UNIVERSITY OF SZEGED, FACULTY OF PHARMACY
DEPARTMENT OF PHARMACODYNAMICS AND BIOPHARMACY

PHARMACOLOGICAL CHARACTERIZATION OF NEWLY SYNTHESIZED OXYTOCIN ANTAGONISTS IN VITRO AND IN VIVO

JUDIT HAVASS

Ph.D. THESIS

SZEGED

2003

I dedicate this work to my family

ANNEX

Full papers and abstracts related to the Ph.D. thesis

I. I. Zupkó, G.K. Tóth, K. Bakos, F. Fülöp, I. Pávó, J. Havass, G. Falkay: In vivo effect effects of 2-substituted [Mpa^{1}Sar^{7}Arg^8]-oxytocin antagonists on postpartum rat. Peptides (20) 1999: 749-751

II. J. Havass, K. Bakos, G.K. Tóth, F. Fülöp, G. Falkay: Új potenciális oxitocin antagonisták szintézise és in vitro receptoranalitkai vizsgálata. Acta Pharmaceutica Hungarica 70, 2000: 168-174

III. K. Bakos, J. Havass, F. Fülöp, L. Gera, J. M. Stewart, G. Falkay, G.K. Tóth: Synthesis and receptor binding of oxytocin analogs containing conformationally restricted amino acids. Letters in Peptides science (8) 2002: 35-40

IV. J. Havass, K. Bakos, Á. Márki, R. Gáspár, I. Zupkó, L. Gera, J.M. Stewart, F. Fülöp, G.K. Tóth, G. Falkay: Non-competitive nature of oxytocin antagonists with general structure Mpa^{1}Xxx^{2}Sar^{7}Arg^8. Peptides 2002, 223: 1419-1425

V. J. Havass, Á. Márki, K. Bakos, G.K. Tóth, G. Falkay: Pharmacological characterization of oxytocin antagonists containing conformationally constrained amino acids in position two Human Reproduction 15, Abstract book 1, 2000, P290

VI. J. Havass, K. Bakos, Á. Márki, R. Gáspár, G.K. Tóth, G. Falkay: Receptor binding and inhibitory effects of newly synthesized oxytocin antagonists on isolated late-pregnant guinea-pig uterus. Fundamental and Clinical Pharmacology 15(1), 2001, 9P240 (abstract)

VII. Havass J., Bakos K.: Merevvázas és nagy térkitöltésű aminósavakat tartalmazó oxitocin antagonisták szintézise és farmakológiai jellemzése. XXXV. Rozsnyai Mátyás emlékverseny, 2000 (abstract)

VIII. Havass J., Bakos K.: Új potenciális oxitocin antagonisták szintézise és farmakológiai jellemzése in vitro. V. Clauder Ottó emlékverseny, 2000. (abstract)

Full papers and abstracts not related to the Ph.D. thesis.

I. R. Gáspár, I. Földesi, J. Havass, Á. Márki, G. Falkay: Characterization of late-pregnant rat uterine contraction via the contractility ratio in vitro, significance of α_1-adrenoceptors. Life Sciences 68, 2001: 1119-1129

II. Á. Márki, J. Havass, R. Gáspár, G. Falkay: Distribution of α-adrenergic receptor subtypes in pregnant uterus. Human Reproduction 15, abstract book 1, P261 2000

I. LIST OF ABBREVIATIONS

Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical nomenclature: Nomenclature and symbolism for Amino Acids and Peptides (J Biol Chem 1984: 219 :345-373)

Atc	aminotetraline-2-carboxylic acid
BSA	Bovine serum albumin
Car	1,2,3,4-tetrahydro-β-carboline-1-carboxylic acid
Cpa	parachloride-phenylalanine
Cys	cysteine
Dbr	3,5-dibromide-phenylalanine
Dmf	2,4-dimethylphenylalanine
GPCR	G protein-coupled receptor
Igl	indanylglycine
K_i	Inhibition constans
Mca	β-mercapto-β,β-cyclopentamethylenepropionic acid
MeOAtc	6-methoxy-2-aminotetraline-2-carboxylic acid
MeTcc	1,2,3,4-tetrahydro-1-methyl-β-carboline-3-carboxylic acid
Mpa	mercaptopropionic acid
OT	oxytocin
OTR	oxytocin receptor
Pen	β,'-dimethylcysteine
Pff	pentafluorophenylalanine
Phe	phenylalanine
PMSF	phenylmethyl-sulfonyl fluoride
Tcc	1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid
Tic	1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid (Tic)
Tmf	2,4,6,-trimethyphenylalanine
Trp	tryptophan
Tyr	tyrosine
VP	vasopressin
VPR	vasopressin receptor

II. INTRODUCTION

According to the recommendation of the WHO preterm birth is defined as a gestational age less than 37 completed weeks of pregnancy or less than 259 days from the first day of the last menstrual period. Effective methods for treatment of preterm labor have become major goals in modern obstetrics because preterm birth is the leading cause of infant morbidity and mortality. The average duration of gestation in women is 40 weeks, it is estimated that there are nearly 13 million preterm babies born worldwide each year, with an incidence of between 5,8 % (Oceania) and 10,6 % (North America) of all birth. In Europe, the overall incidence of preterm birth is approximately 5,9 %, or an expected annual population of 375,000. Of these pregnancies approximately 100,000 each year are "potentially preventable". [Villar, J., 1994] Premature infants are more at a risk for development of such problems as respiratory distress syndrome, hyaline membrane disease, necrotizing enterocolitis and sepsis [McCombs, J., 1995]. Morbidity resulting from prematurity often causes lifelong disabilities and therefore impacts on the family, society, and healthcare economics. Despite the routine use of tocolytic agents in the recent decade, the incidence of premature delivery has not declined. Therefore a major priority in obstetric research is the prevention of prematurity.

Probably the most important risk factor known is a previous preterm birth, which is associated with about a three-fold increase in the risk of a further preterm delivery [Mercer, M.C., 1996]. Other risk factors, such as minority ethnic origin, low socio-economic class, work during pregnancy and smoking have a significant association with preterm labor. Maternal physiological problems that may predispose a patient to preterm labor include serious renal or cardiovascular disease, severe anaemia, severe hyperthyroidism, infection or poorly controlled diabetes [Caritis, S.N., 1988].

Term and preterm labor are considered to have fundamentally similar processes. They share a common terminal pathway consisting of uterine contractility, cervical dilation, and activation of the amniotic membranes [Romero, R., 1994]. This involves anatomical, biochemical, endocrinological and clinical events that occur in the fetus and in the mother. The principal difference between term and preterm labor is how they are activated. It has been proposed that term labor results from the physiological activation of the common terminal pathway, whereas preterm labor is a pathological condition caused by multiple aetiologies which activate one or more of the components of this pathway [Romero, R., 1994].

III. TREATMENT OF PRETERM LABOR

III/1. β_2-adrenergic receptor agonists

Several pharmacological approaches focus on the inhibition of preterm uterine contractility. The major form of currently applied treatment is the administration of selective β_2-adrenergic agonists that inhibit myometrial contractility by activating of the receptor-mediated adenylcyclase cascade. The first β-adrenergic agonist to be administrated clinically to treat preterm labor was isoxuprin [Higby, K., 1993]. Although it was somewhat successful, it produced significant cardiovascular adverse effects. To decrease the adverse effects, ritodrin was licensed specially for obstetric use. Nowadays, worldwide used selective β_2-agonists in obstetrics include fenoterol, terbutalin and hexoprenalin. Presently only hexaprenalin is used in Hungary.

The β_2-agonists are initially administered by intravenous infusion. Following parenteral administration, the patient receives oral medication until delivery. If the labor recurs, the parenteral medication can be reinstated [McCombs, J., 1995]. Although this therapy can prolong pregnancy for 48 hours, it does not reduce the incidence of preterm birth or perinatal morbidity or mortality. Moreover, long-term (1 week) administration of these drugs tends to cause down-regulation and desensitisation of the β_2-receptors, possibly affecting the ability of the medication to inhibit uterine contraction. Besides desensitisation adverse effects are of the major consideration in the use of the β_2-agonists, as well. They are associated with significant maternal and foetal side-effects. The risks of the tocolysis with β_2-agonists include fluid overload (possible stimulation of the renin-aldosterone system, increase in antidiuretic hormone), and pulmonary oedema. The precise mechanism by which pulmonary oedema develops in pregnant women treated with β-agonists is unknown and most likely multifactorial, fluid retention is one possible contributory factor [Pisani R.J., 1989; Clesham G.J., 1994]. Other complications include increased heart rate (as much as 30-40% increase), myocardial ischaemia (isolated systolic hypertension, increased myocardial oxygen consumption, decreased coronary blood flow, hypoxia, and hypokalaemia. Agitation, tremor and nervousness also are frequently reported in patient receiving these medications [Caritis, S.N., 1988]. These drugs also have diabetogenic effect, they increase blood glucose with a resultant increase of insulin secretion [McCombs, J., 1995]

This broad side effect spectrum leads to contraindication in a part of the patients.

III./2 Magnesium sulphate

Side effect and clinical conditions that contraindicate the use of β-sympatomimetics have served to focus of attention on the use of Magnesium sulphate given intravenously as an alternative tocolytic drug. In 1959 Hall et al. demonstrated that strips of human gravid uterine muscle showed reduced contractility in the presence of magnesium ion [Hall D.G., 1959]. Magnesium sulphate is able to inhibit the contraction of the uterus and to influence appropriately the eclampsia. It can delay the delivery for at least 48 h and generally occurred in more than 90% of the patient. Magnesium therapy is usually initiated with intravenous bolus of magnesium followed by an infusion of 2-4 g/h until contractions ceased [Wilkins IA., 1988]. Although similar efficacy is reported for magnesium sulphate and β-agonists, their adverse effect profile differs. The adverse effects reported most often with magnesium sulphate are lethargy, flushing and nausea. Serious consequences, like respiratory depression and cardiac arrest, as well as foetal bradycardia and loss of heart rate variability do not usually occur until the serum magnesium concentration exceeds 5-8 mg/dL [Wilkins IA., 1988]. One disadvantage with magnesium sulphate is the lack of an effective oral dosage form for maintenance use following parenteral therapy.

III./3. Prostaglandin Synthetase Inhibitors

It is generally believed that prostaglandins are of major importance in the regulation of contractility of both the nonpregnant and pregnant uterus. The prostaglandins produced by the decidua and myometrium have similar effect to OT, however reducing the PG- synthesis with nonsteroidal anti-inflammatory agents (NSAID) as COX inhibitors is an obvious possibility for tocolysis. Prostaglandin $F_{2\alpha}$ is able to stimulate uterine contractility during each phases of the menstrual cycle as well as all stages of pregnancy, in contrast to OT that acts only in late pregnancy. Among the NSAID indometacin has been studied most extensively for the treatment of preterm labor [Morales WJ., 1993]. (The salicylate derivates can not be administered with this indication, because of their irreversible binding to COX.). The risks of indomethacin administration are both maternal (peptic ulceration, gastrointestinal bleeding) and foetal. The foetal complications involve oligohydramnion [Hendricks SK., 1990] and intrauterine constriction of the ductus arteriosus, which may result in pulmonary hypertension [Macones, G.A., 1997; Moise KJ., 1988; Norton ME., 1993].

III./4 Calcium channel blockers

Another possibility of the relaxation of the uterus is the administration of the Ca^{2+}-channel blockers. Nifedipine and other dihydropiridines have direct effect on uterine smooth muscle, they decrease the Ca^{2+}-influx, intracellular Ca^{2+} level, thus the motoractivity of the uterus. Therefore, the Ca^{2+}-channel blockers are also used for tocolysis, but an obvious side effect is that they can cause significant hypotension. Mainly nifedipine is used for supplementary medication of preterm contractions. Their maternal side effect profile is better than β-agonists but effects on the foetus have caused concern [Impey, L., 1993]. Of particular concern is the interaction of the Ca^{2+}-channel blockers with magnesium sulphate, being reported as causing respiratory failure [Ben-Ami, M., 1994].

Premature labor continues to be a therapeutic dilemma for obstetricians and pharmacists. The above mentioned drugs available for the treatment of preterm labor are not consistently effective. All have serious potential effects on the mother, the foetus, or both. Therefore, the greatest challenge in the management of preterm labor is the lack of a specific pharmacologic agent for treatment. Perhaps when the cause of premature labor is identified, a more specific and less harmful agent can be developed.

IV. ROLE OF OXYTOCIN ANTAGONISTS

IV/1. Physiology and pharmacology of OT

Although the causes of preterm labor are multifactorial, it is becoming more apparent that oxytocin (OT) has an important role in the preterm birth. It is well accepted that both OT itself and the sensitivity of the uterus to OT play crucial role in the initiation of both normal and pathologically early deliveries [Fuchs AR., 1982; Takahashi K.,1980].

