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Design of Oxytocin Antagonists with Prolonged Action: Potential Tocolytic Agents for the Treatment of Preterm Labor[1]

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ABSTRACT

In our continuing effort to produce more potent and specific oxytocin (OT) antagonists that may have value as tocolytic agents, we have synthesized a number of new OT antagonists. Our previous studies have shown that rigid conformational structure and restricted dynamic properties are associated with antagonistic activity of the [1-penicillamine]OT ([Pen^1]OT) analogs. We therefore synthesized a series of structural analogs of [Pen^1,] OT; [Pen^1,Thr^4]-OT and [Pen^1,Phe^2,Thr^4]OT with greater restricted conformational features. They are [Pen^1,$\Delta^{3,4}$-Pro^7]OT; [Pen^1,Thr^4,$\Delta^{3,4}$-Pro^7]OT; [Pen^1,Phe^2,Thr^4,$\Delta^{3,4}$-Pro^7]OT; [Pen^1,Orn^8]OT; [Pen^1,Phe^2,Thr^4,$\Delta^{3,4}$,-Pro^7,Orn^8]OT; [Pen^1,Tyr(OMethyl)^2,-Thr^4,Orn^8]OT; [Pen^1,Tyr(OEthyl)^2,Thr^4,Orn^8]OT; [Pen^1,Phe(Methyl)^2,Thr^4,Orn^8]OT and [Pen^{1}Phe(Ethyl)^2,Thr^4,Orn^8]OT. As expected, all were found to be potent OT antagonists, with *in vitro* pA_2 values ranging from 5.32 to 7.67. They were also effective OT antagonists *in vivo* in the term pregnant rats. Structural modifications in the above analogs produced various and interesting effects. Dehydroproline substitution for 7-proline in [Pen^1]OT increased antagonistic potency, whereas in [Pen^1,Thr^4]OT and in [Pen^1,Phe^2,Thr^4]OT decreased antagonistic potency. Most significantly, analogs with O-alkyl-Tyr^2, Orn^8 and *p*-alkyl-Phe^2,Orn^8 substitutions were found to have prolonged action both in the isolated rat uterus assays and in the term pregnant rats. Generally, substitution of the alkyl groups resulted in a reduction in anti-OT potency, and increasing the size of the alkyl substituent from a methylene group to an ethyl group diminished antagonistic potencies markedly. The 2-ethyl,8-ornithine analogs, however, retained their prolonged action. This dissociation between potency and prolonged action gives further support to our hypothesis that potency (binding and transduction) and duration of action (reversibility) may each have different molecular requirements and that OT analogs can be designed rationally based on conformational considerations for potency, specificity and long action.

Although the mechanism of initiation of labor is not understood fully, it has become increasingly apparent from recent studies that OT plays an important role in labor. We have proposed in a previous paper (Chan *et al.*, 1982) that parturition may be initiated by the dual action of OT, namely its direct myometrial stimulating action and its prostaglandin releasing action. Similar suggestion has also been made based on human studies (Fuchs *et al.*, 1982). Clinically, both term and preterm labor are associated with an increase in sensitivity of the uterus to OT, whereas postterm labor is associated with a delayed development of OT sensitivity (Takahashi *et al.*, 1980). These laboratory and clinical observations suggest that specific OT antagonists may therefore be effective for the prevention of preterm labor.

OT peptides have very short duration of action because of their rapid clearance rate. To be useful clinically as tocolytic agents, more specific, potent and, in particular, longer-acting antagonists will be required. One of the objectives of our research program on the chemistry and pharmacology of neurohypophysial peptides is to discover such long-acting OT antagonists.

Our previous studies have shown that OT agonists and OT antagonists have different conformational and dynamic properties and that the agonists and the antagonists may each utilize different structural and conformational features of their own for interacting with uterine receptors (Hruby *et al.*, 1980, 1983a,b). OT antagonists with a Pen residue in position 1 have more rigid structure and restricted dynamic features than OT agonists. Sufficient examination of the structural and conformational requirements for OT agonists and antagonists at the uterine receptor (Hruby, 1986; Hruby and Smith, 1986) shows that new analogs can be designed rationally based on conformational and structural considerations.

