

## Water-Soluble Progesterone Analogues Are Effective, Injectable Treatments in Animal Models of Traumatic Brain Injury

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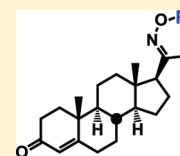
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### S Supporting Information

**ABSTRACT:** After more than 30 years of research and 30 failed clinical trials with as many different treatments, progesterone is the first agent to demonstrate robust clinical efficacy as a treatment for traumatic brain injuries. It is currently being investigated in two, independent phase III clinical trials in hospital settings; however, it presents a formidable solubility challenge that has so far prevented the identification of a formulation that would be suitable for emergency field response use or battlefield situations. Accordingly, we have designed and tested a novel series of water-soluble analogues that address this critical need. We report here the synthesis of C-20 oxime conjugates of progesterone as therapeutic agents for traumatic brain injuries with comparable efficacy in animal models of traumatic brain injury and improved solubility and pharmacokinetic profiles. Pharmacodynamic analysis reveals that a nonprogesterone steroidal analogue may be primarily responsible for the observed activity.

**KEYWORDS:** Traumatic brain injury, traumatic brain injury, progesterone, neurosteroid, progesterone analogues



Despite decades of effort, scientists have not found a pharmacological agent that consistently improves functional outcomes after traumatic brain injury (TBI). After more than 30 failed clinical trials,<sup>1</sup> there is still no safe and effective neuroprotective treatment for TBI, and most major pharmaceutical companies have suspended research and development in this field. However, an innovative, safe, and effective approach to TBI treatment is now providing the promise of a breakthrough.

Preclinical and clinical research demonstrates that the hormone progesterone (PROG) is a potent neurosteroid which, when acutely administered, can dramatically reduce cerebral edema, inflammation, tissue necrosis, and programmed cell death, while at the same time providing and stimulating trophic (growth and survival) support to damaged nerve cells.<sup>2–11</sup> Unlike many other potential neuroprotective agents, PROG is pleiotropic; that is, it acts simultaneously at a number of different receptor sites in both neurons and glial cells.<sup>11</sup> It also plays a protective role in reducing inflammation and cellular damage in other body tissues directly affected by TBI, thus addressing the multiple organ damage that typically occurs after severe traumatic injuries.<sup>12–19</sup> A recently completed phase II, single-center, double-blind, randomized, controlled trial in 100 moderate-to-severe TBI patients showed that 3 days of PROG, administered by continuous intravenous (iv) infusion, reduced mortality by over 60% and significantly improved functional outcomes at 30 days postinjury.<sup>20</sup> A second, independent clinical trial has recently replicated these findings.<sup>21</sup> On the basis of these positive outcomes, two

multisite phase III clinical trials are currently being conducted throughout the United States and Europe.<sup>22,23</sup>

TBI produces a complex succession of molecular events in addition to the immediate loss of nervous tissue caused by concussions, contusions, and ballistic injuries. The “brain injury cascade” initiates rapidly after the initial trauma and unfolds over days, weeks, and even months. Therefore, a key tenet of brain injury treatment is that the sooner one can treat/prevent edema, inflammation, and neuronal loss, the better the functional outcome will be.<sup>18</sup> Unfortunately, current clinical protocols for the use of PROG require patients to be transported to a hospital setting, thus losing valuable time before the treatment can be administered.

PROG is insoluble in aqueous-based formulations, so it must be delivered in a freshly prepared lipid formulation which requires a fairly complicated and time-consuming preparation. Furthermore, the plasma half-life of PROG is only 25 min,<sup>24</sup> so treatment requires continuous iv drip or multiple injections with an oil-based formulation that delays release to the systemic circulation. A treatment for TBI that can be administered easily and rapidly in the field remains elusive.

