A Differential Pharmacokinetic Analysis of the Erythropoietin Receptor Population in Newborn and Adult Sheep


The Colleges of Pharmacy (P.V.-P., N.H.A.-H.) and Medicine, Departments of Pediatrics (R.L.S., E.M.S., J.A.W.) and Internal Medicine (R.J.H.), University of Iowa, Iowa City, Iowa; and Drug Metabolism and Pharmacokinetics, Aventis Pharmaceuticals, Bridgewater, New Jersey (S.C.)

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ABSTRACT

Strong evidence indicates that erythropoietin (Epo) is eliminated via Epo receptors (EpoR). Epo receptors may be classified as erythropoietic receptors that are largely located on erythroid progenitor cells in the bone marrow (BM) and nonerythropoietic receptors present in most tissues. Epo’s elimination kinetics was studied using a very sensitive tracer interaction method (TIM) before and after chemical ablation of BM as an indirect way of evaluating the EpoR through an assortment of pharmacokinetic parameters (V_M, K_M, K, and CL) used in differentiating the EpoR population in newborn and adult sheep. TIM identified a parallel nonlinear Michaelis-Menten (V_M, K_M, K, and CL) used in differentiating the EpoR population in the bone marrow to augment the differentiation of the EpoR population. EpoRs in the body are not known in addition to how these EpoRs but also their binding characteristics. Because the latter approach enables an in vivo analysis of not only the quantity of erythropoietic receptors present in most tissues but also their binding characteristics, the objective of this study is to further our understanding of the EpoR population in vivo. We present a pharmacokinetic (PK) analysis that looks at the macroscopic PK effect of the EpoRs in vivo using a sensitive, tracer-based methodology that we refer to as the tracer interaction method (TIM) (Veng-Pedersen et al., 1997). The TIM approach enables an in vivo analysis of not only the quantity of EpoRs but also their binding characteristics. Because the latter differ among distinct receptor populations the TIM may be used to distinguish these populations. In the present study, we have combined the TIM methodology with chemical ablation of the EpoR population in the bone marrow to augment the differentiation of the EpoR population.

Materials and Methods

Animals

All procedures with animals received prior approval from the local University Animal Care and Use Committee. Animals were housed

ABBREVIATIONS: Epo, erythropoietin; EpoR, erythropoietin receptor; PK, pharmacokinetics; TIM, tracer interaction method; r-HuEpo, recombinant human erythropoietin; RIA, radioimmunoassay; CFU-E, colony-forming unit-erythroids; BFU-E, burst forming units erythroids; BM, bone marrow; CL, clearance; ERD, Epo receptor development.

Erythropoietin (Epo) is a 34-kDa glycoprotein hormone the primary function of which is to regulate erythocyte production by stimulating the proliferation and differentiation of erythroid progenitors (Fisher, 2003). Epo exerts this effect by binding to specific cell-surface receptors (EpoRs) on erythroid progenitor cells located primarily in the bone marrow (Sawada et al., 1990). In recent years, EpoRs have been identified in numerous nonhematopoietic tissues outside the bone marrow (Juul et al., 1998). An increasing number of recent reports have demonstrated and postulated Epo’s nonhematopoietic role in the development and protection of the central nervous system, and possibly other effects (Juul et al., 2001). The relative proportions and quantities of the erythropoietic and nonhematopoietic EpoRs in the body are not known in addition to how these quantities may change during development. Epo receptor mRNA analysis of isolated heterogeneous tissue samples provides only a microscopic local picture that cannot be extrapolated to consider the body as a whole. Also, gene expression does not always equate with protein expression. The objective of this study is to further our understanding of the EpoR population in vivo. We present a pharmacokinetic (PK) analysis that looks at the macroscopic PK effect of the EpoRs in vivo using a sensitive, tracer-based methodology that we refer to as the tracer interaction method (TIM) (Veng-Pedersen et al., 1997). The TIM approach enables an in vivo analysis of not only the quantity of EpoRs but also their binding characteristics. Because the latter differ among distinct receptor populations the TIM may be used to distinguish these populations. In the present study, we have combined the TIM methodology with chemical ablation of the EpoR population in the bone marrow to augment the differentiation of the EpoR population.