In our continuing effort to find more potent and, in particular, long-acting OT antagonists, we have synthesized a new series of OT analogs related to [Pen^1]OT and [Pen^1,Thr^4]OT

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ABBREVIATIONS: OT, oxytocin; Pen, penicillamine; AVP, arginine-vasopressin; VP, vasopressin; Tyr(OMe), O-methyl-Tyr; Tyr(OEt), O-ethyl-Tyr; Phe(Me), *p*-methyl-Phe; Phe(Et), *p*-ethyl-Phe.

with more rigid conformational features and restricted dynamic properties. Three series were synthesized and studied: the 7-dehydroproline analogs, the 8-ornithine analogs and the 2-O-alkyl-Tyr or 2-*p*-alkyl-Phe,8-ornithine analogs. All were found to be potent OT antagonists. Moreover, the 8-ornithine series was found to have prolonged activity. Significantly, prolonged action and potency can be decoupled. This suggests that OT peptides may utilize different conformational features for potency (receptor binding and transduction) and duration of action (reversibility). The synthesis and chemical-physical studies of these OT antagonists will be presented elsewhere. In this paper, we report our pharmacological studies of these potent and long-acting OT antagonists. A preliminary report of some of this work has appeared (Chan and Hruby, 1983).

Materials and Methods

The OT analogs were bioassayed for oxytocic or antioxytocic activities on the isolated rat uterus and in pregnant rats. The time-action of anti-OT effect was determined in pregnant rats. Antivasopressor activity was determined in male rats.

***In vitro* oxytocic assays.** *In vitro* oxytocic assays were performed on isolated uteri from rats (Wistar) in natural estrus, as described by Chan *et al.* (1967), with the use of Mg^{++}-free van Dyke-Hastings solution. The antioxytocic potencies of the OT antagonists were quantitated in this *in vitro* system by the pA_2 method of Schild (1947), using synthetic OT as the agonist.

***In vivo* oxytocic assays.** *In vivo* oxytocic or antioxytocic activities were determined in 21- and 22-day pregnant rats according to the method of Chan *et al.* (1974). In brief, the technique is an isometric contraction recording of a segment of the uterus *in situ*. The rat was anesthetized with urethane. The uterus was exposed through a midabdominal incision. A segment of the uterus, approximately 50 mm, was isolated and ligated with a suture at both ends. The incision was then closed with wound clips leaving the sutures exteriorized. The proximal suture was fixed to a post and the distal suture attached to a force-displacement transducer. Uterine contractions were induced by i.v. injections of OT. The standard OT doses used in this series of experiments were between 50 and 80 mU/rat. The contractile response to OT injection was expressed by the area under the contractile curve for the 10-min interval immediately after drug administration. The *in vivo* anti-OT potency of OT antagonists was determined by injecting an appropriate dose (micrograms) of the OT antagonist 20 to 30 sec preceding the standard OT dose so that the OT response was reduced by 50 to 80%. Time-action of the inhibitory response was measured by monitoring the recovery of OT response. OT injections were repeated at 30-min intervals. The time-action curves were plotted and the maximal inhibition of OT response determined. The time for 50% recovery from the maximal inhibition was determined by interpolation from the time-action curves. This time is designated as the recovery $T_{½}$. In the absence of OT inhibitor, the contractile responses to the standard OT injections at 30-min intervals were relatively reproducible over a 2-hr period. There was no evidence of desensitization to OT.

Antivasopressor assays. Pressor or antivasopressor activities of OT antagonists were determined in urethane-anesthetized and phenoxybenzamine-treated male rats (Wistar). Vasopressor responses to AVP injections were recorded *via* a carotid artery cannula with a pressure transducer. The standard doses of AVP used for anti-VP tests were between 4 and 8 mU. The effects of OT antagonists on the blood pressure and on the pressor response to AVP were determined at only one dose level, the effective anti-OT dose determined in the pregnant rats.

Statistical analysis of data. All data, pA_2 values and recovery $T_{½}$s were expressed as sample means ± S.E.M. and analyzed by analysis of variance. Significant differences between sample means were analyzed by the student's *t* test, both tails at .05 P level.