As a potential approach toward delivering a field-ready treatment for TBI, we have adopted a strategy of preparing novel water-soluble PROG prodrugs and analogues. We previously reported an initial set of such compounds with

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modifications at the C3 center of PROG that allowed incorporation of water solubilizing groups.<sup>25</sup> After surveying numerous chemotypes, analogue P1-185 (**1**, Figure 1) was

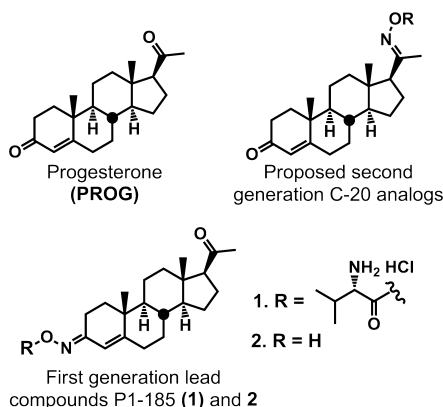


Figure 1. Progesterone and water-soluble analogues.

identified as our first generation lead compound. P1-185 possesses an improved solubility profile and shows activity similar to that of PROG in *in vitro* and *in vivo* models of brain injury TBI. The structure of **1** was designed to allow introduction of a solubilizing group that is susceptible to hydrolysis in the bloodstream. This was then attached to the PROG skeleton by an oxime linker, which can undergo formal hydrolysis *in vivo*.<sup>26</sup> A pharmacokinetic experiment was carried out in rats using the iv route of administration to follow the disappearance of **1** and the potential appearance of its related free oxime (**2**) and/or PROG. The graph in Figure 2A shows that **1** is hydrolyzed rapidly to provide large concentrations of the free oxime **2** and smaller concentrations of PROG. While this study provided a possible explanation for the observed *in vivo* efficacy, it left open the question of whether the free oxime

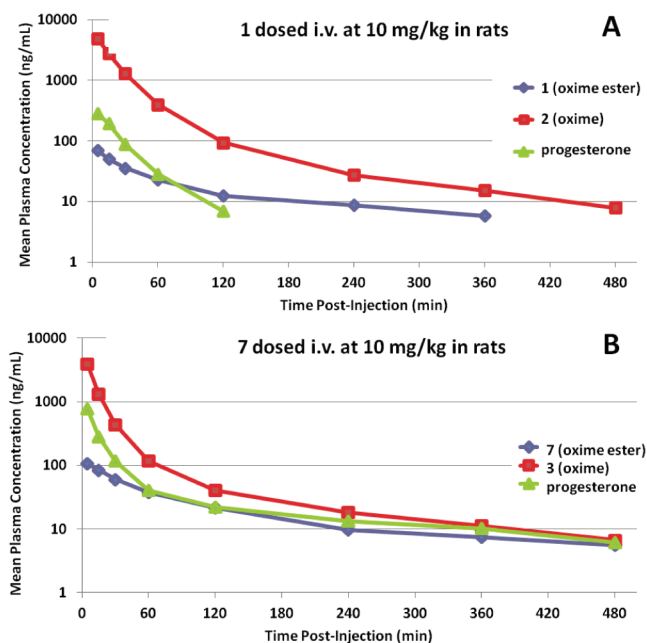


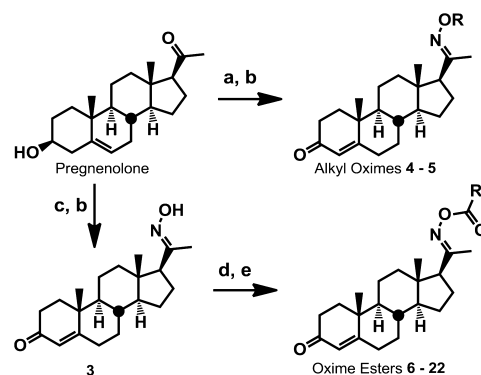
Figure 2. Pharmacokinetic and metabolic profiles of **1** and **7** when dosed iv at 10 mg/kg in rats. (See Supporting Information for error analysis of the data.)

may also contribute intrinsic therapeutic activity. It also raised the question of whether PROG arises directly by metabolism of **1** or in a stepwise pathway via the free oxime **2**.

