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Review: Josef Rudinger Memorial Lecture 2002‡

Peptide Related Drug Research

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Abstract: Drug discovery directed peptide research has been pursued at the IVAX Drug Research Institute (formerly Institute for Drug Research) (IDR) since the mid 1950s. Outlined are the main projects and the most significant results, which include the first synthesis of human ACTH, the discovery of GYKI-14 166, the prototype of peptide inhibitors of thrombin, a stable anticoagulant, efegatran GYKI-14 766, and their dual acting analogues. The identification of an agonist analogue of LHRH leading to Cetrorelix, an LHRH antagonist now in clinical use, is also presented. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: corticotropin; anticoagulants; LHRH analogues; enkephalin analogues; D-amino acid residues; caspases; Cetrorelix

INTRODUCTION

I was particularly pleased to receive this Award because I knew Josef Rudinger, liked him, and had admired his professional excellence and scientific insights since our first meeting in 1959.

In my memorial lecture, my plan was to outline the drug discovery directed peptide research in my laboratory at the IVAX Drug Research Institute (formerly Institute for Drug Research) (IDR) since the late 1950s. Here I give a somewhat extended version of my lecture.

THE BEGINNING OF PEPTIDE SYNTHESIS AT IDR

The isolation and sequencing of the vast number of naturally occurring peptides started in the early 1950s with the identification of two neurohypophyseal hormones, the two closely related octapeptides: oxytocin [H-c(Cys-Tyr-Ile-Gln-Asn-Cys)-Pro-Leu-Gly-NH_2] and vasopressin [H-c(Cys-Phe-Ile-Gln-Asn-Cys)-Pro-Arg-Gly-NH_2]. Of these, oxytocin — as an extract of farm animal pituitary — had been used since the 1930s for medical purposes, namely for inducing the onset of labour. The successful first synthesis of oxytocin by du Vigneaud in 1953 [1] initiated research on synthetic peptides all over the world including Hungary.

Miklos Bodanszky, a leading scientist at the IDR at that time, immediately realized that the pharmaceutical industry could benefit from the results of du Vigneaud: Synthetic oxytocin could substitute the mixture of oxytocin and vasopressin extracted from bovine pituitary produced by Gedeon Richter as Glanduitrin. Furthermore, the fact that closely related peptide structures exhibited distinct hormonal activities with fairly good specificity might indicate that peptide synthesis would open new avenues to drug design. Thus, Bodanszky's group immediately reproduced the original procedure [cf. ref. 2], and made some improvements,

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‡ 27th European Peptide Symposium 31 August 2002. The Award was shared with Kálmán Medzihradszky, whose lecture is the following article.

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BIOGRAPHY

Sándor Bajusz has been the leader of synthetic peptide research at the Institute for Drug Research (IDR) (present name: IVAX Drug Research Institute) in Budapest since 1958. During those years he has investigated peptide hormones, opioid peptides and enzyme inhibitors. Dr Bajusz obtained his diploma in chemistry and physics at the University of Szeged, Hungary in 1954. In 1955 he joined Miklos Bodanszky's peptide group at the Institute for Drug Research, Budapest. His research in peptide chemistry led to a Ph.D. degree in 1968 and to a D.Sc. degree in 1980. From 1985 to 1988 he was working as visiting professor with Dr A.V. Schally at the Endocrine, Polypeptide and Cancer Institute at the Department of Medicine, Tulane University School of Medicine, New Orleans, LA, USA. In 1986 he was appointed to titular professor of Medicinal Chemistry at the University of Szeged. To date he has published over 180 research papers. Awards: State Prize for the synthesis of human ACTH, 1970; Széchenyi Prize for the synthesis of anticoagulant peptides, 1992.

elaborating a modified process for Richter. As a result, Richter was the second company in the world to produce synthetic oxytocin for therapeutic purposes.

In 1955 when I had the privilege of joining Dr Bodanszky's team, the development of an improved synthesis of oxytocin was in progress and the p-nitrophenyl ester coupling [3] had already been invented. I enjoyed very much the invaluable education in peptide chemistry I had from him until January 1957, when Dr Bodanszky, my highly esteemed mentor, fled the country.

From the mid 1950s IDR was involved in various projects on synthetic peptides. These together with the most significant results achieved are shown in Table 1, which are discussed below with a more detailed account of our recent observations on the dual acting anticoagulants and the discovery of Cetrorelix.

FIRST SYNTHESIS OF HUMAN ACTH

Treatment with ACTH preparations of animal origin frequently caused anaphylactic reactions. The synthetic human-ACTH [4,5] with the structure published by Lee *et al.* [18], was found, as expected, to be well tolerated by patients sensitive to other ACTH preparations [19]. A few years later, it turned out that the structure of ACTH of each species had been erroneously determined within the variable sequence 25–33. Correction of the human and porcine sequences (Figure 1) was first published by

Table 1 Main Projects and the Most Significant Results Achieved at the IDR

Pituitary hormones
1. First synthesis of human corticotropin, α_h-ACTH, a 39-residue polypeptide—for human therapy [4, 5]