Materials. All of the OT antagonists were synthesized in our laboratories. Details of the synthesis of the peptides and their properties will be reported elsewhere (T. W. Rockway, V. J. Hruby, J. Ormberg, J. Hlavacek and W. Y. Chan, manuscript in preparation). The analogs examined in these studies were [Pen^1]OT; [Pen^1, Thr^4]OT; [Pen^1,Phe^2,Thr^4]OT and their respective 7-$\Delta^{3,4}$-Pro analogs; [Pen^1,Orn^8]OT; [Pen^1,Phe^2,Thr^4,$\Delta^{3,4}$-Pro^7,Orn^8]OT; [Pen^1, $Tyr(OMe)^2$,-Thr^4,Orn^8]OT; [Pen^1,$Tyr(OEt)^2$,Thr^4,Orn^8]OT; [Pen^1, $Phe(Me)^2$,Thr^4,Orn^8]OT; and [Pen^1,$Phe(Et)^2$,Thr^4,Orn^8]OT. The OT used was Pitocin (Parke-Davis, Morris Plains, NJ) and the VP used was Pitressin (Parke-Davis).

Results

Anti-OT Activities

Effects of 3,4-dehydroproline substitution for 7-Pro. [Pen^1]OT, [Pen^1,Thr^4,]OT and [Pen^1,Phe^2,Thr^4]OT are potent OT antagonists. Their *in vitro* anti-OT potencies have been determined previously and reported (Chan *et al.*, 1982). Substitution of 3,4-dehydroproline for Pro in position 7 of these three OT antagonists produced different effects. [Pen^1,$\Delta^{3,4}$-Pro^7]OT was found nearly twice as potent as [Pen^1]OT as an OT antagonist in the isolated uterus assay. Similar substitution of 3,4-dehydroproline for 7-Pro in [Pen^1,Thr^4]OT and in [Pen^1,Phe^2,Thr^4]OT, however, resulted in a loss of antagonistic activity. The *in vitro* pA_2 values of these six OT antagonists are shown in table 1.

Effects of 8-Orn substitution. 8-Orn substitution in [Pen^1]OT and in [Pen^1,Phe^2,Thr,^{4}-$\Delta^{3,4}$-Pro^7]OT did not result in a significant change in OT antagonistic potency. The duration of inhibitory action of the 8-Orn analogs, however, was prolonged. In the pregnant rats, the $T_{½}$ of recovery of inhibitory response for [Pen^1]OT and [Pen^1,Phe^2,Thr^4,$\Delta^{3,4}$-Pro^7]OT was less than 30 min and for [Pen^1,Orn^8]OT and [Pen^1,Phe^2,Thr^4,$\Delta^{3,4}$-Pro^7,Orn^8]OT the recovery $T_{½}$ were increased 2- and 3-fold, respectively. The *in vitro* pA_2 and the *in vivo* recovery $T_{½}$ values of these four OT antagonists are compared in table 2.

Effects of 2-alkylation and 8-Orn substitution. Substitution of the Tyr^2 residue by O-alkyl-Tyr^2 or by *p*-alkyl-Phe^2

TABLE 1

Effects of dehydroproline substitution on the antioxytocic potency, pA_2, of OT antagonists

Values shown are means ± S.E.M.; *n* = at least 6.

Compound	pA_2 (*in vitro*)
[Pen^1]OT	6.86 ± 0.03
[Pen^1,Thr^4]OT	7.55 ± 0.04
[Pen^1,Phe^2,Thr^4]OT	7.67 ± 0.02
[Pen^1,$\Delta^{3,4}$-Pro^7]OT	7.12 ± 0.02
[Pen^1,Thr^4,$\Delta^{3,4}$-Pro^7]OT	7.21 ± 0.06
[Pen^1,Phe^2,Thr^4,$\Delta^{3,4}$-Pro^7]OT	7.46 ± 0.03

TABLE 2

Effects of 8-Orn substitution on anti-OT potency and duration of antagonistic activity

Values shown are means ± S.E.M. For pA_2, *n* = at least 6. For recovery $T_{½}$, *n* = at least 4.