MATERIALS AND METHODS

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in an indoor, light- and temperature-controlled environment and were maintained in good health throughout the study period. Jugular venous catheters for blood sampling were surgically placed days before the first TIM study under pentobarbital anesthesia, and ampicillin (1 g b.i.d.) was administered the first three postoperative days. Four adult sheep [weight = 23.8 (CV% 11) kg], and four newborn sheep [6.45 (CV% 15) kg] with postnatal ages from 8 to 9 days were used in the experiments. Each animal underwent two TIM experiments (Veng-Pedersen et al., 1997). The first TIM experiment was conducted before chemical ablation of the bone marrow with busulfan. The second TIM experiment was conducted 8 to 10 days after the start of the busulfan ablation. Thus, the analysis involved a total of 32 sets of plasma level data, namely, 16 sets of $^{125}$I-r-HuEpo tracer data, and 16 sets of “cold” r-HuEpo data. Each of the 16 tracer data sets consisted of approximately 36 plasma level versus time data points, and each of the 16 cold data sets consisted of approximately 22 data points. Overall, the analysis used approximately 930 plasma level data points.

**Blood Level Assay**

Plasma samples were assayed for $^{125}$I-r-HuEpo using a sensitive and specific, double antibody immunoprecipitation assay developed in our laboratory (Widness et al., 1992a) with a lower level of detection of 0.004 mU/ml. Plasma r-HuEpo concentration was measured in triplicate using a double-antibody radioimmunoassay (Widness et al., 1986). Linear assay values for r-HuEpo concentrations are obtained between 10 and 450 mU/ml in the sheep RIA. To reduce assay variability all samples were measured in the same assay. To assess CPU-E and BFU-E, samples from the bone marrow before and after busulfan treatment were cultured as described previously (Clapp et al., 1995). Colonies derived from CFU-Es were counted after 6 days of incubation, whereas BFU-E colonies were counted after 9 days of incubation.

**Study Protocol**

**Ablation by Busulfan.** Busulfan was administered orally twice a day in a dose of 11 mg/kg/day for three consecutive days. Ampicillin (1 g b.i.d.) was administered daily for the first 3 days before the

**Fig. 1.** TIM experiment conducted in an adult sheep before and after busulfan treatment. The curves shown fitted simultaneously to the $^{125}$I-r-HuEpo tracer data (squares) before and after busulfan treatment (top and bottom panels) are given by eqs. 1 and 2. The nontracer r-HuEpo data (+) are fitted by a general cross validation cubic spline function. The arrow indicates the time when r-HuEpo is injected.

**Fig. 2.** TIM experiment conducted in a newborn sheep before and after busulfan treatment. The curves shown fitted simultaneously to the $^{125}$I-r-HuEpo tracer data (squares) before and after busulfan treatment (top and bottom panels) are given by eqs. 1 and 2. The nontracer r-HuEpo data (crosses) are fitted by a general cross-validation cubic spline function. The arrow indicates the time when r-HuEpo is injected.

busulfan treatment, and again daily after the start of the treatment. Animals were clinically monitored for adverse effects of the chemotherapy such as weight loss, hair loss, blood in urine or stools, fever, unusual bleeding or bruising, and loss of appetite.

**TIM Experiments.** A detailed description of the theory and principles of the TIM methodology has been published previously (Veng-Pedersen et al., 1997). Briefly, each TIM experiment consists of two stages: 1) an initial 0.10 U/kg i.v. bolus dose of an $^{125}$I-r-HuEpo tracer (specific activity, 1.42 $\times 10^6$ cpm/U) immediately followed by an i.v. infusion at 0.70 mU/kg/min of the tracer to the end of the experiment. 2) An i.v. bolus of the r-HuEpo nontracer administered at the plateau of the tracer (around 4 h).

The immediate, abrupt rise in the plasma tracer level seen after the bolus dose of the r-HuEpo nontracer, as seen in Figs. 1 and 2, top, is a result of the nonlinear Epo elimination. Similarly, the absence of such a perturbation in the tracer level, as seen in Figs. 1 and 2, bottom, after the BM ablation signifies a lack of nonlinearity, i.e., demonstrates linearity in the disposition kinetics.