While C-3 oxime conjugates such as **1** meet our initial goals of improved water solubility and therapeutic efficacy, their syntheses are somewhat challenging. Installation of the oxime moiety at the C-3 position requires a protection/deprotection at the C-20 position, and the oxime itself forms in very low *E/Z* selectivity, necessitating a tedious chromatographic separation, which results in low yields of the desired geometric isomer. To avoid these problems, we designed a second generation of C-20 oxime conjugates (Figure 1) that applies a similar design principle to **1** but utilizes the more sterically biased C-20 ketone as a synthetic handle to stereoselectively attach a solubilizing group onto the oxime linker.

The C-20 analogues were synthesized by the general methods shown in Scheme 1. Starting from readily available

Scheme 1. General Synthesis of C-20 PROG Analogues<sup>a</sup>



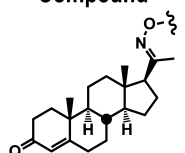


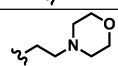

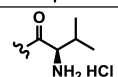

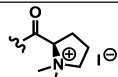

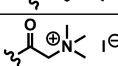
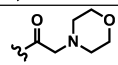
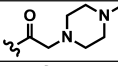

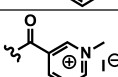
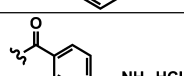
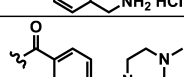
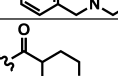
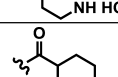
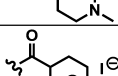
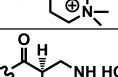
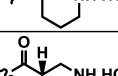
<sup>a</sup>Methods and reagents: (a) NH<sub>2</sub>OR-HCl, pyridine, Δ; (b) *N*-methylpiperidone, Al(O*i*Pr)<sub>3</sub>, PhMe, Δ; (c) NH<sub>2</sub>OH-HCl, pyridine, Δ; (d) RCO<sub>2</sub>H, *i*Pr<sub>2</sub>NEt, EDCI, cat. DMAP, CH<sub>2</sub>Cl<sub>2</sub>, rt; (e) deprotection and salt formation (TFA then HCl) or methylation (MeI, acetone) if necessary.

pregnenolone, condensation with an appropriate O-substituted hydroxylamine, followed by an Oppenauer oxidation, furnished C-20 O-alkyl oximes stereoselectively in 53–66% overall yields in only two steps. Oxime esters were synthesized through the common intermediate **3**, which was performed with modifications to known procedures on up to 50 mmol scale in 75% overall yield over two steps. EDCI-promoted coupling of **3** with the appropriate carboxylic acid, followed by deprotection or methylation of any amino groups as needed, resulted in oxime esters **6–22**. These routes allowed us to construct multiple analogues in a rapid fashion (Table 1).

Analogues with adequate aqueous solubility were screened in our first-pass *in vitro* assay, which measures the analogues' capacities to reduce neuronal cell death after a glutamate challenge. Most of the soluble oxime-ester conjugates tested had a maximum efficacy of 20–27% reduction in cell death at a 5 μM dose. These values compared favorably with our first-generation lead **1**. In contrast, PROG requires a 20 μM dose to surpass this efficacy and is much less potent than many of our compounds at a 5 μM dose. Because the oxime analogues are not metabolized in this assay, these values suggest that the compounds likely possess inherent neuroprotective abilities.

To assess the relative properties of the C-20 and C-3 oxime series, the valine conjugate at C-20 (**7**) was prepared as a direct