OT Antagonists	pA_2 (*in vitro*)	Recovery $T_{½}$ in min (*in vivo*)
[Pen^1]OT	6.86 ± 0.03	~20
[Pen^1,Orn^8]OT	6.75 ± 0.07	50 ± 4
[Pen^1,Phe^2,Thr^4,$\Delta^{3,4}$-Pro^7]OT	7.46 ± 0.03	~20
[Pen^1,Phe^2,Thr^4,$\Delta^{3,4}$-Pro^7,Orn^8]OT	7.45 ± 0.04	93 ± 2

derivatives and the Leu[8] residue with an Orn[8] residue in [Pen[1],Thr[4]]OT and [Pen[1],Phe[2],Thr[4]]OT yielded OT antagonists with prolonged action, with recovery $T_{1/2}$ ranging from 55 to 160 min. However, the substitution in position 2 resulted in an apparent loss of antagonistic potency. The 2-methyl analogs were found to have a lower pA_2 value than their respective parent molecules. Extending the alkyl chain from a methyl group to an ethyl group caused a marked loss in antagonistic potency, a two-order of magnitude lower in pA_2. The prolonged action of the 2-methyl analogs occurred also in the isolated uterus assays. Due to the difficulty of washing out the inhibitory effect, precise determination of the pA_2 of these long-acting OT antagonists was not possible. The pA_2 values listed for these long-acting OT antagonists (table 3) are, therefore, only best estimates. The pA_2 and the *in vivo* recovery $T_{1/2}$ values of these four 2-alkylated and 8-Orn substituted OT antagonists are presented in table 3.

Potency of anti-OT action and duration of action in the pregnant rat. A dissociation between the *in vitro* pA_2 values and the *in vivo* recovery $T_{1/2}$ values was demonstrated clearly as shown in table 3. A similar dissociation between potency and duration of action was also found when both anti-OT action and duration were determined in the 21- and 22-day pregnant rats. The *in vivo* anti-OT potencies of these long-acting OT antagonists could not be determined precisely as in the pA_2 method because of their long-lasting inhibitory action. We determined the dose range of the antagonist needed to cause a 50 to 80% inhibition of the response to the OT standard and expressed *in vivo* anti-OT potencies in relative ratios. The relative *in vivo* anti-OT potencies and the *in vivo* recovery $T_{1/2}$ values of these OT antagonists are shown in table 4.

Anti-VP activities. In the pregnancy rats, doses of the OT antagonists that caused marked anti-OT effect on the uterus had no significant effect on the blood pressure. In the pressor assay system, using anesthetized phenoxybenzamine-treated male rats, the OT antagonists produced no significant effect on the blood pressure at dose levels twice their respective anti-OT doses. When the OT antagonist was injected with 8 mU of VP, anti-VP effect was observed in some but not all analogs [Pen[1],$\Delta^{3,4}$-Pro[7]]OT and [Pen[1],Thr[4],$\Delta^{3,4}$-Pro[7]]OT had little or no anti-VP activity. [Pen[1],Phe[2],Thr[4],$\Delta^{3,4}$-Pro[7]]OT and [Pen[1],Phe(Et)[2],Thr[4],Orn[8]]OT inhibited the VP response by 25 to 40%. [Pen[1],-Tyr(OMe)[2],Thr[4],Orn[8]]OT and [Pen[1],Phe(Me)[2], Thr[4],Orn[8]]OT inhibited the VP response by 40 to 70%. In most experiments, full recovery of VP response returned between 45 and 60 min after the OT antagonist injection.

TABLE 3
Effects of 2-alkyl and 8-Orn substitutions on anti-OT potency and duration of antagonistic activity
Values shown are means ± S.E.M. For pA_2, n = at least 6. For recovery $T_{1/2}$, n = at least 4.

OT Antagonists	pA_2 (*in vitro*)	Recovery $T_{1/2}$ in min (*in vivo*)
[Pen[1],Thr[4]]OT	7.55 ± 0.04	~20
[Pen[1],Tyr(OMe)[2],Thr[4],Orn[8]]OT	~7.3[a]	130 ± 16
[Pen[1],Tyr(OEt)[2],Thr[4],Orn[8]]OT	5.32 ± 0.02	55 ± 12
[Pen[1],Phe[2],Thr[4]]OT	7.67 ± 0.02	~20
[Pen[1],Phe(Me)[2],Thr[4],Orn[8]]OT	~7.3[a]	160 ± 14
[Pen[1],Phe(Et)[2],Thr[4],Orn[8]]OT	5.80 ± 0.03	110 ± 9

[a] Estimated values only. See text for explanation.