In 1906 Sir Henry H. Dale demonstrated that the posterior pituitary gland contains a substance that has uterine stimulating properties [Higuchi T., 1995]. The application of such an extract to induce labor was reported as early as 1911. Further investigations cleared up that posterior pituitary extracts contain two main biologically active peptides, which were named after their physiological effects: oxytocin delivered from a Greek world meaning "swift-birth" and vasopressin (VP) ("vasoconstrictor"). Since the posterior pituitary is part of the central nervous system and it releases these substances to the blood via median eminence and so they can reach the site of the action in the body they become classified as neuronhormones [Szentágotai JRM., 1994].

In the 1950s Du Vigneaud and co-workers established the structure of OT. Du Vigneaud awarded Nobel Prize in 1955 for the first successful synthesis of a biological active peptide in the test tube. OT is a nine-amino acid peptide consisting of a six-member ring formed by disulfide bounds between position 1 and 6 and three amino acids tail (Figure 1). It is structurally similar to vasopressin (VP), differing by only 2 amino acids and thought to evolve from a common ancestral gene.

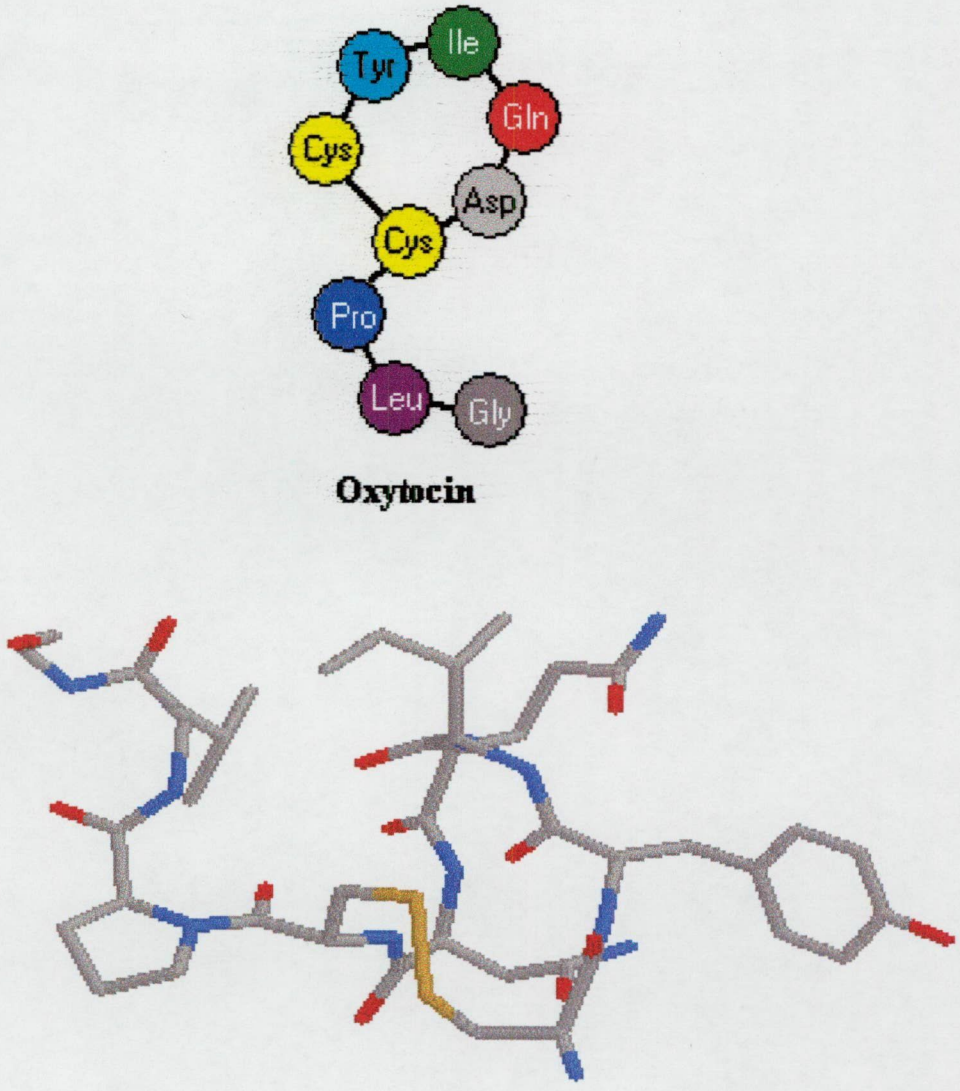


Figure 1.

Chemical structure of the OT molecule

The aminosequency of the OT is: Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2. Recent findings demonstrate that this hormon acts in human as a neurohormone (it stimulates smooth muscle contraction in the pregnant uterus during delivery and enhance milk ejection), as a neuroregulator (it plays role in the maternal behaviour, stress, anorexia, in the modulating cerebrovascular responses to hypoxia) and as a paracrine substance in the decidua which may trigger the onset of the parturition.

Genes for both OT and VP reside on chromosome 20 in opposite transcriptional orientation separated by intergenic sequences. OT is initially synthesized as a 125-amino acid preprohormone in the magnocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus. The prohormone is first cleaved by a serine endoprotease to form oxytocin-Gly-Lys-Arg. This is further cleaved by convertases and carboxypeptidase E in the secretory granules to oxytocin-Gly which is –amidated to OT by peptidylglycine. The rate-limiting step is the conversion of oxytocin-Gly to OT. The cleavage of the precursor to OT occurs in the secretory vesicule during axonal transport to the neurohypophysis.

OT is rapidly metabilized in the liver, kidney and the placenta. In additional, the plasma from pregnant women metabolises OT to biologically inactive peptides due to the action of an enzyme produced by the placenta and released into the blood, cystine aminopeptidase (oxytocinase). These enzyme cleaves the Cys^{1}-Tyr^{2} bond and then cleaves Tyr. Other enzymes known to metabolize OT are postproline endopeptidase, which cleaves the Pro^{7}-Leu^{8} bond [Knobil E., 1998].

OT is known to act through specific OT receptor (OTR). Kimura et al. first isolated and identificated the human OTR using an expression cloning strategy [Kimura T., 1992], (Figure 2).

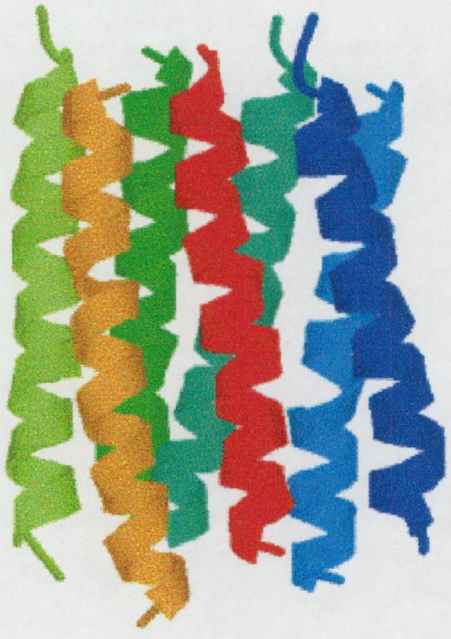


Figure 2.

The 7 transmembrane domain of the OTR

The encoded receptor is a 389 amino acid polypeptide with 7 transmembrane domains and belongs to the class I G protein-coupled receptor (GPCR) family. The OTR is a typical member of the rhodopsin-type (class I) GPCR family. The seven transmembrane α-helices are the most highly conserved among the GPCR family members. Conserved residues among the GPCRs may be involved in a common mechanism for activation and signal transduction to the G protein. The major second messenger system that mediates uterine contraction is the phospholipase C, inositol triphosphate pathway. OTR binding results in activation of G-proteins, in particular the G_q and G_i families.

On the basis of studies with model GPCRs it is assumed that the switching from the inactive to the active conformation is associated with a change in the relative orientation of the transmembrane domain 3 and 6, which then unmasks binding sites [Gimpl, G., 2001]. In the case of the OT binding characterisitic, the NH_2 terminus of the OTR takes part in hormone binding and probably interacts with the hydrophobic leucyl residue in position 8 of the ligands. The NH_2 terminal domain and the first extracellular loop of the OTR are proposed to interact with the linear COOH-terminal tripeptidic part of the OT, whereas the second intracellular loop of the OTR could be identified to interact with the cyclic hormone part (Figure 3) [Postina R., 1996]. Several studies indicate that the binding sites of the OT antagonists are different from the agonist binding site. The binding site for the peptide OT antagonists [Elands J., 1988] was formed by the transmembrane helices 1,2 and 7 with a

major contribution to binding affinity by the upper part of helix 7. These regions did not participate in OT binding [Postina R., 1996].

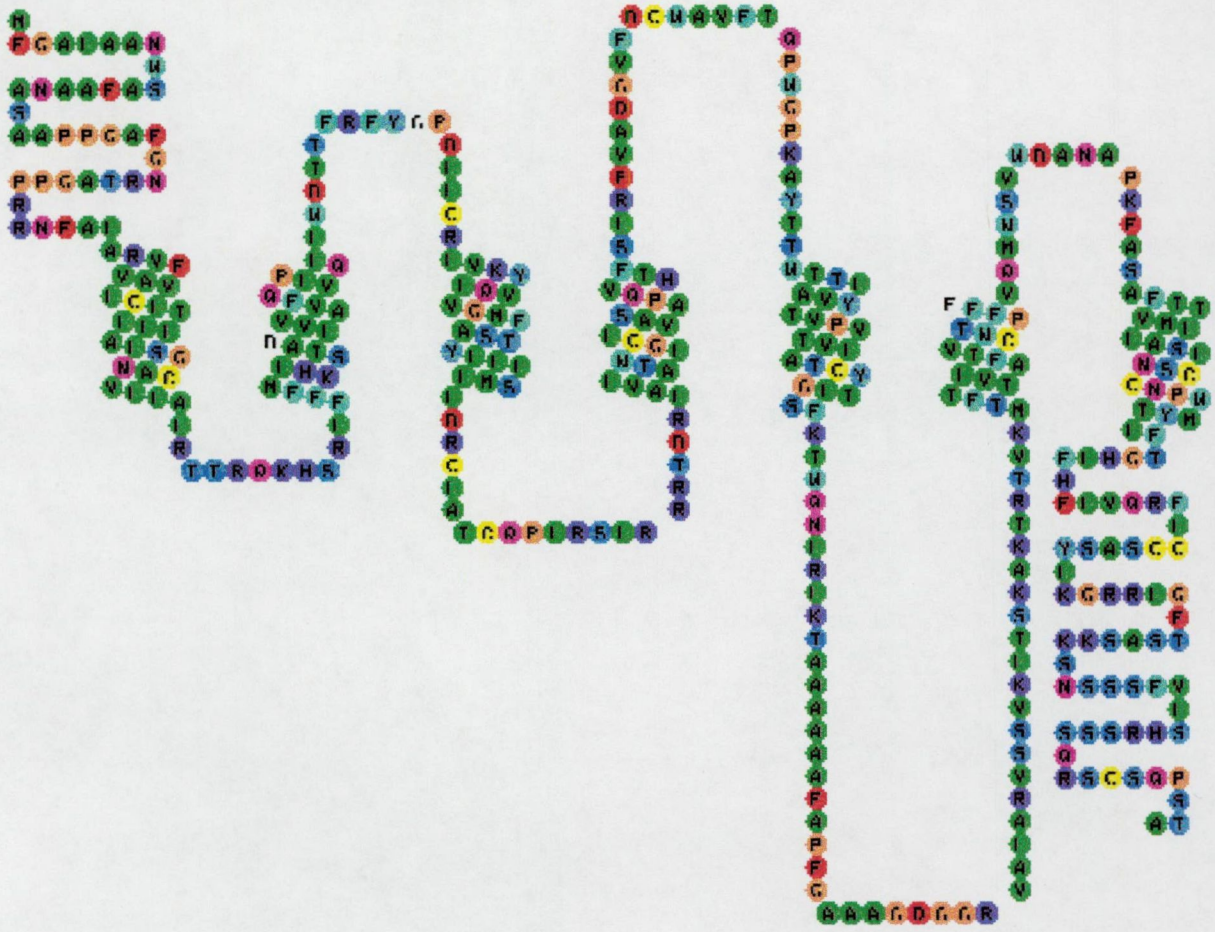


Figure 3.