Antithrombotic peptides
2. Discovery of the prototype of peptide inhibitors of thrombin (GYKI-14 166) using D-amino acid incorporation for a novel purpose [6, 7]
3. Development of a stable anticoagulant, efegatran GYKI-14 766, an analogue of GYKI-14 166 [8]
4. Discovery of the first noncovalent peptide inhibitor of thrombin (GYKI-14 525) [9]
5. Development of dual acting anticoagulants: efegatran analogues targeting both thrombin and factor Xa [10, 11]

Opioid peptides
6. Preparation of the first enkephalin analogue (GYKI-14 238) possessing analgesic activity upon systemic administration [12]

Caspases: cysteine proteases with aspartate specificity
7. Interpretation of the tolerance of caspase-1 (ICE) for D-stereochemistry at P_1 [13]
8. Finding that peptidyl β-homo-aspartals are potent inhibitors of caspase 1 and caspase-3 [14]

Hypothalamic hormones
9. Development of an agonistic analogue of LHRH (GYKI-14 201) [15] that provided a model for Cetrorelix, SB-75, an LHRH antagonist [16,17], now in clinical use

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25 26 27 30 31 33

old | α_h-ACTH - Asp-Ala-Gly-Glu-Asp-Gln-**Ser**-Ala-Glu

corrected | α_h-ACTH - *Asn-Gly-Ala*-Glu-Asp-*Glu*-**Ser**-Ala-Glu

corrected | α_p-ACTH - *Asn*-Gly-Ala-Glu-Asp-*Glu*-**Leu**-Ala-Glu

old | α_p-ACTH - Asp-Gly-Ala-Glu-Asp-Gln-**Leu**-Ala-Glu

Figure 1 Sequence 25–33 of α_h- and α_p-ACTHs: corrections are in italics, residues characteristic of the species in bold.

Gráf *et al.* from the IDR [20]. Thus, our synthetic 'human ACTH' also had the wrong residues in four positions but Ser-31, the only characteristic residue of human ACTH, was correct. This explained the lack of an anaphylactic side effect. Thereafter human ACTH with the corrected sequence 25–33 was also synthesized [21].

These results were achieved in collaboration with Kálmán Medzihradszky of Eötvös Loránd University and Lajos Kisfaludy of Gedeon Richter. Our team assembled from the three institutions worked under the supervision of the late and greatly respected Professor Victor Bruckner, a pioneer of peptide research, who isolated and characterized the capsular substance of *Bacillus anthracis* [22,23].

DISCOVERY OF THE PROTOTYPE OF THE PEPTIDE INHIBITORS OF THROMBIN, GYKI-14 166 (D-Phe-Pro-Arg-H)

GYKI-14 166 was the first highly potent peptide inhibitor of thrombin. This highly specific serine protease is the central mediator of thrombus formation in the pathogenesis of thrombotic diseases. The pharmacological control of thrombosis is based on thrombin inhibition. The following considerations led to the structure of D-Phe-Pro-Arg-H [6,7].

It was known that substrate related peptide aldehydes could inhibit serine proteases. Thus the structure of thrombin inhibitors was derived from the thrombin cleavage sites of its substrates, the clotting factors, of which fibrinogen (factor I), prothrombin (factor II) and factor XIII had been described at that time (Figure 2).

It was observed that thrombin cleaves substrates after both Val-Arg and Pro-Arg sequences. Based on this we, unlike others, prepared peptidyl arginals with both Val and Pro at P_2, and examined their inhibitory activity/effect on the thrombin-fibrinogen reaction (thrombin induced coagulation of citrate plasma). The P_2 Pro-containing congeners were the more inhibitory, and Phe-Pro-Arg-H was the most potent.

It should be mentioned here that a number of reversible and irreversible inhibitors of thrombin have been derived from the sequence of Phe-Pro-Arg [24]. In spite of its structural difference from the thrombin cleavage site of fibrinogen it is frequently referred to as a 'fibrinogen-like sequence' [25,26] (Figure 2). Pro-Arg in P'_2–P'_3 is of no importance in thrombin active site recognition: no cleavage occurs after the Pro-Arg sequence of fibrinogen. It is very likely that the interaction between the *negative charge cluster* of fibrinogen and the *anion binding exosite* of thrombin directs the cleavage site to the Arg-Gly bond.

To increase the inhibitory potential of Phe-Pro-Arg-H, we substituted Phe with D-amino acid residues, i.e, D-Ala, D-Val, D-Ale, D-Phe, in the hope that the unnatural D residue incorporated into the P_3 would provide an unnatural, extra binding site for the enzyme. We expected an increased stability of the thrombin–inhibitor complex in the presence of fibrinogen, the native substrate. Of these analogues, D-Phe-Pro-Arg-H (GYKI-14 166) proved to be the most inhibitory as though the *N*-terminal residue was involved in a hydrophobic interaction with some hydrophobic side chain of thrombin. Some 15 years later x-ray studies with D-Phe-Pro-Arg-CH_2-Cl/thrombin [27] revealed such an interaction between the benzyl side chain of D-Phe and a hydrophobic cavity in thrombin.