TABLE 4
Relative *in vivo* anti-OT potency and recovery $T_{1/2}$ of OT antagonists in term pregnant rats
Values shown are means ± S.E.M.; n = at least 4.

OT Antagonists	Recovery $T_{1/2}$ in min
Group I: Potency = 1.00 (anti-OT dose range, 2–5 μg)	
[Pen[1],Phe(Me)[2],Thr[4],Orn[8]]OT	160 ± 14
[Pen[1],Tyr(OMe)[2],Thr[4],Orn[8]]OT	130 ± 16
Group II: Potency = 0.50 (anti-OT dose range, 5–10 μg)	
[Pen[1],Phe[2],Thr[4]]OT	~20
[Pen[1],Phe[2],Thr[4],$\Delta^{3,4}$-Pro[7]]OT	~20
[Pen[1],Phe[2],Thr[4],$\Delta^{3,4}$-Pro[7],Orn[8]]OT	93 ± 2
[Pen[1],Tyr(OEt)[2],Thr[4],Orn[8]]OT	55 ± 12
Group III: Potency = 0.10 (anti-OT dose range, 10–50 μg)	
[Pen[1],Orn[8]]OT	50 ± 4
[Pen[1],$\Delta^{3,4}$-Pro[7]]OT	~20
[Pen[1],Thr[4],$\Delta^{3,4}$-Pro[7]]OT	~20
[Pen[1],Phe(Et)[2],Thr[4],Orn[8]]OT	110 ± 9

Discussion

Based on structures and conformational considerations, we have designed and synthesized three series of OT analogs with restricted conformational features. As expected, all were found to be potent OT antagonists in the *in vitro* pA_2 assays. They were also effective *in vivo* antagonists against the OT-induced uterine contractions in the term pregnant rats. Most significantly, the 2-O-alkyl,4-Thr,8-Orn analogs of [Pen[1]]OT were found to have a prolonged action, having a $T_{1/2}$ for recovery as long as nearly 3 hr. This is in contrast to the short $T_{1/2}$ of OT peptides, generally measured in minutes. The discovery of these long-acting OT antagonists holds out promise that clinical deployment of OT antagonists as tocolytic agents for the treatment of premature labor may be realized with the development of further improved OT antagonists.

The experimental findings here also give support to our hypothesis proposed in previous papers (Hruby *et al.*, 1980, 1983a) that potency (hormone-receptor binding and transduction) and duration of action (reversibility of reaction) may each have different molecular requirements.

In the dehydroproline series, it is interesting to note that the introduction of a double bond in 7-Pro enhanced the antagonistic potency of only [Pen[1]]OT. In [Pen[1],Thr[4]]OT and [Pen[1],Phe[2],Thr[4]]OT, the two most potent OT antagonists in our series, introduction of the double bond in 7-Pro slightly reduced the antagonistic potency. It was shown some time ago that substitution of 3,4-dehydroproline for 7-Pro in OT resulted in doubling the agonist potency (Moore *et al.*, 1977). Inasmuch as position 7 is believed to be important primarily for recognition of the receptor, the different effects of dehydroproline on the agonist and the antagonist potencies, suggest that the agonist-receptor and the antagonist-receptor states have different conformational requirements. This would imply that they also have different energy requirements depending on other interactions of the hormone with the receptor in its agonist *vs.* antagonist conformations. On the basis of the data available thus far, it would appear that when the double bond-containing Pro derivative, $\Delta^{3,4}$Pro replaces Pro in position 7 of OT, a more energetically favorable interaction within its agonist state occurs relative to the native hormone and hence increased potency occurs. In most cases of antagonist interactions with the OT receptor, it would appear that when the $\Delta^{3,4}$-Pro replaces

Pro in position 7 a less favorable interaction with the receptor occurs, and hence antagonist potency generally is reduced.

8-Orn substitution in [Pen^1]OT and [Pen^1,Phe^2,Thr^4,$\Delta^{3,4}$-Pro^7]OT had no effect on potency but prolonged the duration of action of the peptides.