The structure and the amino acids sequence of the OTR

OT is the most potent known parturient. The action OT is mediated by binding to specific OTR present in the uterus and other target tissues. Around the onset of labor, uterine sensitivity to OT markedly increases. This is due to both the upregulation of OT receptor mRNA levels and strong increase in the density of myometrial OT receptors, reaching a peak during early labor. This has been demonstrated both in rats and in human species, in which receptor levels rise during early labor to 200 times that in nonpregnant state [Fuchs AR., 1984]. Thus, at the onset of the labor OT can stimulate uterine contractions at levels that were ineffective in the nonpregnant state. OTR is also found in the decidua, where OT can stimulate the production of $PGF_{2\alpha}$. At the end of pregnancy, an increased secretion of $PGF_{2\alpha}$ leads to luteolysis and causes progesteron withdrawal and initiation of labor. After parturition, the concentration of OTR rapidly decline. In rats, the uterine OTR mRNA level decreased

more than sevenfold within 24 h [Zingg H., 1995]. Possibly, the down-regulation of the OTRs is necessary to avoid unwanted contractile responses during lactation when OT level is raised. Clinically, the primary drug used to induce labor in women is synthetic OT. OT is infused iv. beginning at about 2 mU/min and the dose is increased every 20-30 min until effective uterine contractions are archived (the maximal OT dose is 12 mU/min). The uterus is more sensitive to OT during the last trimester of pregnancy due to the increase in the number of myometrial OTR (Knobil E.,1998)

The widespread use of synthetic OT in clinical obstetric practice speaks to its effectiveness in initiating parturition, and aroused a question whether OT antagonists are able to prevent preterm labor. (Goodwin et al., 1994). According to the all of the above mentioned it is well accepted that the OT system may be regarded as a key regulator of parturition, with the induction of OTR as a trigger event. Therefore, the blockade of the uterine OTR is one of the logical approaches to prevent preterm labor.

IV/2. OT antagonists

The parturition process can be divided into two phases: the endocrine and the paracrine phase. The endocrine phase begins with an increase in the oestrogen/progesterone ratio that brings about the release of OT and nocturnal uterine contractions. They contribute to the pushing of the fetal head into the ripening and opening the cervival os. As the cervix opens, this exposes the chorioamnion membranes to vagina bacteria and this, along with the trauma of the head pushing into the cervix, induces an inflammatory-like reaction that brings blood elements into the lower uterine segment and cervix. Cytocines are released locally and stimulate PG production and the control of the uterine contractions switches from those dominated by OT to paracrine and autocrine factors such as PGs. Thus, depending on the phase of the delivery process, different tocolytics may be needed to stop labor. Based on this concept, antagonists to OT will be the most effective in the endocrine phase, whereas inhibitors to PG production or inflammatory reactions will be more effective during the paracrine phase of parturition. Thus, the current research focus is on selecting factors in the early stages of the parturition process that indentify patients at risk and treating with agents such as OT antagonists that will stop uterine activity in the endocrine phase.

This is the reason that, there has recently been an increasing tendency toward the rational design of highly active and selective analogues of different peptides. In the past few decades, hundreds of OT analogues has been synthesised in an attempt to obtain selective and active

analogues, and to know more information about the structure-activity relationship. Although the first OT analogues with antagonistic properties were described by Law and du Vigneaud in 1960, but only one of the synthesised analogues, atosiban (1-deamino-D-Tyr(OEt)^2-Thr^{4}Orn^8-OT) have been licensed in human medical practice for the treatment of preterm labor [Melin P., 1994]

The specificity for OT receptors is the key to the clinical application of any OT antagonist, since the major problem with all current tocolytic agents is their systemic activity, which causes potentially harmful multi-organ side-effects. The efficacy and safety of the atosiban was investigated in a randomised, double-blind, placebo-controlled trial with tocolytic rescue (Romero, R., 2000). In this study the treatment of patients in preterm labor with atosiban resulted in prolongation of pregnancy for up 7 days for those at a gestational age >28 weeks with a low rate of maternal-fetal adverse effects. In addition, at a gestational age >28 weeks, infant morbidity and mortality of atosiban-initiated standard care were similar to those with placebo-initiated standard care. The side effect profiles of atosiban and placebo were comparable, with exception of a higher incidence of injection-site reactions during the maintenance among patients receiving atosiban. The safety results associated with atosiban treatment compare favourably with published data on the β-sympathomimetics, including ritodrine, the only tocolytic agent approved in the United States. The superiority of atosiban maternal-fetal safety compared with that of β-agonists has also been demonstrated in 2 randomized clinical trials. Although the delay in delivery was comparable between the atosiban and β-agonist group, maternal/fetal outcomes were substantially better with atosiban. Adverse events were reported more frequently in women administered β-agonists, particularly maternal cardiovascular adverse events.

During the development of the OT antagonists, modification of the OT molecule at positions 1, 2, 7 and 8 produced analogues with higher receptor affinity and selectivity for the myometrium in vitro (Figure 4).

Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly

OT

Deamino-Cys-Xxx-Ile-Gln-Asn-Cys-Sar-Orn-Gly

Synthesised peptides

Figure 4.

General structure of the synthesised OT analogues

Most of the OT antagonists describe to contain Mpa, Mca, Pen instead of Cys at position 1. Lacking of the N-terminal amino group of the cystein in the first position protects the peptide molecule against the eliminating oxytocinase enzyme [Gronzka, Z., 1991]. It is also known that the substitution of the Pro with Sar at position 7 results in highly potent OT agonist with weak antidiuretic activity [Gronzka, Z., 1983; Pávó I., 1994]. This considerable selectivity is a characteristic feature of OT analogues with antagonist potency. Another possibility for the modification is the Leu at position 8. The amino acid at position 8 also has a role in the binding of the antagonists to the OT receptor. The most potent OT antagonists contain basic amino acid (Arg/Orn) instead of apolar Leu at position 8 [Lebl, M., 1987]. The results of Hurby et al. and Manning et al. pointed the importance of the amino acids at position 2 for antagonist activity together with the presence of the basic amino acids in position 8 [Hruby VJ., 1990; .Manning M., 1995]

V. AIM OF THE STUDY

The data summarized previously illustrate that the tocolytic therapy is not a solved problem, while it is one of the most important problems of the healthcare. In spite of the fact, that theoretically the spontaneous activity of the uterus can be influenced in many ways, still, in practice the effective agents in use do not correspond to the expectations of the ideal medicines. The current classes of tocolytic agents are far from the ideal because all available agents have documented serious adverse effects for either the mother or the infant, or both.

The aim of our study was the in vitro, in vivo pharmacological characterization of more effective and selective OT antagonists in cooperation the Department of Pharmacodynamics and Biopharmacy, Department of the Medical Chemistry and the Department of the Pharmaceutical Chemistry of the University of Szeged. We set out to establish the optimal size of the apolar side-chain at position 2 and in parallel to find the optimal conformational constraints. Our approaches in the design of more effective OT antagonists are based mainly on the incorporation of bulky apolar side-chain amino acids with a conformationally constrained restricted feature at position 2.

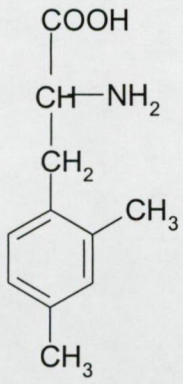
These synthesized peptides are 1-deamino-7-sarcosine-8-arginine analogues. The appropriate modification of the Tyr at position 2 plays a crucial role in determining the antagonistic property.

This study reports the *in vitro* and *in vivo* pharmacological investigations of the new synthesized OT antagonists with general structure [Mpa^{1}Xxx^{2}Sar7Arg8]-OT, substituted at position 2 with conformationally constrained and bulky amino acids, and the concluded structure-effect relationships as guidelines for the further design of OT antagonists.

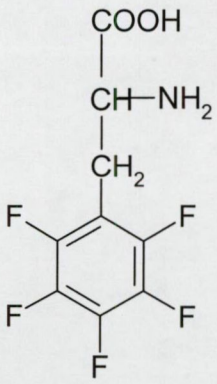
According to the amino acid at position 2, the newly synthesised OT analogous can be divided into two groups:

A) differently substituted Phe analogues with conformational constrains: D- and L-pentafluorophenylalanine (Pff), parachloride-phenylalanine (Cpa), 3,5-dibromide-phenylalanine (Dbr), 2,4-dimethylphenylalanine (Dmf), 2,4,6,-trimethyphenylalanine (Tmf

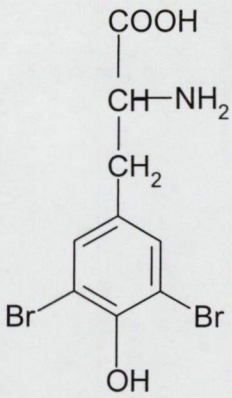
A.



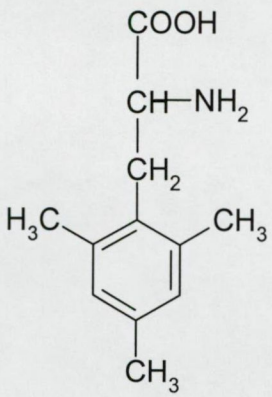
Dmf



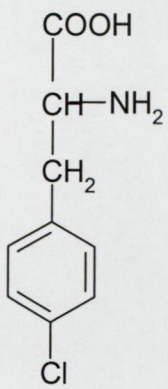
Pff



Dbr



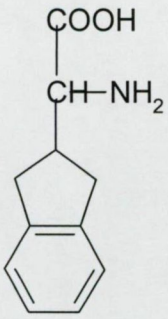
Tmf



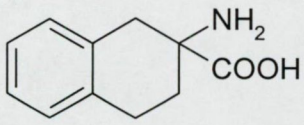
Cpa

B) rigid structure – containing Phe, Trp and O-methyl Tyr analogous: indanylglycine [D-, and L-Igl], aminotetraline-2-carboxylic acid (Atc), 6-methoxy-2-aminotetraline-2-carboxylic acid [MeOAtc], conformationally constrained derivatives of tryptophan: 1,2,3,4-tetrahydro-1-methyl-β-carboline-3-carboxylic acid (L- and D-MeTcc), 1,2,3,4-tetrahydro--β-carboline-3-carboxylic acid (L- and D-Tcc), 1,2,3,4,-tetrahydro-β-carboline-1-carboxylic acid (Car-I, and II), 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid (Tic) and tryptophan (Trp)

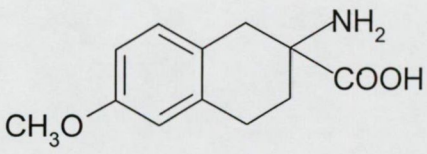
B.



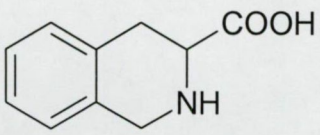
Igl



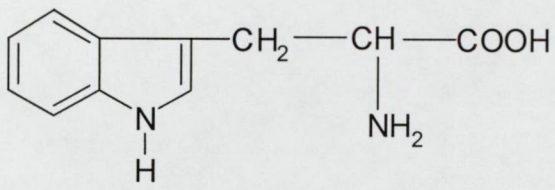
Atc



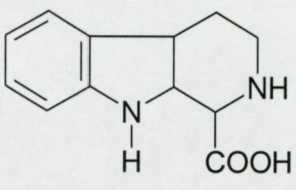
MeOAtc



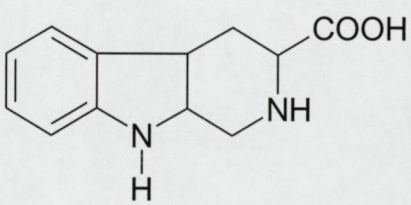
Tic



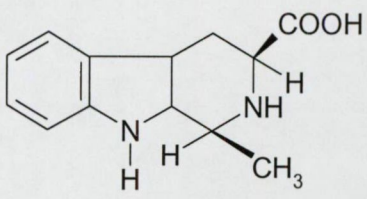
Trp



Car



Tcc



MeTcc

VI. MATERIALS AND METHODS

VI/1. Mating of animals

Animal investigations were carried out with the approval of the Ethical Committee for Animal Research, University of Szeged (registration number 23/1999), in accordance with the Guiding Principles for the Care and Use of Research Animals. Sexually mature female Sprague-Dawley rats (body mass: 200-300 g) and Charles River guinea-pigs were used in the experiments. Mating with males was carried out in the early morning hours. Copulation was justified by the presence of a copulation plug or spermatozoa in the vagina. The day of conception was considered to be the first day of pregnancy. Animals were housed in temperature and humidity-controlled, light-regulated (12 h of light, 12 h of dark) rooms, with water and food intake ad libitum.

VI/2. Membrane preparation

The affinities and the selectivities of the synthesized peptides for the OTR and VP receptor (VPR) were determined by radioligand binding assay.