It had been known since 1966 that D-amino acid substitution might increase the biological activity of peptide hormones. In the first examples, substitution of the *N*-terminal Ser^1 of ACTH fragments (1–25) [28] and (1–24) [29] with D-Ser resulted in a 5–6-fold increase of the activity. This elevated activity of the analogues was explained by increased

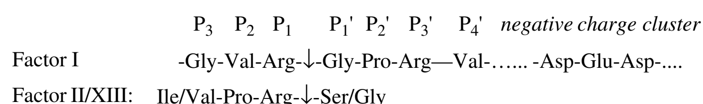

Figure 2 Thrombin cleavage sites of clotting factors I, II and XIII.

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enzyme resistance [28] or structural alterations that influenced the rate of adsorption, metabolism or transport to the target organ [29]. A further example [30] was the replacement of Gly^6 with D-Ala in LHRH, pGlu-His-Trp-Ser-Tyr-Gly^6-Leu-Arg-Pro-Gly-NH_2, which resulted in a 3.5–4.5-fold increase of the activity, while the L-Ala^6 analogue was almost inactive. Since [D-Ala^6]LHRH showed high activity both *in vitro* and *in vivo* the increased enzyme resistance could not be an explanation, rather it was interpreted by conformational changes, i.e. D-Ala^6 could stabilize a conformation favourable for binding, and hence activity at the receptor. It was also noted that 'the methyl group of the D-alanine side chain could not likely be implicated in a process involving functionality'.

In our opinion, contrary to the note cited above, *the side chain of any suitable D-amino acid substituent could be implicated in a process involving functionality*. Thus, we suppose that even D-Ala^6 of LHRH can bind to a normally dormant side chain of the receptor and the formation of such an extra bond will result in increased activity. Just as we speculated for the design of D-Phe-Pro-Arg-H.

ANTICOAGULANT EFEGATRAN (GYKI-14 766) (D-MePhe-Pro-Arg-H, LY294468)

Stability studies revealed that D-Phe-Pro-Arg-H was prone to spontaneous inactivation by intramolecular condensation leading to 5,6,9,10,10a-hexahydro-2-(3′-guanidino-propyl)-5-benzyl-6-oxoimidazolo[1,2*a*] pyrrolo[2,1-*c*]pyrazine [8]. Since both H atoms of the terminal NH_2 seemed to participate in this transformation, N-alkyl derivatives were prepared. Of these, D-MePhe-Pro-Arg-H possessed high and selective thrombin inhibiting activity, even *in vivo*. These observations made it of potential clinical importance. Later, this compound was licensed to Eli Lilly Company, named as efegatran with a code number LY294468, and entered clinical trials [31].

Efegatran was well tolerated at all dose levels adminstered and no significant prolongation of bleeding time was observed. Efegatran infusions produced rapid onset, dose-dependent prolongation of clotting time with no accumulation of anticoagulant effect (observed with hirudin [32]), and a rapid return to baseline activity after termination of infusion, i.e. without rebound phenomenon (found with argatroban [33]). Adjunctive efficacy of efegatran appeared to be similar to that of heparin, however, efegatran is obviously free of *heparin-induced thrombocytopenia*, the severe complications of heparin therapy, that appear in about 5% of heparin-treated patients [34].

As is usual in clinical studies, evaluation was based on data on the primary end-point, which means a consideration of the events occurring — death, reinfarction or the development of heart failure at 30 days. These data did not show greater than a 30% difference between heparin and efegatran and, therefore, the clinical trials were terminated.

THE FIRST NONCOVALENT INHIBITOR OF THROMBIN: D-Phe-Pro-Agm (GYKI-14 525)

Peptide D-Phe-Pro-Agm GYKI-14 525 is an agmatine (Agm, des-carboxy arginine) analogue of D-Phe-Pro-Arg-H GYKI-14 166. Although this compound has no aldehyde function, it showed significant anticoagulant activity both *in vitro* and *in vivo* [9]. This indicated that an appropriate structure, like side chain combination in this compound, could be inhibitory even without a serine trap. Most of the modern inhibitors of thrombin are of this type [35, 36]; sad to say, the significance of D-Phe-Pro-Agm was not explored at the time of its discovery, some 20 years ago.

DUAL ACTING ANTICOAGULANTS: EFEGATRAN ANALOGUES TARGETING BOTH THROMBIN AND FACTOR Xa

During blood coagulation, the operating enzymes are incorporated into the clots. Thus plasma clots can hydrolyse the substrates of entrapped enzymes if they are clot permeable, such as Tos-Gly-Pro-Arg-pNA and Moc-D-Chg-Gly-Arg-pNA ($\leq$500 $Å^3$); Bz-Ile-Glu-Gly-Arg-pNA ($>$625 $Å^3$) is not cleaved. Upon disintegration of the clots, the entrapped enzymes are released into the plasma where *both thrombin (factor IIa) and factor Xa will induce the formation of secondary clots*, i.e. thrombolysis requires the simultaneous inhibition of both factors IIa and Xa. Recently we have found [10,11] that some efegatran analogues (P_3-Pro-Arg-H) having ethoxycarbonyl D-amino acid or D-α-hydroxy acid residues at P_3 can inhibit the amidolytic activity of both factors IIa and Xa. Examples of this are the cyclobutylalanine (Cba) and cycloheptylglycolic acid (cHga) analogues:

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Eoc-D-Cba-Pro-Arg-H (GYKI-66 319) and D-cHga-Pro-Arg-H (GYKI-66 323).