Substitution of the Tyr^2 residue by O-alkyl-Tyr^2 or by *p*-alkyl-Phe^2 in combination with 8-Orn substitution in [Pen^1,Thr^4]OT produced the most dramatic long-acting OT antagonists. As shown in table 3, [Pen^1,Tyr(Me)^2,Thr^4,Orn^8] OT had a recovery $T_{½}$ of 130 min and [Pen^1,Phe(Me)^2,Thr^4,-Orn^8]OT a recovery $T_{½}$ of 160 min. The prolonged action of these analogs cannot be explained by resistance to metabolic degradation or clearance as the long-lasting effect was also observed in the isolated rat uterus assays. The prolonged *in vivo* activity, therefore, would appear to be the result of some direct interaction of these analogs with the receptor. This is supported further by the observation that this prolonged activity also is generally seen in the *in vitro* assay as well. Because the prolonged activity cannot be attributed to higher receptor affinity in most cases, these results support our hypothesis (Hruby *et al.*, 1983a,b; Hruby, 1986) that prolongation can result from favorable hormone-receptor interactions that occur *subsequent* to hormone-receptor binding and transduction (for agonists) and inhibition (for antagonists), and hence prolong the biological effect.

It is interesting to note that increasing the size of the 2-alkyl substituent from a methyl group to an ethyl group resulted in a marked loss in antagonistic potency. Bankowski *et al.* (1980) have reported similar findings. Although [Pen^1,Tyr(OEt)^2, Thr^4,Orn^8]OT and [Pen^1,Phe(Et)^2,Thr^4,-Orn^8]OT had pA_2 values nearly two-orders of magnitude lower than their respective 2-methyl counterparts, they retained their long action. It is therefore clear that potency and long action can be dissociated. This finding gives further support to our hypothesis that long action has different molecular requirements independent of those for potency.

Our most potent and long-acting OT antagonists have 2-methyl, 4-Thr and 8-Orn substitutions. It is evident that 4-Thr substitution increased only antagonistic potency but not the duration of action. [Pen^1,Thr^4]OT and [Pen^1,Phe^2,Thr^4]OT appear to be the most potent OT antagonists in our series but do not exhibit prolonged action. The relative importance of O-alkyl-Tyr^2 (or *p*-alkyl-Phe^2) and 8-Orn substitutions in contributing to the long action cannot be assessed from our data at this point. [Pen^1,Orn^8]OT was our least substituted analog that possessed long action. However, in contrast to other reports (Bankowski *et al.*, 1980; Sawyer *et al.*, 1980), we did not find an enhancing effect of 8-Orn substitution on antagonistic potency. [Deamino-Pen^1,Tyr(OMe)^2]OT and [deamino-Pen^1,Tyr(OMe)^2,Thr^4]OT have been synthesized and studied by Lowbridge *et al.* (1979) in nonpregnant rats. These authors reported high anti-OT activities for the two OT antagonists but did not describe duration of action. However, it appears that optimal molecular features for long action involves modifications at both positions 2 and 8 as represented by the analogs reported in this paper.

The long-acting OT antagonists also possessed anti-VP effects when tested for vasopressor activity in the anesthetized, phenoxybenzamine-treated male rats. It appears that there was a correlation between *in vivo* anti-OT potencies and anti-VP activities. For [Pen^1,Tyr(OMe)^2,Thr^4,Orn^8]OT and [Pen^1, Phe(Me)^2,Thr^4,Orn^8]OT the two most potent and long-acting OT antagonists, anti-OT doses also produced marked inhibition of the vasopressor response to VP. In their studies on Tyr(OMe)^2 and Orn^8 substituted [deamino-Pen^1]OT, Bankowski *et al.* (1980) also found nearly equal antioxytocic and antivasopressor potencies for the OT antagonists. This suggests that OT receptors in uterine smooth muscles resemble the V_1 receptors of VP in vascular smooth muscles. Whether OT receptors in uterine smooth muscle cells may be a sub-type of V_1 receptors can only be speculated. Although the anti-VP effect of these OT antagonists does not appear to be a problem in their use as tocolytic agents, effort should be made and can be made to improve their specificity.

The discovery of these potent and long-acting OT antagonists should encourage further research effort to develop clinically useful tocolytics. The availability of these specific OT antagonists would also provide valuable tools for studying the mechanism of parturition.

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