The OTR binding assays were carried out on a uterine plasma membrane fraction of term pregnant guinea-pig uterus. The plasma membrane was prepared by a modification of the method described by Fuchs et al.[Fuchs AR., 1992]. Uterine tissue was minced and homogenized in 10 vol ice-cold buffer containing 10 mM Tris-HCl, 1.5 mM EDTA, 0.5 mM dithiotreitol and 1 mM PMSF; pH 7.5. After homogenization with an Ultra Turrax homogenizer, the suspension was centrifuged at 1000 x *g* for 10 min at 4 °C. The resultant supernatant was centrifuged at 100 000 x *g* for 60 min at 4 °C. The pellets were washed and resuspended for storage in Ca^{2+}-free Hanks' salt solution with 1 mM PMSF at a protein concentration of approximately 10 mg/ml at –70 °C.

Rat kidney inner medulla containing VP_2 receptors and the rat liver plasma containing VP_1 receptors were prepared by the following method [Marchingo AJ., 1988]. Adult male Sprague-Dawley rats (200-300 g) were killed by stunning and decapitation. The medullopapillary region of the kidneys and the livers were homogenized with an Ultra Turrax in 20 vol ice-cold buffer containing 10 mM Tris-HCl and 0.1 mM PMSF; pH 7.4. The homogenate was centrifuged at 500 x *g* for 5 min to remove all debris and nuclei. The supernatant was centrifuged at 20 000 x *g* for 20 min, and the pellet was resuspended in ice-cold incubation buffer, containing 100 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 mg/ml

bacitracin, and 100 IU/ml aprotinin and recentrifuged at 20 000 x *g*. The final pellet was resuspended for storage in 10 vol incubation buffer.

VI/3. Receptor assay

The affinities of OT and its analogues for the OTR and VPRs were determined by homologue and heterologue displacement analyses. In the OTR assay, the assay buffer consisted of 25 mM Tris-HCl, 1 mM $MnCl_2$, 1 mM PMSF and 0.1% BSA; pH 7.5. The affinity of the OT analogue was investigated in the presence of 1 nM [^{3}H]oxytocin (44 Ci/mmol) at 22 °C for 60 min. In the case of VPR assay, the assay buffer contained 100 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mg/ml bacitracin, 100 IU/ml aprotinin and 0.1% BSA. The competition binding assays were carried out in the presence of 1 nM [^{3}H]vasopressin (75 Ci/mmol) at 25 °C for 120 min. In both cases, the non-specific binding was determined in the presence of 10^{-5} M unlabelled OT and VP. The bound ligand was separated from the free ligand by rapid filtration through a Whatman GF/C glass fibre filter, using a Brandel M24R cell harvester. Specific binding was determined by subtracting the non-specific binding from the total binding.

VI/4. Measurement of uterine contraction *in vitro*

Uteri were removed from 60-65-day pregnant guinea-pig, and trimmed of fat, the foeto-placental units were removed and the decidua was scraped off. A uterine muscle strip (10 mm long and 10 mm wide) was suspended vertically in a 10 ml organ bath containing Van Dyke-Hastings solution. Van-Dyke Hasting solution was used in these experiments, the composition of which was a follows (in milligrams per liter): sodium chloride 113, potassium chloride 6.2, calcium chloride 0.44, magnesium chloride 0.47, sodium bicarbonate 29.8, sodium-dihydrogen-phosphate 0.83, disodium-hydrogen-phosphate 0.26, and glucose 5. The solution was equilibrated with a mixture of 95 % oxygen and 5 % carbon dioxide at pH 7.4 and the standard bath temperature was 37 °C. The initial tension was set to about 1.5 g, which was relaxed to about 0.5 g at the end of the equilibration.

Before the onset of the experiments uterine strips were equilibrated for 60 min and allowed to contract spontaneously and display stable rhythmic contractions. Repeating washing of the tissue was use during this period. At the end of the adaptation period, the antagonists (10^{-8} M) were added to the tissue bath in a volume of 50 μl. After a 30-min incubation in the presence of the antagonist increasing doses ranging from 10^{-9} to 10^{-3} M OT were administered in cumulative manner in a volume of 50 μl. The spontaneous contractility of the uterine strip

was registered as control for 180 s. The OT responses were measured as the area under the curve during a 3 minute period after administration of each dose of OT. The contractions were measured with a strain gauge transducer (SG-02, Experimetria U.K. Ltd.) and recorded by an ISOSYS Data Acquisition System (Experimetria U.K. Ltd.).

VI/5. Measurement of uterine contraction *in vivo*

In in vivo experimental pharmacological animal method the experiments were carried out on 24 hours postpartum rat uterus. During *in vivo* preliminary experiments the spontaneous contraction were the strongest after 24 hours, though the values after 12 and 48 hours were also significantly increased as compared to the 1-hour value [Zupkó, I., 1998] (Figure 5).
24 hours after spontaneous delivery, female Sprague-Dawley rats were anaesthetized with urethane (1 g/kg, intraperitoneally). After cannulation of the jugular vein, a Millar catheter (Millar Instruments Inc., Houston, Texas, USA) fitted with a liquid-filled latex microballoon was inserted into the uterine horn through a small section above the cervical part (Figure 6).
At the end of the 30-min equilibration period, the intrauterine pressure was recorded and the effects of the intravenously administered compounds were assessed by expressing the integrated tension relating to a 5-min period after the administration of each dose as a percentage of the average for three 5-min periods before the first administration. The maximum inhibition and the ED_{50} values were calculated.

VI/6. Statistical analyses

In the cases of the receptor binding experiments all assays were carried out at least three times in duplicate and values are given as means ± S.EM. Binding parameters were calculated by nonlinear regression, Graph Pad Prism 2.01 Computer Program, Graph Pad Software, USA.
In the *in vitro* and *in vivo* contractility studies the contractile response was measured as the area under the curve during a 3-min or 5-min period. Areas under curves were evaluated with the computer program as mentioned above. In the case of the in vitro studies for statistical evaluations, data were analysed by means of the ANOVA, followed by Neuman-Keuls post-hoc test.

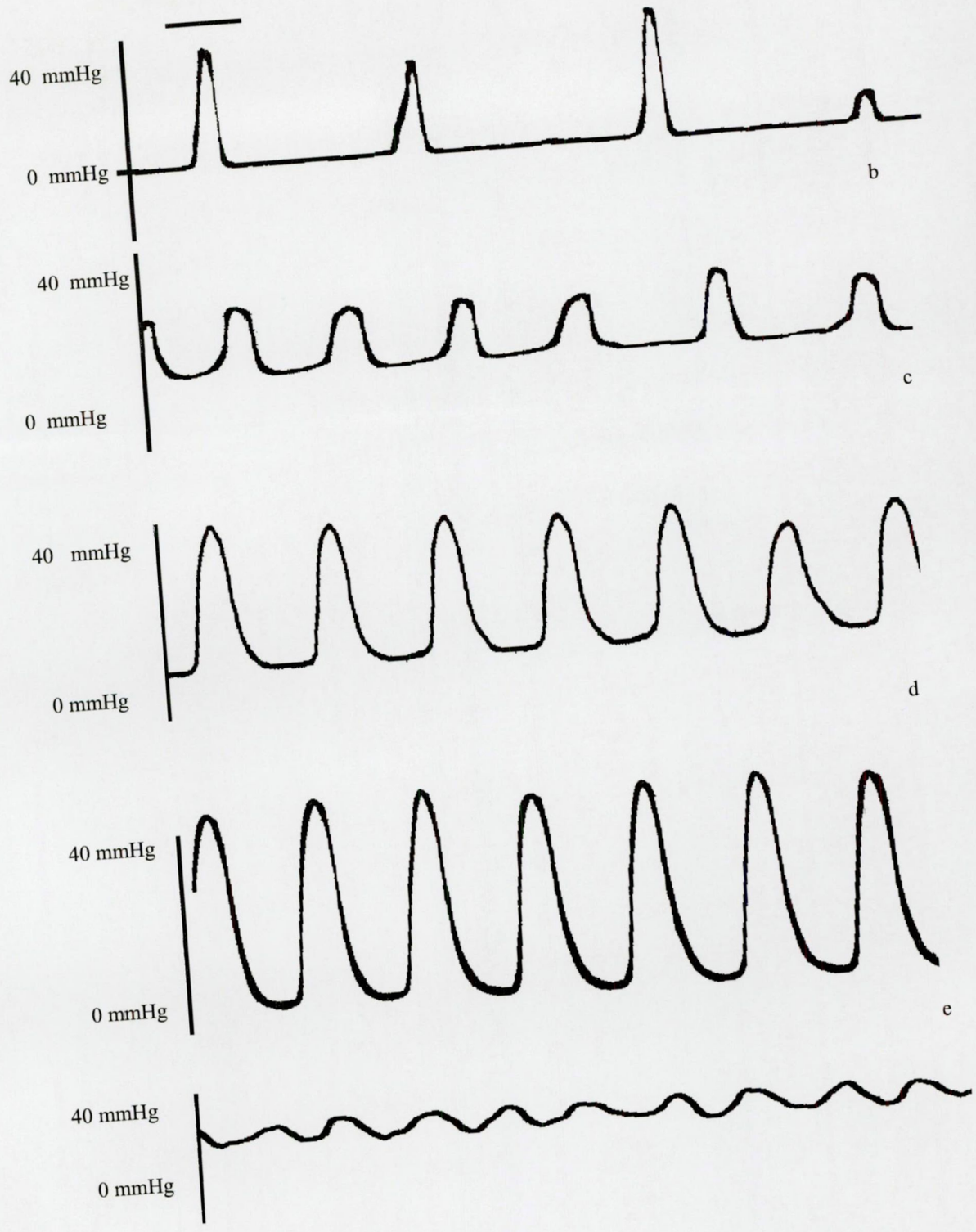


Figure 5.

Characteristic patterns of intrauterine pressure (a) 1, (b) 8, (c) 12, (d) 24, (e) 48 h after delivery.

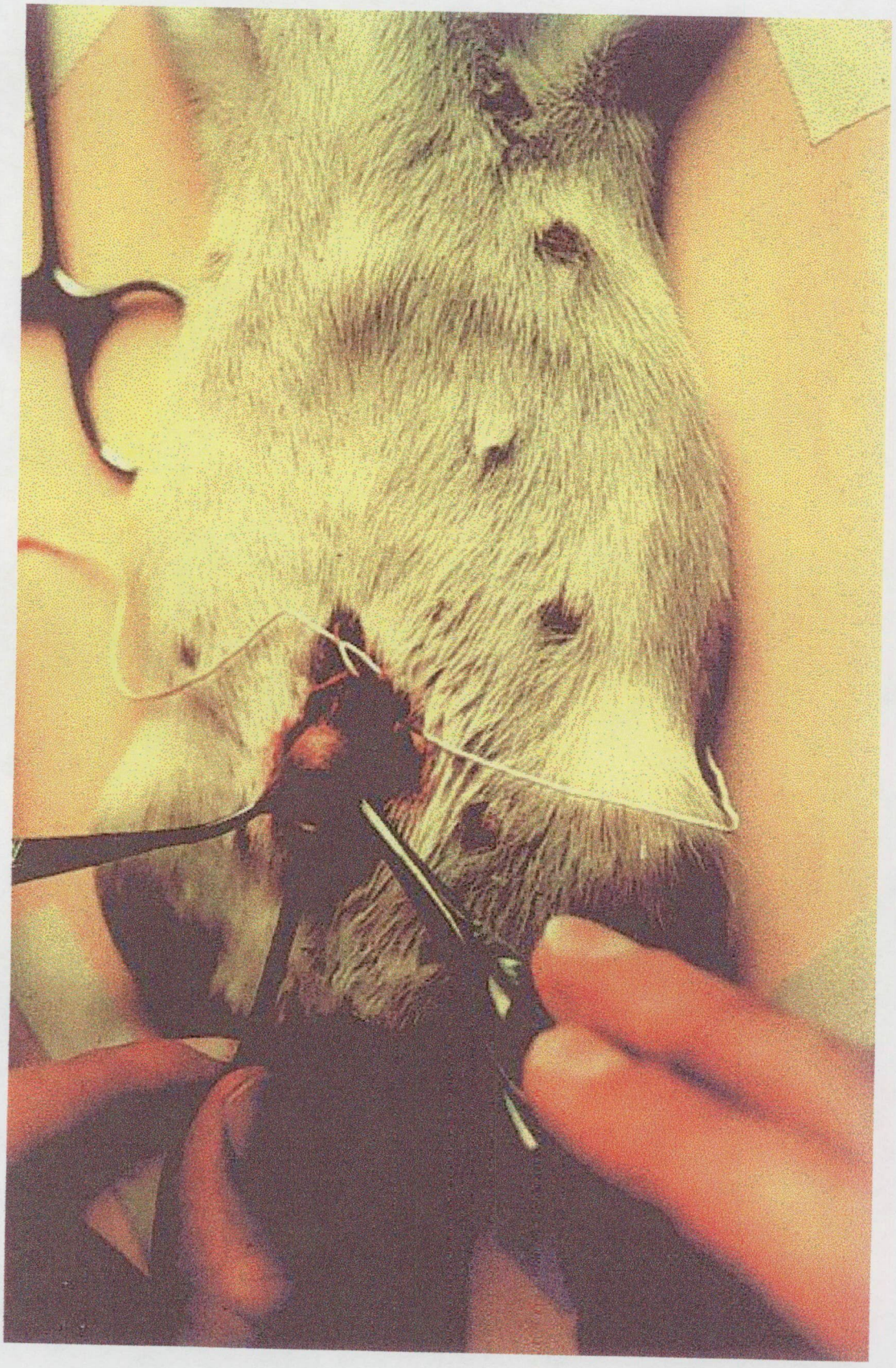


Figure 6.