Dual Acting Anticoagulants and Disseminated Intravascular Coagulation

Efegatran (D-MePhe-Pro-Arg-H, **1**) our thrombin-specific anticoagulant and some of its dual acting analogues have recently been examined for their effects on disseminated intravascular coagulation (DIC). This is a systemic thrombohaemorrhagic disorder in which both plasmin and thrombin are present in the circulation. Plasmin, being a global protease, degrades various plasma proteins such as fibrinogen, other coagulation factors, hormones, etc. Thrombin induces coagulation in the circulating blood producing microclots that may cause multiorgan failure. DIC can be provoked experimentally by lipopolysaccharide (LPS) in animal experiments.

During the administration of peptide anticoagulants **1**–**4** to rabbits treated with a sublethal dose of LPS, we observed that most compounds increased the lethality in the animals (Table 2). The one that reduced lethality, **4**, was a good anticoagulant in the APTT assay, and greatly inhibited thrombin and factor Xa within the plasma clots, i.e. it was an efficient dual acting anticoagulant, which also showed inhibitory activity on the fibrinolytic enzymes.

Further studies included peptidyl arginals as well as non-covalent inhibitors with a *C*-terminal agmatine (Agm) or a 4-aminomethylbenzamidine (Pab) moiety (see Table 3). Melagatran [37] and the related peptide **11** were included as references. The van der Waals volume of each compound was <500 Å^3.

The findings presented in Table 3 indicate that replacement of Pab or Agm for Arg-H improved the anticoagulant activity, particularly in the TT assay, which only measures thrombin inhibition on fibrinogen. However, inhibition of a peptide substrate favours Arg-H. In the case of the pair of compounds, **4** and **5**, the arginal was 2.2 times less active than the Pab-peptide in the TT, but 18.9 times more active in fIIa inhibition in buffer (it is a further example of the substrate dependence of protease-inhibiting efficacy of small-molecule peptide inhibitors [7]). Pab- and Agm-peptides are virtually not inhibitory against factor Xa and the fibrinolytic enzymes, and are not clot permeable. Accordingly, melagatran, like the related peptide **11**, is an exception and has an activity pattern more or less similar to those of arginals **4**, **6** and **8**.

Analysis of the anticoagulant effect in the APTT assay, the inhibition of thrombin and factor Xa within the plasma clot, and the inhibitory effects on fibrinolytic enzymes presented in Table 3 showed that **4**, **6** and **8** are more efficient than melagatran (or efegatran, no. **1** in Table 2). Accordingly, **4**, **6** and **8** could be expected and were found to be active in the rat DIC model using a lethal dose of LPS.

Table 2 Effect of Efegatran (1) and Analogues (2–4) on Lethality in LPS-treated Rabbits (31%), and their Inhibitory Activities on Plasma Coagulation, Thrombin (fIIa) and Factor Xa (fXa) in Buffer and within Plasma Clot, and on Fibrinolytic Enzymes[a]

No.[b]	Lethality	Coagulation CT_2 (nM)		Factor inhibition, IC_{50} (nM)				Fibrinolytic enzymes LA_{50} (μM)		
				Buffer		Plasma clot				
		TT	APTT	fIIa	fXa	fIIa	fXa	PL	tPA	UK
1	36[c]	87	622	2	9000	375	1000	54	132	82
2	42	114	349	3	90	390	702	39	27	120
3	56	69	429	9	42	2400	300	75	41	43
4	16	147	331	54	97	103	118	18	10	14

[a] CT_2, concentration needed to double clotting time in the TT (thrombin time) and APTT (activated partial thromboplastin time) assays; IC_{50} values were determined by chromogenic substrates Tos-Gly-Pro-Arg-pNA (thrombin) and Moc-D-Chg-Gly-Arg-pNA (factor Xa); LA_{50}, concentration required to reduce the lytic area to 50% of the control on a fibrin-plate; PL, plasmin; tPA, tissue plasminogen activator; UK, urokinase.
[b] **1**, Efegatran: D-MePhe-Pro-Arg-H; P_3 in analogues: **2**, D-cyclobutylalanine; **3**, D-homophenyllactic acid; **4**, ethoxycarbonyl-D-cycloheptylalanine.
[c] Treatment with heparin results in a similar slight increase of lethality.

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Comparative data on **8**, melagatran and efegatran in this assay are shown in Table 4.

The activity profile of compound **8** may indicate that there is a link between DIC-inhibition and good anticoagulant activity as measured by the APTT assay, including significant inhibitory effects on clot bound thrombin and factor Xa, and a certain inhibitory action on the three fibrinolytic enzymes measured in the fibrin plate assay. (These latter results may reflect one or more proteolytic reaction(s), which is (are) not related to fibrinolysis).

ENKEPHALIN ANALOGUE Tyr-D-Met-Gly-Phe-Pro-NH_2(GYKI-14 238)

In 1975 peptide ligands for the opiate receptor were isolated, namely two pentapeptides of pig brain named Leu and Met enkephalin in allusion to their origin [38], and a 31-peptide of camel pituitary (β-endorphin) suggesting that it would be an endogenous pain-killer [39].