**Data Analysis.** The tracer plasma level data were analyzed according to the TIM-based PK model given by eqs. 1 and 2 using the interactive WINFUNFIT computer program for general nonlinear regression, written for the Windows (Microsoft, Remond, WA) platform, evolved from the original FUNFIT program (Veng-Pedersen, 1977):

$$\frac{dc_a}{dt} = - \left( \frac{K_m}{K_m + c_a} + \frac{G}{\gamma} \right) c_a + Ge^{-\gamma c_a} + R/V \quad \text{for } 0 < t \leq T$$

$$\frac{dc_a}{dt} = - \left( \frac{K_m}{K_m + c_a} + \frac{G}{\gamma} \right) c_a + Ge^{-\gamma c_a} + R/V \quad \text{for } t > T \quad (1)$$

$$\frac{dc_a}{dt} = - \left( \frac{G}{\gamma} \right) c_a + Ge^{-\gamma c_a} + R/V \quad (2)$$

The convolution type (+ denotes convolution), integro-differential equations (eqs. 1 and 2) describe the tracer plasma level in the TIM experiments in the presence and absence of a nonlinear elimination pathway, respectively, corresponding to the “before” and “after” ablation conditions. Equation 1 applied before the busulfan marrow
ablation considers an elimination via two routes, namely, a nonlinear route corresponding to the Michaelis-Menten terms \( V_M(K_M + c_a) \) and \( V_M(K_M + c_b) \), and a linear route corresponding to the first-order elimination rate constant \( K \). The terms \( c_a \) and \( c_b \) denote the plasma concentration profiles of the \(^{125}\text{I}\)-r-HuEpo tracer and the cold r-HuEpo, respectively. The rate of infusion of the tracer is denoted by \( R \) and the volume of distribution by \( V_{HuEpo} \), respectively. The rate of infusion of the tracer is denoted by concentration profile, \( r-\text{HuEpo} \) data were used to represent the cold \( r-\text{HuEpo} \) plasma cubic spline (Hutchinson and deHoog, 1985) fitted to the unlabeled the exact same effect as the convolution term in eqs. 1 and 2.

Pharmacokinetic parameters determined from simultaneous fitting of eqs. 3 and 4 to plasma level data acquired before and after bone marrow ablation. Equation 2 is the function used to describe the tracer kinetics of \(^{125}\text{I}\)-r-HuEpo as determined by the TIM experiments. This effect is clearly shown in the representative plots in Figs. 1 and 2, where the top panel (preablation TIM data) shows a significant perturbation in the tracer data (square symbols) caused by the bolus injection of the nontracer (+ symbol) at about 4 h. The pronounced perturbation is absent in the bottom panel in the ablated state. This phenomenon was consistently observed in all eight animals. The finding that the bone marrow ablation changes the elimination kinetics from nonlinear to linear, corresponding to an elimination of the nonlinear elimination pathway (eqs. 1 and 2), was further tested. This was done in the fully ablated state 9 days after the start of the busulfan treatment by two additional experiments using significantly larger bolus doses of the nontracer \( r-\text{HuEpo} \). In these experiments, the standard \( r-\text{HuEpo} \) dose of 100 U/kg was increased to 400 and 4000 U/kg. Doing so did not result in a detectable perturbation in the tracer level (data not shown), further confirming the linear elimination kinetics in the ablated state. Nonlinearity may still exist at higher concentrations; however, a dosing as high or higher than 4000 U/kg is not met in current clinical practice.

### Effect on Volume of Distribution (V).

The volume of distribution was not found to change significantly (\( p > 0.05 \)) as a result of the ablation, with before/after ablation values [mean (CV%)] for newborns of 74.4 (5.24)/78.2 (15.0) ml/kg and for adults of 47.5 (20.5)/58.0 (20.0) ml/kg.

### Effect on CL.

The ablation resulted in a substantial reduction in the tracer clearance in both adult and newborn sheep (\( p < 0.01 \); Table 1).