Millar chateter fitted with latex microballoon inserted into the postpartum rat uterus

VII. RESULTS

VII/1. Results of receptor binding assay

During a modern drug development the determination of the affinity and selectivity of the newly synthesised OT analogues to the OT and VP receptors is the first step. These parameters were measured by radioligand binding assay on pregnant guinea pig uterus, rat liver and kidney membrane. Figure 7. shows the representative displacement curves of tested analogues on pregnant guinea-pig uterus membrane in the presence of [^{3}H]OT. Each drug displaced the [^{3}H]OT binding in a concentration-dependent manner.

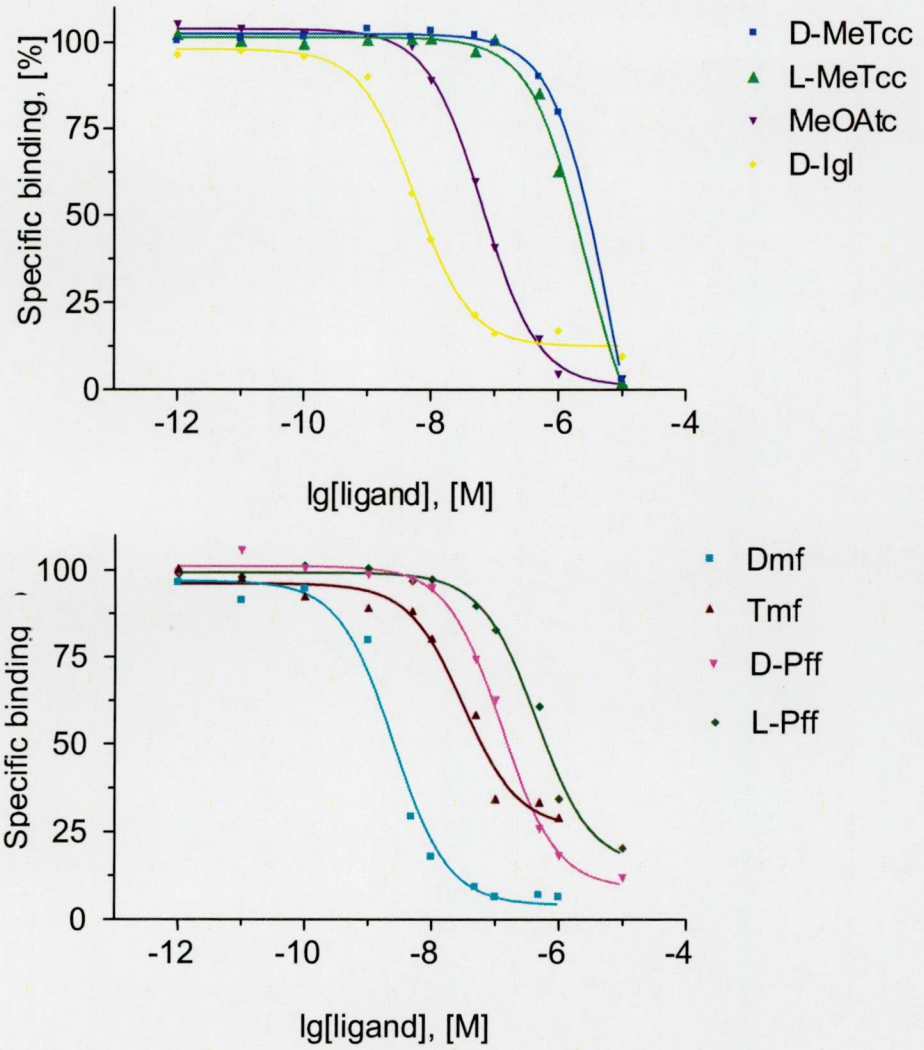


Figure 7.

Representative displacement curves of OT analogues on pregnant guinea-pig uterus in the presence of 1nm [^{3}H]OT. All assays were carried out at least three times in duplicate.

Data were analysed by an iterative nonlinear regression program. In each case the program determined the inhibition constants (K_i) of the tested peptides to the OT and VP receptors. This constant can describe the affinity of the molecule to the receptor. The inhibition constant is a corrigated IC_{50} value. The IC_{50} value was defined as the concentration of the antagonists required obtaining 50% inhibition of the specific binding. The K_i is a better parameter compare to the IC_{50} because it is independent from the concentration of the radioligand. Therefore the inhibition constant is used to compare the affinity of the different compounds. Calculation of the K_i was performed according to the Cheng and Prusoff equation.

$$K_i = \frac{IC_{50}}{1 + [L]/K_d}$$

where L is the concentration of the labelled ligand and K_d the apparent dissociation constant of the radioligand for the receptor.

The lower K_i value of a compound is the higher affinity of the compound to the receptor. The inhibition constant values of the tested OT antagonists relative to the OT, VP_1 and VP_2 receptors are listed in Table 1. For comparison, atosiban was tested in the same system.

Table 1

K_i values of the tested peptides for the OT, VP_1 and VP_2 receptors

		Tested peptides	K_i values (nM±S.E.M.)		
			OTR	**VP_1R**	**VP_2R**
		Oxytocin	2,51 ± 0,6		
		AVP		1.5 ± 0.5	0.65 ± 0.04
		Atosiban	5,76 ± 1,48	1412 ± 145	667 ± 163
	1.	Mpa[1]**I-Dmf**[2] Sar[7]AVT	1,49 ± 0,31	48,4 ± 25,7	70,8 ± 15,8
	2.	Mpa[1]**I-Tmf**[2] Sar[7]AVT	46,5 ± 19,6	254 ± 128	384 ± 99,3
I.	3.	Mpa[1]**D-Cpa**[2] Sar[7]AVT[a]	9 ± 2	8 ± 2.5	2.8 ±0.7
	4.	Mpa[1]**D-Pff**[2] Sar[7]AVT	52,9 ± 20,5	5420 ± 2490	2250 ± 820
	5.	Mpa[1]**L-Pff**[2] Sar[7]AVT	230 ± 83,9	3420 ± 620	9950 ± 2470
	6.	Mpa[1]**L-Dbr**[2] Sar[7]AVT[a]	3300	2700	152 ± 40
	7.	Mpa[1]**L-Igl**[2] Sar[7]AVT	7,32 ± 0,37	215,4 ± 0,57	173,9 ± 0,25
	8.	Mpa[1]**D-Igl**[2] Sar[7]AVT	3,81 ± 0,18	263 ± 36,9	425 ± 29,1
	9.	Mpa[1]**S-Atc**[2] Sar[7]AVT[a]	369.5 ± 87	17600 ± 1838	2290 ± 410
	10.	Mpa[1]**R-Atc**[2]Sar[7]AVT[a]	1917 ±1682	5640 ± 163	5340 ± 640
	11.	Mpa[1]**MeOAtc**[2] Sar[7]AVT	56,2 ± 9,56	1940 ± 1230	1640 ± 1100
	12.	Mpa[1]**I-Car**[2] Sar[7]AVT[a,b]	8545 ± 3330	$> 10^4$	63,5 ± 4.1
II.	13.	Mpa[1]**II-Car**[2] Sar[7]AVT[a,b]	57450 ± 1061	$> 10^4$	17,8 ± 4.1
	14.	Mpa[1]**S-Tcc**[2] Sar[7]AVT	1227±157	1300±26	1450±113
	15.	Mpa[1]**R-Tcc**[2] Sar[7]AVT	692,8±157	1715±569	6190±269
	16.	Mpa[1]**S,S-MeTcc**[2] Sar[7]AVT	2020 ± 124	3740 ± 2850	5400 ± 590
	17.	Mpa[1]**R,R-MeTcc**[2] Sar[7]AVT	4700 ±745	9564 ± 3412	55100 ± 6200
	18.	Mpa[1]**D-Trp**[2] Sar[7]AVT[a]	37.6 ± 23.2	888 ± 210	4960 ± 510
	19.	Mpa[1]**S-Tic**[2] Sar[7]AVT[c]	*	*	*

[a] K_i values of the labelled OT antagonists were measured by Imre Pávó

[b] determination of the absolute configuration has not been done

* the parameters were not measured

Table 1 summarizes the K_i values of the tested OT analogues to the OT, $VP_{1\ and}$ VP_2 receptors. In the first part of the Table contains the K_i values of the differently substituted phenylalanine derivatives.

1. Peptides containing **Dmf** at position 2 resulted in compound with a high affinity. It was similar to that of atosiban. This peptide has lower affinity to the different types of VP receptors. Incorporation further methyl group at position 6 of the phenylalanine ring (**Tmf**) decreased the affinity to both receptors.
2. In case of the halogenizated phenylalnine derivatives, the **Cpa** (phenyalainine ring is substituted with chloride at position 4) has a highest affinity to the OTR, but it has also similar affinity to the VPRs; it can not differentiate these receptors.
3. The peptides incorporated pentafluoro phenylalanine derivatives (**L, and D-Pff**) have lower affinity to the OTR than the previously mentioned. As concern **Pff**, the incorporation of the L and D configuration of the amino acid resulted in analogues with different affinity. Building of the D configuration resulted in higher affinity and selectivity to the OTR.
4. The compound containing **Dbr** at position 2 (phenylalanine ring is substituted with a hydroxyl group and two bromide) has a weak affinity to the OTR, and VP_1 receptor, but it has one order of magnitude higher affinity to the VP_2 receptor.

The second part of the Table 1 contains the K_i values of the peptides containing different rigid structure at position 2.

1. Substitution at position 2 with the L and D configuration of the **Igl** resulted in the compound with the highest affinity. They have two orders of magnitude lower affinity to the VPRs, but they can not identify the two types of VPR.
2. Substitution of the aminotetralin-2-carboxylic acid with a methoxy group (**MeOAtc**) resulted in analogue with two order of magnitude higher affinity to the OTR than the mother compound (**Atc**). This modification of the molecule did not prove the affinity to the VPRs.
3. The uterotonic receptor affinity of the OT analogue incorporated Trp derivatives (**D-Trp**) was found to be higher than the affinity to the two types of VPRs. It can also differentiate the VPRs, it has higher affinity to the VP_2 receptors than VP_1 receptor.
4. The modification of the Trp analogue led us to introduce Car, which is a constrained analogue of Trp. The **I-Car, II-Car** displayed practically no binding to the uterotonic receptor, and the VP_2 receptor affinity was negligible in both cases. The VP_1 affinities

were relatively low in both cases. Thus, with a 3 order of magnitude decrease in the OT and VP_2 binding, these analogues are selective VP_1 receptor agonists.

The selectivity is one the most important parameters of a molecule, because it determines the side effect profile of a molecule. The selectivity ratio is a parameter, which can characterize a compound. The selectivity ratio was calculated from the K_i values for the OTR and the different types of VPRs. The lower selectivity ratio of a compound represents the better selectivity to the OTR. The Table 2 gives the selectivity ratios of the newly synthesized OT antagonists.

Table 2.