As the data of Table 5 demonstrate the peptides showed the typical *in vitro* activities of morphine in the GPI (guinea-pig ileum) and MVD (mouse vas deferens) assays, which measure the effects mediated by receptor subtypes μ and δ, respectively, although with a different intensity and activity pattern. It should be noted that β-endorphin was more analgesic than morphine, particularly in mice, and the analgesic activity of enkephalins was very weak and only appeared if given centrally. This phenomenon was explained by the rapid metabolism of enkephalins. In our opinion, such an explanation was untenable because several peptides of similar size are active upon systemic administration. Peptides while being in the blood will not be hydrolysed because plasma does not contain proteases but rather protease inhibitors (>60 μM) [40]. Instead are considered the inept binding properties; the relative receptor binding for Met-enkephalin, β-endorphin and morphine were 0.28, 9.6 and 1, respectively [41].

To improve the binding properties — and thereby the opiate activity of enkephalin — we substituted Gly^2 and then Gly^3 with D-Ala while Met was replaced with Ile and Pro, which were known to form stronger hydrophobic bonds than Met. D-Ala in place of Gly^2 and Pro at the *C* terminus proved to be favourable, and amidation of the peptide further increased activity. Therefore [D-Ala^2,Pro^5]enkephalin amide was further modified. D-Phe or branched amino acids were found unfavourable, straight side chain residues, in

Table 3 Inhibitory Effects of Peptide Inhibitors P_3-Pro-P_1 (**4**–**10**), Melagatran, and Related Peptide **11** on Plasma Coagulation, Thrombin (fIIa) and Factor Xa (fXa) in Buffer and Plasma Clot, and on Fibrinolytic Enzymes[a]

No.	Peptide inhibitors P_3-Pro-P_1[b]		Coagulation CT_2 (nM)		Factor inhibition IC_{50}, nM				Fibrinolytic enzymes LA_{50} (μM)		
					Buffer		Plasma clot				
	P_3	P_1	TT	APTT	fIIa	fXa	fIIa	fXa	PL	tPA	UK
4	Eoc-D-cHpa	Arg-H	141	331	54	97	103	118	18	10	14
5	Eoc-D-cHpa	Pab	64	209	965	5700	19 000	12 000	2000	794	1330
6	D-cHla	Arg-H	86	281	38	156	95	117	21	9	8
7	D-cHla	Pab	24	55	62	4210	16 300	8630	1522	574	867
8	N-Me-D-cHpa	Arg-H	118	315	40	87	118	102	16	14	18
9	N-Me-D-cHpa	Agm	66	904	38	1170	8980	2320	412	485	602
10	N-Me-D-cHpa	Pab	15	319	66	1200	4500	2850	2000	512	1353
	Melagatran: HOOC-CH_2-D-Chg-Aze-Pab		30	500	6	295	175	360	38	68	102
11	D-Chg-Aze-Pab		31	337	8	285	345	795	29	57	83

[a] See Table 2, footnote a.
[b] cHpa, L-cycloheptylalanine; cHla, L-cycloheptyllactic acid; Pab, 4-aminomethylbenz-amidine; Agm, agmatine; Eoc, ethoxycarbonyl; Aze, L-azetidine-2-carboxylic acid.

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Table 4 Effect of Peptide Anticoagulants on the Survival Rate in a Rat DIC Model using Lethal Dose of LPS[a]

Time (h)	Survival rate (%) at doses of 0.75, 1.0 and 1.5 mg/kg									
	Control	**8**			Melagatran			Efegatran		
	0	0.75	1.0	1.5	0.75	1.0	1.5	0.75	1.0	1.5
0	100	100	100	100	100	100	100	100	100	100
4	76	100	100	100	93	83	100	100	92	100
5	72	100	100	100	100	92	100	73	83	100
6	37	100	100	100	100	92	91	67	66	100
7	24	90	100	100	64	92	91	33	58	92
8	20	90	92	100	64	82	92	27	50	92

[a] Male rats were treated with an i.v. bolus 30 mg/kg LPS (lethal dose). Test compounds were given as an initial bolus followed by an i.v. infusion for 8 h immediately after administration of LPS. Lethality was recorded at 4, 5, 6, 7 and 8 h post LPS.

Table 5 Opiate Activity of Morphine and Opioid Peptides: Leu- and Met-enkephalin, β-endorphin and GYKI-14 238

Opiate	GPI[a] (μ)	MVD[a] (δ)	MVD/GPI	MTF[b]/RTF[c] icv	MTF[b]/RTF[c] iv
Morphine	14.4	2.03	0.14	1.00[bc]	1.00[bc]
Leu-enkephalin: Tyr-Gly-Gly-Phe-Leu	2.1	128.2	61.05	<0.01[bc]	0[bc]
Met-enkephalin: Tyr-Gly-Gly-Phe-Met	5.4	87.2	16.15	<0.01[bc]	0[bc]
β-endorphin[d]	13.9	17.9	1.29	34.7[b]/5.3[c]	4.4[b]/0[c]
GYKI-14 238: Tyr-D-Met-Gly-Phe-Pro-NH_2	46	44	0.96	48.8[c]	5.5[c]