#### Table 1

Pharmacokinetic parameters determined from simultaneous fitting of eqs. 3 and 4 to plasma level data acquired before and after bone marrow ablation

<table>
<thead>
<tr>
<th></th>
<th>( V_M/K_M )</th>
<th>( K )</th>
<th>( K )</th>
<th>( CL )</th>
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<tbody>
<tr>
<td></td>
<td>1/h</td>
<td></td>
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<tr>
<td><strong>Newborn</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mean</td>
<td>1.10</td>
<td>0.498</td>
<td>0.309</td>
<td>118</td>
</tr>
<tr>
<td>CV%</td>
<td>15.8</td>
<td>33.2</td>
<td>25.3</td>
<td>10.9</td>
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<tr>
<td><strong>Adult</strong></td>
<td></td>
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<tr>
<td>Mean</td>
<td>1.30</td>
<td>0.128</td>
<td>0.0895</td>
<td>67.8</td>
</tr>
<tr>
<td>CV%</td>
<td>3.81</td>
<td>17.9</td>
<td>18.4</td>
<td>19.3</td>
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<tr>
<td>H (_2) Newborn = Adult:</td>
<td>( p &lt; 0.05 )</td>
<td>( p &lt; 0.05 )</td>
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<tr>
<td>( H (_2) CL before = CL after: )</td>
<td>( p &lt; 0.01 )</td>
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Newborn Compared with Adult Sheep

**CL before Ablation.** Newborns had a significantly ($p < 0.01$) larger clearance before ablation than adults, with CL values of 118 (10.9) and 67.8 (19.8) mH/kg, respectively (Table 1).

**CL after Ablation.** The newborns also had a significantly ($p < 0.01$) larger clearance after the ablation than adults, with CL values of 39.8 (40.8) and 7.62 (39.8) mH/kg, respectively (Table 1).

**Reduction in CL Resulting from the Ablation.** With the reduction in clearance expressed in a relative manner as (CL after ablation)/(CL before ablation), the newborn showed a significantly smaller reduction resulting from the ablation ($p < 0.05$) with values of 0.333 (37.3) and 0.110 (19.6) for newborns and adults, respectively.

**First-Order Elimination Rate Constant ($K_a$) for the Nonablated Elimination Pathway.** The elimination rate constant for the nonablated elimination pathway was found to be significantly larger for the newborns than the adults with values of 0.498 (33.2) and 0.128 (17.9) h$^{-1}$, respectively (Table 1).

**“First-Order” Elimination Rate Constant ($V_m/K_m$) for the Ablated Elimination Pathway.** The first-order elimination rate constant corresponding to low concentrations ($c < K_m$) for the ablatable elimination pathway was not found to be significantly different between newborns and adults, with values of 1.10 (15.8) and 1.30 (3.81) h$^{-1}$, respectively (Table 1).

**Michaelis-Menten Parameter $K_M$.** The $K_M$ parameter for the ablated elimination pathway was not found to be significantly different ($p > 0.05$) between newborns and adults with values of 425 (17) and 523 (15) mU/ml, respectively. The values are similar to reported in vitro binding affinity $k_a$ values for humans and animal erythroid progenitors cell lines expressing EpoR, i.e., 240 to 2400 mU/ml (Sawyer, 1990).

The maximum plasma concentration of the tracer was consistently less than 4500 cpm/ml, which corresponds to about 3 mU/ml. Thus, the tracer concentration, $c_a$, was all the time well into linear range ($c_a < K_M$). Accordingly, the $V_m/K_m$ term in eq. 3 $t < T$ becomes $V_m/K_m$, and the TIM analysis can be as discussed previously (Veng-Pedersen et al., 1997) be done by measuring the tracer in cpm per milliliter units without the need for converting to milliliters per milliliter. This is a convenient advantage because an accurate determination of the specific activity of biologicals can be a problem.

**Michaelis-Menten Parameter $V_M$.** The $V_M$ parameter for the ablated elimination pathway was not found to be significantly different ($p > 0.05$) between newborns and adults with values of 455 (34) and 682 (24) mU/ml/h, respectively.

**Relative Dominance of the Nonablated Elimination Pathway [$K/(V_m/K_m + K)$].** The relative dominance of the nonablated elimination pathway was found to be significantly larger for newborns than adults, with values of 0.309 (25.3) and 0.0895 (18.4), respectively (Table 1).