Selectivity ratios of the tested peptides

		Tested peptides	Selectivity ratio	
			OT/VP$_1$	**OT/VP$_2$**
		atosiban	0,004	0,008
	1.	Mpa^1**I-Dmf**^{2} Sar^{7}AVT[b]	0,031	0,021
	2.	Mpa^1**I-Tmf**^{2} Sar^{7}AVT[b]	0,183	0,121
I.	3.	Mpa^1**D-Cpa**^{2} Sar^{7}AVT[a]	1,125	3,214
	4.	Mpa^1**D-Pff**^{2} Sar^{7}AVT	0,010	0,023
	5.	Mpa^1**L-Pff**^{2} Sar^{7}AVT	0,067	0,023
	6.	Mpa^1**L-Dbt**^{2} Sar^{7}AVT[a]	1,222	21,71
	7.	Mpa^1**D-Igl**^{2} Sar^{7}AVT	0,014	0,009
	8.	Mpa^1**L-Igl**^{2} Sar^{7}AVT	0,034	0,042
	9.	Mpa^1**S-Atc**^{2} Sar^{7}AVT[a]	0,021	0,161
	10.	Mpa^1**R-Atc**^{2} Sar^{7}AVT[a]	0,726	0,359
	11.	Mpa^1**MeOAtc**^{2} Sar^{7}AVT	0,029	0,034
	12.	Mpa^1**D-Trp**^{2} Sar^{7}AVT[a]	0,042	0,008
II.	13.	Mpa^1**I-Car**^{2} Sar^{7}AVT[a,b]	134,6	
	14.	Mpa^1**II-Car**^{2} Sar^{7}AVT[a, b]	3227	
	15.	Mpa^1**S-Tcc**^{2} Sar^{7}AVT	0,944	0,846
	16.	Mpa^1**R-Tcc**^{2} Sar^{7}AVT	0,404	0,112
	17.	Mpa^1**S,S-MeTcc**^{2} Sar^{7}AVT	0,540	0,374
	18.	Mpa^1**R,R-MeTcc**^{2} Sar^{7}AVT	0,491	0,085

[a] K_i values of the labelled OT antagonists were measured by Imre Pávó

[b] determination of the absolute configuration has not been done

None of the tested analogues had such a high selectivity as atosiban.

1. Six of the synthesised exhibited relatively good selectivities: Mpa^1**D-Trp**^{2}Sar^{7}AVT, Mpa^1**D-Pff**^{2}Sar^{7}AVT, Mpa^1**D-Igl**^{2}Sar^{7}AVT, Mpa^1**MeoAtc**^{2}Sar^{7}AVT, Mpa^1**Dmf**^{2}Sar^{7}AVT, Mpa^1**L-Pff**^{2}Sar^{7}AVT. It means that these compounds two orders of magnitude higher affinity to the OTR than the VPRs.
2. The constrained derivatives of Trp, peptides containing **Car** at position 2 proved to be selective to the VPRs, extremely to the VP_2 receptor.

VII/2. Results of the *in vitro* uterine contractility studies

In vitro contractility studies were performed with 8 OT analogues to characterize the biological activity of these peptides. An initial series of studies was undertaken to determine the effects of the tested peptides on OT-induced contraction in isolated strips of pregnant guinea-pig uterus. OT induced dose-related increases in basal tone, amplitude and frequency of contraction with maximum effects. In the presence of the newly synthesised OT antagonists, the maximum effects caused by OT were significantly decreased $F(9,72)=5.276$, $p=0.0001$ (Figures 8 and 9).

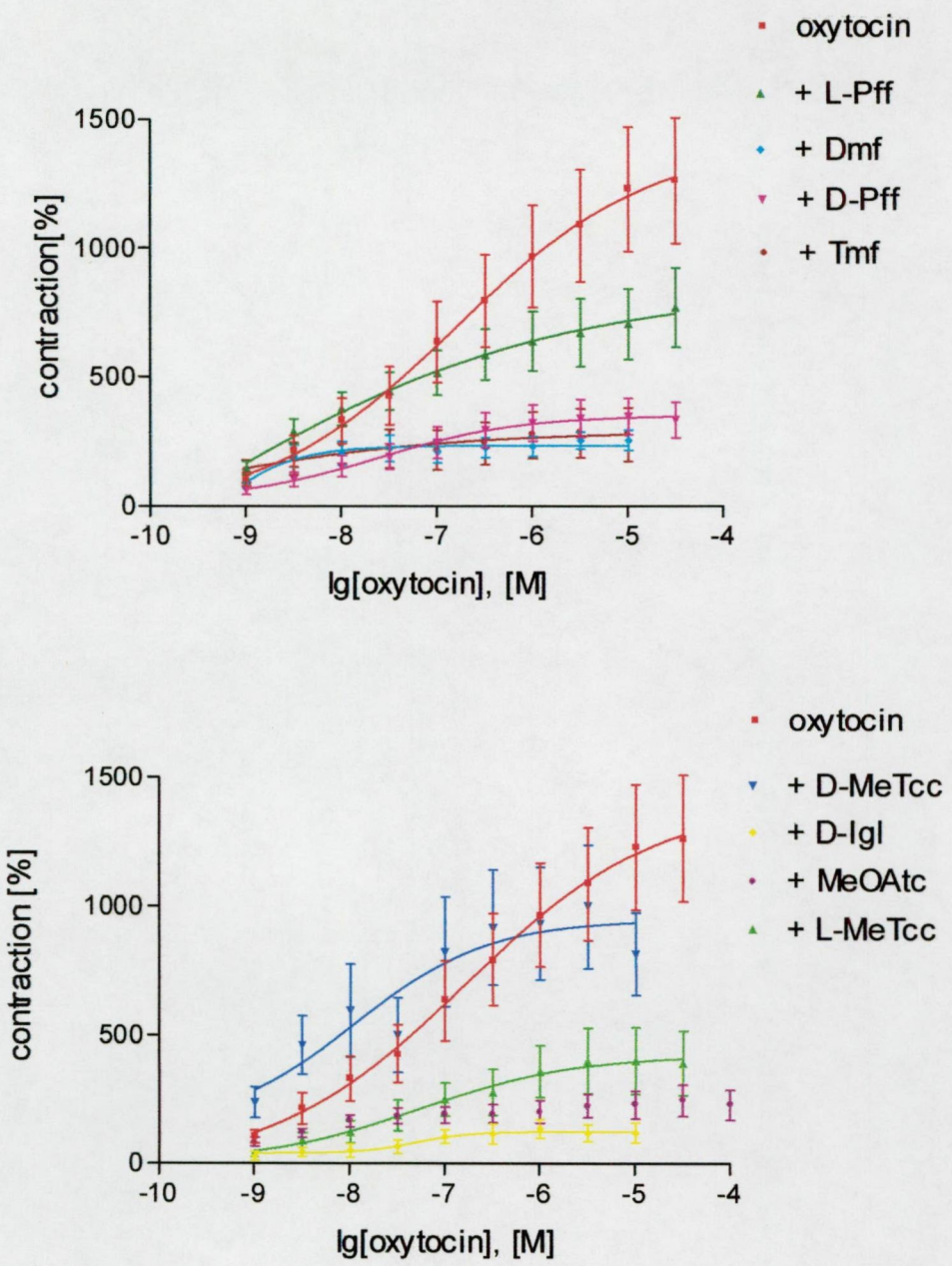


Figure 8.

The dose-response curves represent the effects of OT on isolated guinea-pig uterus alone and in the presence of the newly synthesized OT antagonists. The spontaneous uterine contractility was taken as 100%. All of the tested peptides significantly decreased the maximum effect observed with OT, without any significant change in the ED_{50} value. (Values are given as means ±S.E.M.)

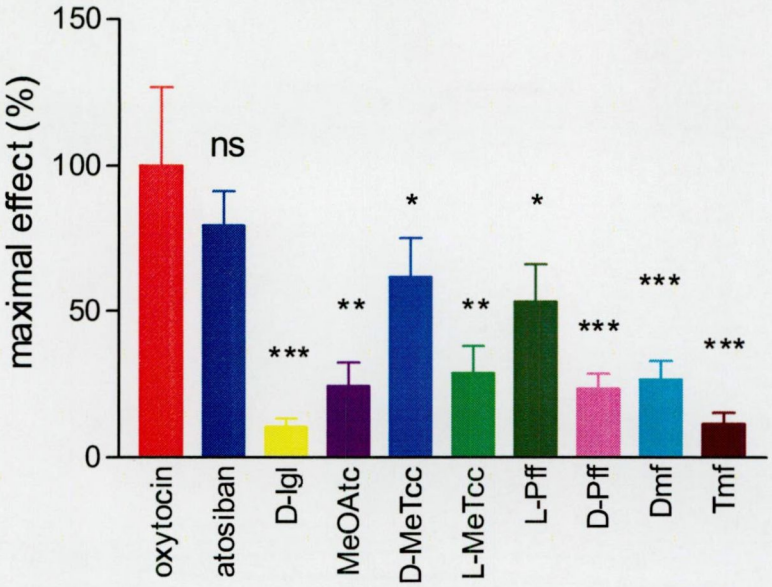


Figure 9.

*Statistical comparison of the antioxytocic effects of the tested peptides. Each bar represents the maximum effects of OT alone and in the presence of the tested peptides in the isolated pregnant guinea-pig uterus (results of Newman-Keuls post hoc test, ns: not significant, *: $p<0.05$; **: $p<0.01$; ***: $p< 0.001$)*

In contrast, in the presence of atosiban, a higher concentration of OT was required to elicit the same degree of myometrial contractility. In isolated guinea-pig uterine strips, atosiban (10^{-8} M) displaced the OT dose-response curve to the right in a parallel fashion, without significant change in the maximum effect (Figure 10).

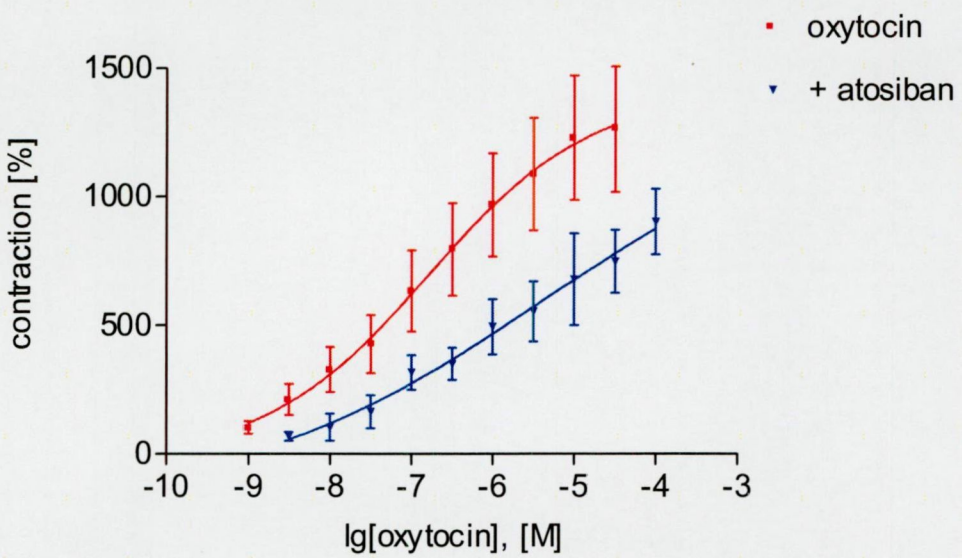


Figure 10.

The dose-response curves represent the effect of OT alone and in the presence of atosiban. The spontaneous uterine contractility was taken as 100%. Atosiban did not decrease the maximum effect of OT, but significantly changed the ED_{50} of OT. (Values are given as means ±S.E.M.)

VII/3. Results of the *in vivo* uterine contractility

The aim of the next series of experiments was to examine the ability of twelve newly synthesized OT antagonists to inhibit the spontaneous contractility in the 24-hour postpartum rat uterus. Four compounds were selected on the basis of their good *in vitro* effectivities. The OT antagonists were administered intravenously, in a cumulative manner (the first dose was 0.001 mg/kg). All the tested peptides markedly suppressed the myometrial contraction in a dose-dependent way (Figure 11).