[a] Activities are given in $10^{-6}/IC_{50}$ values. MVD (mouse vas deferens) and GPI (guinea-pig ileum) measure agonist activity on the δ- and μ-subtype of opiate receptors.
[bc] Analgesic activity was assessed by the mouse/rat tail-flick (MTF/RTF) test after intracerebroventricular (icv) and intravenous (iv) administration, and are related to that of morphine (= 1).
[d] *YGGFM*TSEKSQTPLVTLFKNAIIKNHKKGQ (1–5 fragment = Met-enkephalin).

particular Met appeared to be appropriate. This led to [D-Met[2],Pro[5]]enkephalin amide [12], the first analogue of enkephalin possessing significant analgesic activity upon systemic administration. In clinical studies its efficacy was also proved. Inspection of the *in vitro* activity profile of GYKI-14 238 based on MVD/GPI potency ratios indicates that this compound is neither an enkephalin-like nor a morphine-like opioid. GYKI-14 238 is almost equipotent in the MVD and GPI assays. Thus, it is more similar to ß-endorphin in this respect but substantially differs from that in *in vivo* properties, since ß-endorphin is completely inactive in rats if applied intravenously.

INTERPRETATION OF TOLERANCE OF ICE (CASPASE-1) FOR D-STEREOCHEMISTRY AT P_1

ICE, interleukin-1β (IL-1β)-converting enzyme, processes the IL-1β precursor to mature inflammatory cytokine IL-1β. ICE has been identified as the first member of a new Asp-specific family of cysteine proteases (caspases); hence ICE is named caspase-1. Further members of the family are involved in the initiation or execution of apoptosis (programmed cell death), e.g. apopain/caspase-3.

The most efficient ICE inhibitors are peptidyl aspartals and aspartyl halo- or (acyloxy)methanes. A recent report revealed that both D- and L-Asp are

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accepted by ICE at the P_1 site of these inhibitors and emphasized, 'this tolerance for D-stereochemistry at P_1 is unprecedented for the cysteine protease superfamily' [42]. In this context, we examined the tolerance for D-P_1-residues of papain and trypsin as classic cysteine and serine proteases [13].

The L-P_1-isomers of the diastereomeric pairs of peptide aldehydes **12**–**16** used in the experiments (Table 6) were described in the literature or derived from already known structures, e.g. **15** from Ac-Gly-Phe-Nle-H, a good papain inhibitor, and **14** from Z-Phe-Arg-AMC, a good papain substrate.

The data in Table 6 show that the L-P_1-containing isomers exhibit low IC_{50} values and the D : L potency ratios range from 1.7 (ICE) to 597 (trypsin). The results obtained for inhibitors **13**–**15** suggest that papain, the classic cysteine protease, also accepts D-aldehydes at P_1 in the order of Cys(Et) > Arg > Phe. This rank order is in proportion with the lability of the α-C-H bond of these residues. By contrast, the high ratio found with **16** indicates that serine protease has no tolerance for D-residues at P_1.

In view of these findings, it seems very likely that ICE's tolerance for the D-stereoisomer at P_1 arises from its cysteine protease nature and the aspartyl residue's high tendency for racemization.

When inhibiting a cysteine protease with peptidyl (acyloxy)methanes or peptide aldehydes it is the recognition and binding of the side chain of the P_1-L-residue that guides the α-carbonyl to the thiolate. One may suppose a similar event with a P_1-D-residue that brings the α-proton close to the thiolate moiety — even if for a short period of time. A contact between the thiolate and the α-proton can result in a proton abstraction and SH addition with asymmetric induction yielding P_1-L-residue-containing adducts.

PEPTIDYL β-HOMO-ASPARTALS AS CASPASE-1 (ICE) AND CASPASE-3 (APOPAIN) INHIBITORS

The study cited above on the aspartyl (acyloxy)methane inhibitors of ICE [42], also revealed that the analogues containing β-homo-aspartyl: NH–CH(CH_2COOH)–CH_2–CO^{β}–CH_2–O–CO–Ar or β-homo-aspartol: –NH–CH(CH_2COOH)–CH_2–CH_2^{β}–O–CO–Ar residue are inactive. This finding — as reported — points to the key role of the α-CO of the Asp residue in P1 recognition.

For the mechanism of irreversible inhibition of cysteine proteases by peptidyl (acyloxy) or halo methanes [–NH–CH(R)–CO^{α}–CH_2^{β}–X], it has been assumed [43] that the thiolate reacts directly with the C=O^{α} (path *a)* as well as with the adjacent CH_2^{β}–X (path *b)*. In view of path *b*, one may expect interaction between the thiolate moiety and the CO part of β-homo-aspartyl-containing analogue that results in reversible inhibition of ICE. The lack of such an interaction with the β-homo-aspartyl analogue studied may be due to steric hindrance. If so, the corresponding β-homo-aldehyde will be inhibitory.