**Ablation by Busulfan.** No CFU-E colonies were found after 6 days of incubation for bone marrow aspirates drawn at days 8 and 13 after busulfan treatment. In contrast, pre-busulfan aspirates yielded 29 CFU-E colonies/105 cells in CFU-E cultures. Similarly, when those samples were incubated for 9 days, 29, 3, and 0 colonies/105 cells in BFU-E culture were observed for the samples drawn on day –1, 8, and 13, respectively. Microscopic analysis of bone marrow core biopsies showed no significant cellularity after the busulfan treatment.

**Discussion**

Contrary to most nonprotein type of drugs, the kidneys and liver are not considered important in the in vivo elimination of Epo (Withness et al., 1996; Yoon et al., 1997). Also, the long-term stability of Epo in whole blood at room temperature indicates that no significant biotransformation takes place in blood (Withness et al., 1984; Kendall et al., 1991). However, the possibility of a tissue-based, extrarenal, extrahepatic elimination, e.g., via proteolysis cannot be completely ruled out. An increasing amount of evidence suggests that the metabolic fate of Epo is largely depending on the bone marrow containing EpoR carrying progenitor cells that eliminate Epo through endocytosis of the EpoR-Epo complex followed by lysosomal degradation (Sawyer, 1990; Sawyer and Hankins, 1993; Fisher, 2003). The hypothesis of a receptor-based elimination is consistent with previous findings that most growth factors, neuropeptides, and biologically active endogenous polypeptides are eliminated via receptor-based endocytosis (Gammeltoft, 1991).

Erythroid progenitor mass has been shown to be a determinant of serum Epo concentration at a given hemoglobin level (Cazzola et al., 1998). Subjects infected with human parvovirus (B19) showed a severe loss of EpoR-carrying erythroid precursor cells, resulting in a pronounced increase in the Epo plasma level that was nearly inversely related to the presence of red blood cell progenitors. It was suggested that this relationship reflects Epo’s binding to receptors (Potter et al., 1987). After observing that plasma Epo levels remained abnormally high in sublethally irradiated dogs after the cessation of hypoxia, Stohlman concluded that plasma Epo levels were significantly “influenced by the functional state of the erythrocytic tissue of the marrow” (Stohlman, 1959). Other studies have documented that plasma Epo levels increase after bone marrow ablation in excess of that expected by a reduction in hemoglobin alone (Grace et al., 1991; Davies et al., 1995). Several studies have reported nonlinearity in Epo’s PK in which its plasma clearance decreases with increasing Epo doses (Kato et al., 1997a; Yoon et al., 1997) consistent with a Michaelis-Menten-type elimination kinetics.

Epo’s nonlinear behavior has been documented in various animal species, including rats (Kato et al., 1997b), mice (Kato et al., 1998), rabbits (Yoon et al., 1997), sheep (Veng-Pedersen et al., 1999), and humans (Flaharty et al., 1990; Veng-Pedersen et al., 1995, 1999).

The fact that the ablation of the bone marrow changes the elimination kinetics from nonlinear (linear + nonlinear) to purely linear (eqs. 1 and 2; Figs. 1 and 2) supports the hypothesis that the nonlinear elimination kinetics is due to elimination via the EpoR pool located in the bone marrow. However, the possibility that the nonlinearity present before ablation is due to both a nonlinear and linear binding component of the EpoR in the bone marrow cannot be completely ruled out.
It is well recognized that EpoRs exist in many tissues other than the bone marrow (Juul et al., 2001; Nagai et al., 2001). The role of these nonhematopoietic receptors is not well understood. However, recent reports have indicated that at least some of these receptors seem to have a neuroprotective role in protection from hypoxic-ischemic insults (Juul, 2002). Busulfan ablation is considerably more selective in ablating bone marrow cells than most other tissues’ cells in the body, including tissues where mRNA for EpoR have been detected (Carlini et al., 1999). Thus, it does not seem very likely that such EpoR cells outside the bone marrow will be much affected by the busulfan treatment.