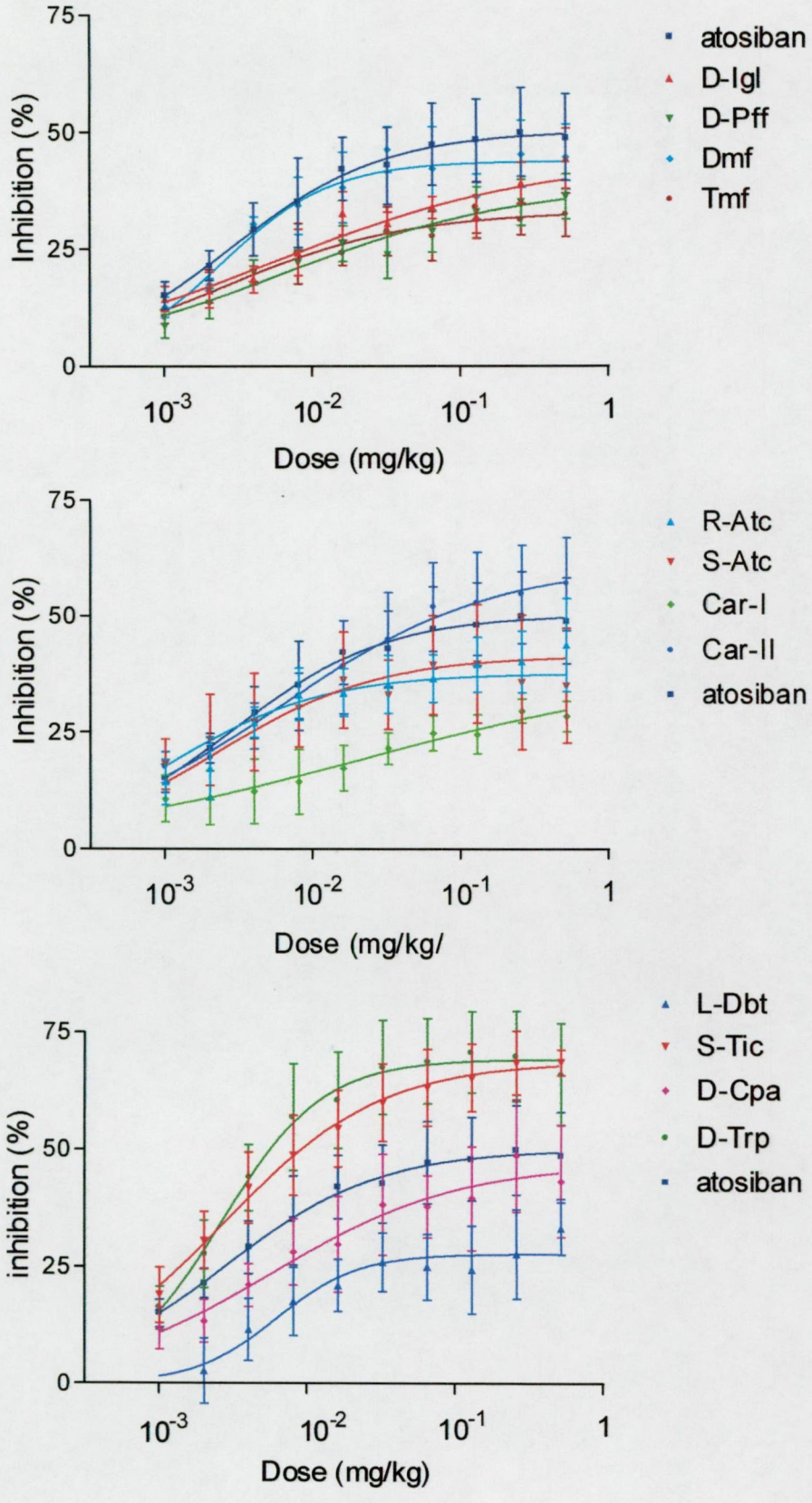


Figure 11.

The dose-response curves represent the inhibitory effects of the tested peptides on the postpartum rat in vivo. The tested peptides were given intravenously, in a cumulative way. (The data are the averages of the results of six independent experiments; values are given as means ±S.E.M.)

The calculated maximum inhibition of the spontaneous uterine motility and ED_{50} values are listed in Table 3.

Table 3.

Calculated maximum inhibition and ED_{50} values of the tested peptides

		Tested peptides	Maximal effect (%)	ED_{50} (μg/kg)
		Fenoterol	93,45	1,001
		Atosiban	50,23	5,568
	1.	Mpa^1**Dmf^2**Sar^{7}AVT	45,57	5,33
	2.	Mpa^1**D-Cpa^2**Sar^{7}AVT	43,34	4,41
I.	3.	Mpa^1**Tmf^2**Sar^{7}AVT	36,18	6,449
	4.	Mpa^1**D-Pff^2**Sar^{7}AVT	35,22	8,29
	5.	Mpa^1**L-Dbr^2** Sar ^{7}AVT	29,62	7,23
	6.	Mpa^1**D-Trp^2** Sar^{7}AVT	71,17	2,72
	7.	Mpa^1**S-Tic^2** Sar^{7}AVT	66,76	2,61
	8.	Mpa^1**II-Car^2** Sar^{7}AVT	54,05	4,23
II.	9.	Mpa^1**R-Atc^2**Sar^{7}AVT	39,99	2,24
	10.	Mpa^1**D-Igl^2**Sar^{7}AVT	38,04	6,28
	11.	Mpa^1**S-Atc^2** Sar^{7}AVT	37,39	1,34
	12.	Mpa^1**I-Car^2** Sar^{7}AVT	27,44	5,13

These data demonstrate that all of the tested peptides are effective in inhibiting the spontaneous contractility of the rat uterus *in vivo*. For comparison, in the same system we tested β_2-adrenergic agonist fenoterol. Fenoterol exerts a substantially more pronounced inhibitory effect on postpartum rat uterus.

Part I. of Table 3. contain the differently substituted phenylalanine derivatives.

1. Peptides containing **Dmf** at position 2 proved to be the more potent compound. The antioxytocic activities of these peptides comparable with atosiban. Incorporation of further methyl group to the phenylalanine ring, **Tmf** decreased the inhibitory effect.
2. Incorporation **Cpa** (phenylalanine ring is substituted one chloride) resulted in an analogue with similar potency to **Dmf**.
3. Further halogenization of the phenyalanine ring decreased the potency of the compounds (**D-Pff, L-Dbr**) with lower potency.

The part II. of Table 3. contains the peptides incorporated rigid structure of the amino acids.

1. Substituted with **D-Trp** and **S-Tic** at position 2 proved to be more potent than atosiban, although the differences were not significant statistically.
2. The two configuration of the constrained Trp analogue (**I-Car, II-Car**) resulted in different potency compounds. Incorporation of one enatiomer of **Car** did not lead to significantly effective compound. Peptide containing **II-Car** has similar effectivity than the atosiban.
3. The other rigid phenyalanine derivatives (**D-Igl, R-Atc, S-Atc**) resulted in analogs with similarly moderate activity. Incorporation of the two configuration of **Atc** resulted in analogues with similar effectivity.

VIII. DISCUSSION

Preterm labor is one of the major causes (75% to 80%) of perinatal morbidity and mortality. The social and economic consequences of the preterm birth in term of mental anguish and cost of caring for preterm infants and the associated lifelong infirmities are enormous. Currently it is probably the most important problem to be addressed by modern obstetric research. At present, tocolytic treatment is not prophylactic but commences after the onset of labor. The first line of treatment of preterm labor is the use of β-adrenergic agonists, but these agents are not ideal therapy for preterm labor. In fact, their use is associated with a high failure rate and a significant side effect liability. The most common side effects are associated with β-agonist actions on the maternal cardiovascular system and metabolism. Therefore, the development of specific new tocolytic agents without the side effect liability of the β-adrenergic agonists has an important clinical justification.

Although the role of OT in the spontaneous onset of labor remains controversial, it may be concluded that OT antagonists offer potentially useful approach for the management or even the prevention of uncomplicated preterm labor. This primary attraction is their apparent myometrial selectivity and the lack of side-effects as compared with other available therapies. e.g. β-mimetics. A number of studies have dealt with the design and pharmacological investigation of new OT antagonists. During the development of the OT antagonists, modification of the amino acids in the OT molecule at positions 1, 2, 4 or 7 and 8 produced analogues with higher receptor affinities for the myometrium in vitro [Gronka, Z., 1991; Gronzak, Z., 1983; Lebl, M.; 1987; Melin, P., 1986; Pávó, I., 1994]. The most potent of these was atosiban, which subsequently underwent clinical evaluation. Long-term exposure to atosiban in vitro does not affect myometrial sensitivity to OT, therefore it is unlikely that the in vivo administration has any residual effect on uterine sensitivity to OT. Moreover, atosiban does not affect the response of the cells to prostaglandins, suggesting that the cells remain responsive to other G protein-coupled receptors [Phaneuf, S., 1994].

In this study, the synthesised peptides are 1-deamino-7-sarcosine-8-arginine analogues. The position 2 plays a crucial role in determining the antagonistic property. Therefore, Tyr at position 2 was modificated in these compounds.

The nature peptides have an extensive conformational freedom. This is the reason that the same peptide chain can cause different biologic effect. These small, flexible molecules and their three-dimensional structure depend highly on the environment in solution. Modulation of

the flexibility of a peptide backbone from an extended conformation to a β-turn structure is an important breakthrough in the rational design of highly selective and active peptide. We attempted to restrict this relatively extensive conformational freedom in order to stabilize the bioactive antioxytocic conformation. There are different possibilities for the insertion of definite constraints into a peptide backbone: by introduction of a constrained amino acid; the introduction of a cyclic amino acids or side-chain cyclised amino acids; the formation of mono- or polycyclic structures involving disulphide bridges; cyclisation of the side-chain to the backbone, or cyclisation of the C-terminal to the N-terminal.

Our approaches in the design of more effective OT antagonists are based mainly on the incorporation of bulky apolar side-chain amino acids with a conformationally constrained restricted feature at position 2 [Hruby, V.J., 1994; Manning, M., 1989, 1995 a, 1995 b].

The rationale for the development of OT antagonists was based on producing a novel compound that mimicked normal physiological processes with high uterine specificity. The specificity for the OTR in the uterus is the key to the clinical application of the OT antagonists, since the major problem with all current tocolytic agents is their systemic activity, which causes potentially harmful multiorgan side-effects.

The first step in this screening investigation was the determination of the affinity and the selectivity of the newly synthesised OT analogues to the OTR. They were carried out by radioligand binding assay on late-pregnant guinea-pig uterus, and rat kidney and liver membrane preparation in the presence of tritiated OT and VP. The results of receptor analyses indicate that generally those OT antagonists that contain Phe derivatives at position 2 have higher affinity and selectivity for the OTR than those do containing substituted Trp or Tyr analogues. It is conceivable that the incorporation of a substituted Trp analogue into the peptide results in a molecule that is too large and rigid to bind to the apolar side-chain binding pocket of the OTR. However, the incorporation of the D configuration of the Trp resulted in a high affinity and selectivity analogue. The good binding parameters of this molecule led us to introduce Car, which is a constrained analogue of the Trp. The compound containing Car has low affinity and selectivity to the OTR. It proved to be selective to the VP_1 receptor. Further modification of this amino acid, replacement of the carboxylic acid into the position 3 of the phenyalalanine ring (Tcc) and building a methyl group into the position 1 of the phenylalanine ring (MeTcc) improves the affinity and selectivity to the OTR.

In order to achieve better affinity, different phenylalanine derivatives were synthesised for incorporation. Of these compounds, Dmf, Cpa, D, and L-Igl resulted in the compounds with the highest affinities, similar to that of atosiban, but they had no such good selectivity as

atosiban. In case of Dmf, incorporation of a further methyl group into position 6 of the phenylalanine ring (Tmf) decreased the affinity for the OTR and destroyed the selectivity, too. Substitution of the phenylalanine ring with halogen atoms decreased the affinity for the OTR. Peptide containing Cpa has a highest affinity to OTR, but it is not selective. Incorporation five fluoride atoms into the phenylalanine ring resulted in a compound with the highest selectivity. The binding to the OTR of the D isomer of this phenylalanine derivative is higher than in the case of the L isomer.

The MeOAtc was designed by modification of the OT analogue containing Atc at position 2. The MeOAtc, has higher affinity and selectivity than those of the original molecule. The presence of the methoxy at position 6 of the tetraline ring improves the interaction of the analogue and the OTR.

Summarizing the results of the receptor-binding experiments, the bulky, conformationally constrained amino acids at position 2 have a great influence on the receptor-ligand interaction. According to the results of these experiments, incorporation of the differently substituted phenylalanine derivatives and the nonsubstituted D-Trp resulted in the compounds with the best binding parameters.

In vitro contractility studies were performed for the determination of the biological activity of these compounds. We measured the effects of the tested peptides on OT-induced contraction in isolated strips of pregnant guinea-pig uterus. Eight OT analogues were selected to these studies on the base of their binding parameters. The results of the *in vitro* contractility study indicate that all the tested peptides are non-competitive antagonists, in contrast with atosiban, which is a competitive one [Paneuf, S., 1994]. This pharmacological characteristic can not be explained by the chemical features of these peptides. Washing experiments demonstrated that none of the synthesised OT antagonists can produce an irreversible interaction to the binding sites of the OTR and VPRs. Pregnant guinea-pig uterine membrane was preincubated in the presence of the tested peptides for 30 min at 37 °C, and the specific binding was then measured as described in the Methods. The rest of the preincubated membrane fraction was diluted to a 15-folds volume and centrifuged, and the specific binding was measured again. This dilution-centrifugation-binding procedure was performed three times. There was no significant decrease in the number of specific binding sites. Consequently, this non-competitive antagonism is due to the size and structure of the apolar side-chain of the amino acid at position 2. In the new peptides, the amino acids at this position are more conformationally constrained than that in atosiban, which can explain their behaviour.