Considering these findings and speculation we examined the inhibitory activities of peptide β-homo-aldehydes (hXaa-H) in comparison with their parent α-aldehydes (Xaa-H) against ICE (caspase-1), apopain (caspase-3), papain and trypsin [14]. As for the inhibition of the latter two enzymes, the L-β-homo-aldehyde analogues of α-aldehyde inhibitors **13**–**16** (Table 6) were prepared and found to be $>$500 times less inhibitory than the α-aldehyde congeners. For caspase inhibition, the β-homo-aspartal analogues (**18**, **20**) of two known substrate related aspartals (**17**, **19**) were prepared and tested (Table 7).

Accordingly, these caspases, unlike papain, the classic cysteine protease, can be inhibited with substrate related β-homo-aldehydes.

Furthermore these data may suggest that a direct attack of the catalytic thiolate at the X-methyl group of peptidyl X-methanes (path *b)* can only take place in the case of certain caspases. Alkylation of papain-like proteases very likely proceeds on path *a*, thiolate attacks the α-CO of peptidyl X-methanes.

DISCOVERY OF CETRORELIX

Luteinizing hormone-releasing hormone (LHRH): <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, is a hypothalamic decapeptide amide that was first isolated, sequenced and synthesized by Andrew Schally in 1971. LHRH controls the release from the pituitary of gonadotropins, LH and FSH, which stimulate the synthesis of sex hormones in the gonads.

Considering that LHRH or its antagonists might be useful in gynaecology and probably also in birth control and cancer therapy, many laboratories initiated extensive research programmes to prepare this compound and to perform SAR studies on analogues of this molecule. We elaborated a practical synthesis and made a small, but significant contribution to the worldwide SAR studies.

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Table 6 Protease Inhibiting Activity of Peptidyl L-amino Aldehydes Compared with their D-amino Aldehyde Analogues

Protease		Peptide aldehyde	IC_{50} (μM[a*])		D-P_1 : L-P_1
		P_5 P_4 P_3 P_2 —P_1—	L-P_1	D-P_1	
ICE	**12**,	Ac-Tyr-Val-Ala-DL-Asp-H	42.7 ± 6.8[b]	71.7 ± 21[b]	1.7[b]
	13,	Z-Arg-Ile-DL-Phe-H	0.070 ± 0.010	8.50 ± 1.00	121
Papain	**14**,	iBoc-Phe-DL-Arg-H	0.130 ± 0.020	12.50 ± 1.50	96
	15,	Ac-Gly-Phe-DL-Cys(Et)-H	0.036 ± 0.004*	0.30 ± 0.04*	8
Trypsin	**16**,	Boc-D-Phe-Pro-DL-Arg-H	0.003 ± 0.0001	1.79 ± 0.09	597

[a] Measured with substrate **S1**, Ac-Tyr-Val-Ala-Asp-AMC (ICE); **S2**, Z-Phe-Arg-AMC or
* **S3**, Ac-Gly-Phe-Cys(Et)-AMC (papain); **S4**, Tos-Gly-Pro-Arg-pNA (trypsin).
[b] Values of a bioassay (inhibition of IL-1β production in LPS-stimulated human whole blood): L-P_1, 0.8 ± 0.09; D-P_1, 1.1 ± 0.1 μM; D-P_1 : L-P_1 = 1.375.

Table 7 Inhibitory Activities of Peptidyl Aspartals and β-homo-aspartals on the Proteolytic Activity of Caspases: ICE and Apopain

Peptidyl (homo)aspartal	Caspase	Substrate	IC_{50}	hAsp-H : Asp-H
17, Ac-Tyr-Val-Ala-Asp-H	ICE	proIL-1β	2.0 ± 0.6 μM	
18, Ac-Tyr-Val-Ala-hAsp-H	in human whole blood		2.9 ± 1.0 μM	1.45
19, Ac-Asp-Glu-Val-Asp-H	Apopain	PARP[a]	30 ± 3.0 nM	
20, Ac-Asp-Glu-Val-hAsp-H	recombinant	protein	50 ± 10 nM	1.7

[a] PARP, poly(ADP-ribose) polymerase.

During studies on the significance of the constituent amino acids, it was observed that replacement of Arg^8 with Lys preserved 10%–30% activity but substitution with the neutral norvaline led to an inactive analogue. Consequently, the basic character of Arg^8 might be of particular importance.

We supposed that the δ-guanidino group of Arg^8 might bind to a carboxyl group on the receptor through an ionic interaction that is strengthened by two H-bonds. These latter are missing when the lysine-ε-ammonium binds to the carboxylate. We hypothesized that the neutral citrulline (Cit) analogue of Arg might be similarly active because its δ-ureido side chain can bind to a carboxyl group through H-bonds; here the ionic interaction is missing. Indeed, the neutral [Cit^8]LHRH showed 6%–33% activity [15].

Replacement of Gly^6 with various D-residues led to elevated potency, in the case of D-Trp, a 100-fold increase. Repeated administration of such superagonists induces inhibition via down-regulation of the number of receptors. An LHRH antagonist, per se, produces inhibition after the first injection. Highly potent antagonists were obtained by substitutions with hydrophobic D-residues in positions 1, 2, 3, 6 and optionally 10.

