENTIAL ANTIPSYCHOTIC AGENT DISCOVERED THROUGH THE PEPTIDE DRUG DESIGN

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The initial point in peptide design of the new potential antipsychotic group was the assumption that neuroleptic sulpiride in terms of its structural and pharmacological properties could be proposed as a nonpeptide analogue of some endogenous peptide with the neuroleptic-like properties. Then the series of sulpiride topological analogues of peptide nature was developed and Pro-Tyr-NH_2 proved the most active compound in the number of antidopamine tests. This fact taken together with the existence of the fragment Pro^{10}-Tyr^{11} in the sequence of endogenous peptide neurotensin (NT) possessing some pharmacological properties of neuroleptics provides certain reasons to propose that sulpiride is a mimetic of neurotensin. Using the information about SAR of NT analogues and its bioactive conformation on the basis of Pro-Tyr-NH_2 structure the series of N-acylprolyltyrosines, tripeptoid analogues of NT, have been obtained. Inserting of N-acyl group that imitates the side chain Leu^{13} resulted in potency increase. One of the most active compounds, N-caproyl-L-prolyl-L-tyrosine methyl ester, was chosen as a potential antipsychotic agent and studied more comprehendly. This peptide demonstrates the antagonistic activity on apomorphine-induced climbing and on L-DOPA-dependent extrapolatory behavior at doses ranging between 0,4-4,0 mg/kg ip. At this dose interval the compound has no effect on the locomotor activity, coordination, emotional and pain sensitive levels. It did not provoke a cataleptogenic effect or lethality, even at doses much higher than those causing the antidopamine effects (more than 500 mg/kg ip). Antidopamine effect of N-caproylprolyltyrisine methyl ester is stereoselective and the L-configuration of both proline and tyrosine residues is important. It is concluded that N-caproyl-L-prolyl-L-tyrosine methyl ester is a potential atypical antipsychotic agent.

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GROWTH HORMONE-RELEASING HORMONE ANTAGONISTS CONTAINING LACTAM BRIDGE CONSTRAINTS

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The results of recent oncological studies suggest a possible application of antagonists of human growth hormone-releasing hormone (hGH-RH) in the treatment of various insulin-like growth factor (IGF) I- and II-dependent tumors. Previously, we reported the synthesis of various antagonists of GH-RH with high and protracted *in vitro* and *in vivo* activities. Accumulating data of structure-activity relationship of hGH-RH analogues revealed that an amphiphilic helical structure plays an important role in the high receptor affinities and high agonistic activities of GH-RH(1-29) analogues. Based on the fact that *i*-(*i*+4) lactam bridge enhances the helicity of the central region of GH-RH(1-29), 10 new antagonistic analogues were synthesized. The design approach of the new GH-RH antagonists of this series was based on the constrained secondary structure introduced by a lactam bridge into prior generations of GH-RH antagonists. All the new analogs contained D-Arg^2, Cpa^6, Ala^{15} or Abu ^{15}, and Nle ^{27} substitutions in the GH-RH(1-29)NH_2 and were acylated with phenylacetic acid. The *i*-(*i*+4) lactam bridges were formed between residues 8-12, 16-20, 17-21, 25-29, and between both 8-12 and 21-25. The new GH-RH antagonists were synthesized by solid phase methods, then purified, and analyzed. The ability of the analogs to inhibit GH-RH induced GH release was evaluated *in vitro* in a superfused rat pituitary system. Five peptides at 30 nM dose inhibited more powerfully the hGH-RH-induced GH release than the standard antagonist Ac-[D-Arg^2]hGH-RH(1-29)NH_2 at 100 nM dose, but their potencies were less than those of other powerful antagonists synthesized previously. Among the lactam bridge analogs, cyclo^{17,21}[PhAc^0, D-Arg^2, Cpa^6, Ser^8, Ala^{15}, Glu^{17}, Nle^{27}]hGH-RH(1-29)NH_2 and cyclo$^{8,12;\ 21,25}$[PhAc^0, D-Arg^2, Cpa^6, Glu^{8,25}, Abu^{15}, Nle^{27}]hGH-RH(1-28)Agm were found to be the most potent *in vitro* and also showed long-lasting inhibiting potencies. They inhibited GH release 90 min after administration to a similar extent as the standard antagonist at 0 min.

CHARACTERIZATION OF AN INTEGRIN $\alpha_v\beta_3$ BINDING SEQUENCE OF PEX, A DOMAIN OF MATRIX METALLOPROTEINASE MMP-2

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Matrix metalloproteinase-2 (MMP-2) is a matrix-degrading enzyme, which facilitates cell migration in invasive processes, such as tumor metastasis and angiogenesis [1]. Localization of MMP-2 to cell surface by interaction with integrin $\alpha_v\beta_3$ [2], and disruption of angiogenesis by a non-catalytic MMP-2 fragment, called PEX, has been shown [3]. In search for efficient inhibitors of MMP-2 / $\alpha_v\beta_3$ interaction, it was of interest to us, to define the sequence of PEX, which binds to integrin $\alpha_v\beta_3$.

For this purpose, epitope-mapping by the SPOT-technique [4] was used. The PEX-sequence was synthesized as a set of cellulose-bound, overlapping octapeptides via Fmoc-strategy. This technique was extended by the convenient use of Fmoc-fluorescence to determine the course of peptide synthesis without loss of material for analysis. A receptor binding test was developed by using fluoresceine-labelled integrin $\alpha_v\beta_3$, which directly interacted with the cellulose bound peptides. Evaluation of fluorescent spots led to a set of binding peptides, which were synthesized on polystyrene and examined in a covalent microtiter plate based binding assay [5]. As a result, three active peptides were confirmed to bind to $\alpha_v\beta_3$. Two of these peptides are located in a continuous sequence, which is solvent exposed on the surface of PEX, as can be visualized with the x-ray structure of the C-terminal domain of MMP-2 [6].

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