In the *in vivo* studies a Millar catheter fitted with latex microballoon was inserted in the postpartum rat uterus. In spite of the obvious hormonal differences between the postpartum rat and human situation, this preparation can be regarded as an animal model of pregnancy [Csapo, A.I., 1982]. The in vivo experimental pharmacological animal methods the experiments were carried out on 24 hours postpartum rat uterus. During the *in vivo* experiments the spontaneous contraction were the strongest after 24 hours, though the values after 12 and 48 hours were also significantly increased as compared to the 1-hour value [Zupkó, I., 1997]

The results *in vivo* experiments support the pharmacological antagonistic characteristic of these peptides. All of the tested peptides inhibited the intrinsic OT-induced contraction in a dose-dependent manner. The incorporation of D-Trp and a rigid structure of the phenylalanine, Tic resulted in the most effective OT antagonists among the tested peptides. This may be due to the size and the arrangement of the side-chain of the amino acids at position 2. Modification of the Trp resulted in Car destroyed the efficacy of the molecule, but there was a significant differences between the two configuration of the amino acid. Incorporation of the II-Car (the absolute configuration has not been determined) resulted in an antagonist with a strong inhibitory effect. The substituted phenylalanine derivative, Dmf also resulted in a moderate effective compound, similar to atosiban. The phenylalanine ring substituted at positions 2 and 4 with methyl groups is needed for antioxytocic activity. Incorporation of a further methyl group into the phenylalanine ring (Tmf) decreased the efficacy of the peptide. The further phenylalanine derivatives have weaker effect on postpartum rat uterus.

As regards these syntheses of OT antagonists, the incorporation of D-Trp, S-Tic and Dmf at position 2 resulted in a peptide that was nearly equipotent to atosiban. These compounds have similar binding parameters for the OTR and inhibitory effects in the *in vivo* system. However, the atosiban has a better selectivity for the OTR.

IX. FINAL CONCLUSION

The favourable safety profile of selective OT antagonists, in combination with equivalent tocolytic effectiveness would appear to represent an advance over current tocolytic therapy. When treating preterm labor, it is essential to balance the relative risks and benefits of the treatment against expected maternal and foetal outcomes. Today, the decision to administer or continue tocolytic therapy appears to concentrate on the tolerability of current tocolytic agents or more importantly their poor side effect profile.

Exact localization of the OTR was determined in the recent past. OTR can be found the all body, for example kidney, heart and cardiovascular system, thymus, fat cells, pancreas, adrenal gland and central nervous system. In a view of the widespread OT-related actions (sexual, maternal and social behaviour, memory and learning, tolerance and dependence to opiods, central disorder) OT antagonists may not only be regarded as promising candidates to prevent preterm labor, but may together with OT agonists also prove useful for treatment of psychiatric illnesses such as anxiety, drug abuse, sexual dysfunction, and eating disorders.

In consequence of the pseudoirreversible pharmacological properties of the newly synthesised OT antagonists, these peptides comprise a novel group of OT antagonists for potential clinical use. Besides the weak side effect profile, this non-competitive pharmacological nature may be of therapeutic benefit because of the sustained effect on the myometrium. The advantages and possible disadvantages of the non-competitive OT antagonists need to be evaluated thoroughly in further pharmacological and clinical investigations.

In the near future, a well-tolerated, effective, specific and rationally designed tocolytic agent may change the procedure of tocolysis. The non-competitive OT antagonists may be among such promising drug novelties.

X. REFERENCES

Ben-Ami M., Giladi Y., Shalev E.: The combination of magnesium sulfate and nifedipin: a cause of neuromuscular blockade. Br J Obstet Gynecol 1994, 101: 262-263

Caritis SN., Darby MJ., Chan L.: Pharmacologic treatment of preterm labor. Clin Obstet Gynecol 1988, 31: 635-651

Clesham GJ., Whyte M.: β-Adrenergic agonists and pulmonary oedema in preterm labor. Br Med J 1994, 308: 260-262

Csapó AI., Puri CP., Tarro S., Henzl MR.: Deactivation of the uterus during normal and premature labor with calcium antagonist nicardipin. Am J Obstet Gynecol 1982, 142: 483-491

Elands J., Barberis C., Jard S., Tribollet E., Dreifuss JJ., Bankowski K., Manning M., Sawyer WH.: ^{125}I labelled d(CH_2)5[Tyr(Me)2,Thr4,Tyr-NH_2(9)]OVT: a selective oxytocin receptor ligand. Eur J Pharmacol 1988, 147: 197-207

Fuchs AR., Fuchs F.: Endocrinology of human parturition: a review. Br J Obstet Gynecol 1984, 91: 948-967

Fuchs, A.R.; Fuchs, F.; Husslein, P.; Soloff, M.S;. Fernstrom, M.J. Oxytocin receptors and human parturition: a dual role for oxytocin in the initiation of labor. Science 1982;215:1396-8

Fuchs, A.R.; Helmer, H.; Behrens, O.; Liu, H.C.; Antonian, L.; Chang, S.M.; Fields, M.J. Oxytocin and bovine parturition: A steep rise in endometrial oxytocin receptors precedes onset of labor. Biol. Reprod 1992;47:937-44

Gimpl G., Fahrenholz F.: The oxytocin receptor system: Structure, Function and Regulation. Phys Rev 2001, 81(2): 629-683

Goodwin TM., Paul R., Silver H., Spellacy W., Parsons M., Chez R.: The effect of the oxytocin antagonist atosiban on preterm uterine activity in human. Am J Obstet Gynecol 1994, 170: 474-478

Grzonka Z., Kasprzykowski F., Lubkowska L., Darlak K., Hahn TA., Spatola AF.: In vitro degradation of some arginine-vasopressin analogs by homogenates of rat kidney, liver and serum. Peptides Res 1991:4:270-4

Grzonka Z., Lammek B., Kasprzykowski F., Gazis D., Schwartz IL.: Synthesis and some pharmacological properties of oxytocin and vasopressin analogues with sarcosine or N-methyl-L-alanine in position 7. J Med Chem 1983:26:555-9

Hall DG., McGaughey HS., Corey EL., Thorton WN.: The effects of magnesium therapy on the duration of labor. Am J Obstet Gynecol 1959, 78: 27-32

Hendricks SK., Smith JR., Moore DE., Brown ZA.: Oligohydramnions associated with prostaglandin synthetase inhibitors in preterm labor. Br J Obstet Gynecol 1990, 97: 312-316

Higby K., Xenakis EMJ., Pauerstein CJ.: Do tocolitytic agents stop preterm labor? A critical and comprehensive review of efficacy and safety. Am J Obstet Gynecol 1993, 168: 1247-1259

Higuchi T.: Oxytocin: a neurohormon, Neuregulator, Paracrin substance. Jap J Physol 1995, 45: 1-21

Hruby VJ., Chow MS., Smith DD.: Conformational and structural considerations in oxytocin-receptor binding and biological activity. Ann Rev Pharmacol Toxicol 1990, 30: 501-34

Impey L.: Severe hypotension and fetal distress following sublingual administration of nifedipin to a patient with severe pregnancy induced hypertension at 33 weeks. Br J Obstet Gynecol 1988, 100: 959-961

Kimura T., Tanizawa O., Mori K., Brownstein MJ., Okayama H.: Structure and expression of a human oxytocin receptor. Nature 1992, 356: 526-529

Knobil E., D. Neill J.: Encyclopedia of Reproduction. Academic Press, 1998, San Diego, CA, USA

Lebl M.: Analogs with inhibitory properties. In: Jost K. Lebl M. Brtnik F. editors. Handbook of neurohypophyseal hormone analogs. Vol 2. Boca Raton: CRC Press. 1987: 17-74

Macones GA., Robinson CA.: Is there justification for using indometacin in preterm labor? An analyses of neonatal risks and benefits. Am J Obstet Gynecol 1997, 177: 819-824

Manning, M.; Miteva, K., Panchera, S.; Stoev, Wo, N.S.; Chan W.Y. Design and synthesis of higher selectivity in vitro and in vivo uterine receptor antagonists of oxytocin comparison with atosiban. Int J Pept Protein Res 1995;46:244-52

Marchingo, A.J.; Abrahams, J.M.; Woodcock, E.A.; Smith, A.I.; Mendelsohn, F.A.O.; Johnston, C.I. Properties of [^{3}H]1-desamino-8-D-arginine vasopressin as a radioligand for vasopressin V_2 receptors in rat kidney. Endocrinology 1988;122(4):1328-36

McCombs J.: Update on tocolytic therapy. Ann Pharmacother 1995; 29:515-522

Melin P.: Development of an oxytocin antagonist – Atosiban. Res Clin Forums 1994, 16: 155-168

Mercer BM., Goldenberg RL., Das A., et al.: The preterm prediction study: a clinical risk assessment system. Am J Obste Gynecol 1996; 174: 1885-189

Moise KJ. Huhta JC., Sharif DS., Ou CN., Kirshon B., Wasserstrum N.: Indometacin for the treatment of premature labour, effects on the fatal ducts arteries. N Eng J Med 1988, 319: 327-331

Morales WJ., Madhya H.: Efficacy and safety of indometacin compared with magnesium sulphate in the management of preterm labor. Am J Obstet Gynecol 1993, 169: 97-102

Norton ME., Merill MJ., Cooper BAB., Kuller JA., Clyman RI.: Neonatal complications after administration of indometacin for preterm labor. N Eng J Med 1993, 329: 1062-1067

Pávó I., Slaninova J., Klein U., Fahrenholz F.: Enhanced selectivity of oxytocin antagonists containing sarcosine in position 7. J Med Chem 1994, 37: 255-259

Phaneuf, S.; Asbóth, G.; MacKenzie, I.Z.; Melin, P.; López Bernal, A. Effect of oxytocin antagonists on the activation of human myometrium in vitro: Atosiban prevents oxytocin-induced desensitization. Am J Obstet Gynecol 1994; 171(6) 1627-34

Pisani RJ., Rosenow EC.: Pulmonary oedema associated with tocolytic therapy. Ann Intern Med 1989, 110: 714-718

Postina R., Kojro E., Fahrenholz F.: Separate agonist and peptide antagonist binding sites of the oxytocin receptor defined by their transfer into the V2 vasopressin receptor. J Biol Chem 1996, 271: 31593-31601

Romero R., Baha M., et all.: An oxytocin antagonist (atosiban) in the treatment of preterm labor: A randomized, double-blind, placebo-controlled trial with tocolytic rescue. Am J Obstet Gynecol 2000, 182: 1173-1183

Romero R., Mazor M., Munoz H., Gomez R., Galasso M., Shere DM.: The preterm labor syndrome. Ann NY Acad Sci 1994, 734: 414-429

Szentágotai JRM.: Funkcionális anatómia Bp., Semmelweis K., 1994

Takahashi, K.; Diammond, F.; Bieniarz, J.; Yen, H.; Burd, L. Uterine contractility and oxytocin sensitivity in preterm, term, and postterm pregnancy. Am J Obstet Gynecol 1980;136:774-9

Villar J., Ezcurra EJ., Gurtner de la Fuente V., Campodonico L.: Pre-term delivery syndrome: the unmet need. Res Clin Forums 1994; 16(3):9-38

Wilkins IA., Lynch L., Mehalek KE., Berkowitz GS., Berkowitz RL.: Efficacy and side effects of magnesium sulfate for the supression of preterm labor. Am J Obstet Gynecol 1988, 159: 685-689

Zingg HH., Rozen F., Breton C., Larcher A., Neculcea J., Chu K., Russo C., Arslan A.: Gonadal steroid regulation of oxytocin and oxytocin receptor gene expression. Adv Exp Med Biol 1995, 395: 395-404

Zupkó I., Gáspár R., Kovács L., Falkay G.: Are α-adrenergic antagonists potent tocolytics? In vivo experiments on pospartum rats. Life sciences 1997 61(11): 159-163

XI. ACKNOWLEDGEMENT

I would like to express my warmest thanks to Prof. László Kovács, for offering me the possibility to participate in his Ph.D. programme. I am extremely grateful for his kindness, help and for all the advice he provided me with during my work.

I am deeply indebted to my supervisor, Prof. George Falkay, for his leadership, support and management of my work during this three-year period. This work could not have been completed without his encouragement and help.

I would also like to take the opportunity to say thanks to the co-authors, Prof. Gábor Tóth and Krisztina Bakos as well as to my colleagues, Árpád Márki, Róbert Gáspár and István Zupkó, for their successful cooperation and motivating discussion.

Furthermore, I am grateful to Zsuzsa Magyar, Judit Czinkota, Ágnes Csiszár for their technical assistance in carrying out the experiments.

Special thanks are due to Lajos Fekete for taking care of the animals.