Table 8 presents the structure and antiovulatory activity of some LHRH analogues of this type. Compound **21**, having D-Trp in position 6, was the first highly active antagonist, a hydrophobic molecule that could only be applied in corn oil. In order to obtain water-soluble analogues, D-Arg, a hydrophilic residue was introduced in place of D-Trp. Compound **22** thus obtained could be administered in aqueous solution and was somewhat more inhibitory than **21**.

In 1984, I learned from Dr Schally that compound **22** (ORG-30276) caused distinct erythema at the site of injection in human tolerance studies. Other D-Arg^6-containing LHRH antagonists were also allergenic, while compound **21**, the less active antagonist with D-Trp^6, was free of such side effects.

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Table 8 LHRH Antagonists

No.[c]	LHRH analogues[ab] 1 2 3 4 5 6 7 8 9 10	Activity %, Dose, solvent
21	Ac-D-Cpa-D-Cpa-D-Trp-Ser-Tyr-*D-Trp*-Leu-Arg-Pro-D-Ala-NH_2	88, 7.5 μg, corn oil
22	Ac-D-Cpa-D-Cpa-D-Trp-Ser-Tyr-*D-Arg*-Leu-Arg-Pro-D-Ala-NH_2	88, 3.0 μg, water
23	Ac-D-Nal-D-Cpa-D-Trp-Ser-Tyr-*D-Cit*-Leu-Arg-Pro-D-Ala-NH_2	100, 3.0 μg, water
24	Ac-D-Nal-D-Cpa-D-Pal-Ser-Tyr-*D-Cit*-Leu-Arg-Pro-D-Ala-NH_2	100, 2.0 μg, water

[a] Cpa, 4-chlorophenyl-L-alanine; Nal, 3-(2-naphthyl)-L-alanine; Pal, 3-(3-pyridyl)-L-alanine.
[b] Prepared at Schally's institute by Coy *et al.* (**21**,**22**) and Bajusz *et al.* (**23**,**24**), [16,17].
[c] **21**, a D-Trp[6]-containing hydrophobic antagonist; **22**, ORG-30276; **23**, SB-30; **24**, SB-75.
Activity: % inhibition of ovulation in rats at the dose/animal.

Table 9 Systemic Toxic Effects of the D-Arg[6]-containing Parent Compound ORG-30267 and two D-Cit[6]-containing LHRH Antagonists, SB-30 and SB-75, in rats

Peptide, i.v. dose	Observation of alterations with animals for 1 h
22, ORG-30276 1 mg/kg	3 min: intense lethargy, profound respiratory depression, and marked cyanosis 60 min: cyanosis persisted for more than 1 h
23, SB-30 1 mg/kg	10 min: slight cyanosis, little drowsiness 60 min: complete recovery
24, SB-75 1 mg/kg	No apparent reaction
24, SB-75 4 mg/kg 2 × 4 mg/kg	No apparent reaction

SB-75 with the generic name Cetrorelix has been selected for further development at ASTA Medica AG; now it is used in *in vitro* fertilization.

These findings indicated the involvement of the basic character of D-Arg[6], and reminded me of our earlier observation. Namely that neutral Cit was an appropriate substitute for the basic Arg[8] of LHRH.

The D-Cit[6] analogues prepared [16,17] were water soluble, highly active, and free of any oedematogenic effects. Two of these compounds, **23** (SB-30) and **24** (SB-75), are shown in Table 8. Apparently, both are more potent than either **21** or **22**.

As shown in Table 9, ORG-30276 administered to rats in an i.v. dose of 1 mg/kg was toxic, and the early appearing marked cyanosis persisted for more than the observation time of 60 min. SB-30 in an identical dose initiated only slight and transient cyanosis, and SB-75 was free of side reactions even after a second treatment of 4 mg/kg.

SB-75 with the generic name Cetrorelix has been selected for further development, and now it is in clinical use for the prevention of premature LH surges in women undergoing *in vitro* fertilization and embryo transfer.

CONCLUDING REMARKS

During the discussion of the main projects summarized in Table 1 the results derived from speculation (ideas or beliefs) and experiments were presented. For instance, the belief that a D-amino acid residue in peptide ligands would provide a unique site for receptor-binding assisted the development of thrombin inhibitors, including efegatran (GYKI-14 766), and the first intravenously active analgesic peptide (GYKI-14 238). Another hypothesis was that the neutral ureido group would partly surrogate the functions of the basic guanidino group. This idea led to [Cit[8]]LHRH, a neutral and still active LHRH analogue. Furthermore this finding combined with data about the oedematogenic effect of the [D-Arg[6]]LHRH antagonists resulted in the [D-Cit[6]]LHRH analogues free of such toxic effects, and eventually to SB-75 or Cetrorelix, which is now used in *in vitro* fertilization. It is a good feeling that I have contributed to the development of a drug even if it represents only a

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'smaller market' [44]. The speculation on the usefulness of clot-permeable anticoagulants for targeting both thrombin and factor Xa in the treatment of DIC or sepsis resulted in new peptidyl arginals with promising results in animal studies.

Such ideas seem to be helpful as working hypotheses in improving the potency and quality of drug candidates, and hence pave the way for their clinical trials. However, as the fate of efegatran illustrates, it is the market prospects which actually determine whether a drug candidate will ever be made available for sale.

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