The PK parameters obtained by the TIM analysis provides an indirect quantification of the EpoR populations under the well supported assumption that Epo’s elimination is via EpoRs. From this assumption, it follows from simple kinetic principles that the quantity of ablatable EpoR is proportional to \( V_{M}/K_{M} \) and the quantity of receptors remaining after the busulfan ablation is proportional to \( K \). The total ablation of the bone marrow makes it possible to “isolate” the receptors outside the bone marrow. This is of particular interest considering the discovery of the neuroprotective role of Epo (Juul, 2002) and the recent attention to Epo’s possible nonhematopoietic importance in development.

Based on the analysis of the data from this study (Table 1), we propose the following Epo receptor development (ERD) hypothesis: the nonerythroid receptors are most dominant while playing an important role in early development. Their relative importance diminishes later in development when Epo’s role is more directed at ensuring proper tissue oxygenation by production of red blood cells.

The ERD hypothesis suggests that the observation that severe hypoxic-ischemic episodes in the life of mammals occurring at birth, e.g., perinatal asphyxia. The effect of such episodes may be mitigated to a greater extent in newborn relative to adult (Dawes, 1968). As discussed below, our PK analysis supports this ERD hypothesis under the assumptions that 1) a significant fraction of the receptors located outside BM is aimed at neuroprotection, and 2) Epo is primarily eliminated via its Epo receptors. With respect to assumption 1 we propose that the elimination of Epo after the bone marrow ablation is primarily via nonhematopoietic receptors, as quantified by the rate constant \( K \).

Our PK analysis does not provide an analysis of the function(s) of the proposed nonhematopoietic EpoR population; we can only speculate that it involves receptors associated with neuroprotective and other yet to be discovered effects. The large amount of evidence of Epo’s elimination via erythropoietic EpoR in the bone marrow supports the hypothesis that nonerythropoietic Epo receptors outside the bone marrow are also involved in the elimination. Accordingly, the quantification of the elimination by \( V_{M}/K_{M} \) in combination with \( K \) (Table 1) gives a “reference” from which an indirect measure of the relative importance of the nonhematopoietic EpoR population can be obtained. This measure is given by \( K/(V_{M}/K_{M} + K) \) (Table 1), which is the fraction of Epo that is eliminated via the nonhematopoietic pathway. This fraction is significantly \((p < 0.05)\) larger in the newborn than adult sheep, indicating that the linear elimination, i.e., the nonhematopoietic EpoR population, is more dominant in lamb than adult sheep.

The reduction in the total clearance expressed as the ratio (CL after ablation)/CL before ablation) also shows a significantly \((p < 0.05)\) smaller reduction for newborn [0.333 (37.3) versus 0.110 (19.6)], again supporting the ERD hypothesis. Moreover, looking at the nonhematopoietic elimination pathway in an absolute, rather than relative sense, gives the same consistent picture: the value for \( K \) is significantly \((p < 0.05)\) larger for the newborn compared with adult sheep. Our finding of a significantly \((p < 0.05)\) larger total clearance for the newborn in the nonablated state compared with adult sheep is consistent with our previous findings (Widness et al., 1992b). This may be explained by the more pronounced elimination via nonerythropoietic EpoR in newborn as evident by a smaller reduction in the total clearance by the BM ablation.

The use of a tracer in PK analysis is based on the assumption that the tracer behaves in an identical kinetic manner to that of the parent drug, which is never exactly the case. However, the difference is usually so small that the analysis results provide reliable, useful information about the parent drug. In any case, when analyzing the PK disposition with an unknown endogenous production under dynamically variable conditions such as in the present study, there is no alternative to the use of a tracer.

It is difficult to predict whether the current study extrapolates to humans. However, because Epo genetically is well preserved across mammalian species, and because of an excellent similarity in the PK between sheep and humans (Veng-Pedersen et al., 1999), we believe such an extrapolation is indeed possible, but the final answer will have to wait until a safe (nonradioactive) r-HuEpo tracer is developed enabling the ERD hypothesis to be tested in humans.

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References


Address correspondence to: Dr. Peter Veng-Pedersen, College of Pharmacy, University of Iowa, Iowa City, IA 52242. E-mail veng@uiowa.edu