Table 1. Aqueous Solubilities, Stabilities, and In Vitro Efficacy of Analogues

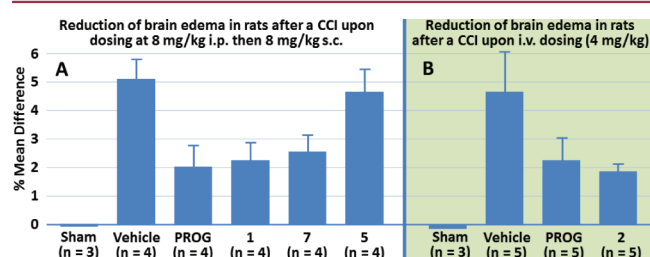
Compound	Aqueous Solubility, $\mu\text{M}^a$		Aqueous Stability <sup>b</sup>	Reduction in cell death, MTT Assay <sup>c</sup>	
	pH 7.4	pH 4.0	$t_{1/2}$ at pH = 7.0	At 5 $\mu\text{M}$ (%)	At best concentration (%)
 PROG	< 25	< 25	s <sup>e</sup>	4	42 (20 $\mu\text{M}$ )
1	172	322	10.1 h	27	27 (5 $\mu\text{M}$ )
2	< 45	< 45	s	21	21 (5 $\mu\text{M}$ )
3					
4		< 25	< 25	s	ns <sup>f</sup>
5		108	> 450	s	26
6		100	180	nd	ns
7		146	> 645	11.3 h	24
8		34	395	52 min	21
9		51	345	50 min	nd
10		171	449	6.2 h	nd
11		45	> 540	56 min	nd
12		215	215	nd	nd
13		145	153	70.6 h	submitted
14		< 35	< 35	ns	20
15		212	349	3.1 h	0
16		< 30	< 30	ns	nd
17		< 30	< 30	ns	nd
18		247	247	94.8 h	4
19		108	> 660	nd	nd
20		> 500	> 500	70.6 h	0
21		428	> 500	38.5 h	nd
22		314	314	40.8 h	nd

<sup>a</sup>As determined by laser nephelometry. <sup>b</sup>Hydrolysis of the C–O bond to give 3. <sup>c</sup>Details about in vitro. <sup>d</sup>nd = not determined. <sup>e</sup>s = stable ( $t_{1/2}$  > 10 days). <sup>f</sup>ns = not soluble; could not be determined.

comparison to the C-3 oxime 1. These compounds were compared head to head for their ability to reduce cerebral

edema in rats following bilateral cortical contusion as described previously.<sup>24</sup> The two compounds produced similar levels of

therapeutic benefit, both of which compared favorably with that of natural PROG (Figure 3A). PROG reduced brain edema

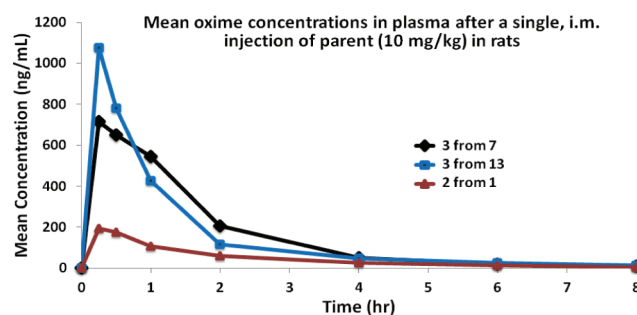


**Figure 3.** *In vivo* efficacy of PROG and various analogues in a rat model of injury induced brain edema.

after 24 h by 62% compared to that in untreated rats, while **1** and **7** reduced brain edema by 59% and 56%, respectively. Subsequently, we examined the pharmacokinetic profile of **7** in rats after a single iv dose at 10 mg/kg. Again, we observed low levels of the parent compound **7**, high levels of the relative free oxime **3**, and therapeutic levels of PROG (Figure 2B). The levels of PROG that were produced were slightly superior to those generated by **1**, so the C-20 oxime series proved to be a good scaffold on which to examine the effect of modifications on the water-solubilizing group.

We anticipate that a field-ready therapy for TBI will likely need to be delivered via an intramuscular (im) injection in an aqueous formulation to allow for rapid release into circulation. We therefore measured the aqueous solubility of all new analogues using nephelometry at pH 7 and 4. We also evaluated their chemical stability in neutral media. These results are summarized in Table 1. The valine conjugate **7** was more soluble (146  $\mu\text{M}$ ) than PROG ( $\ll 25 \mu\text{M}$ ) in neutral aqueous buffer, but it exhibited markedly increased solubility in a cyclodextrin formulation of at least 60 mM. Interestingly, **7** hydrolyzed in phosphate buffer (pH = 7.0) with a half-life of 11.4 h, while it was indefinitely stable at pH < 5. Proline conjugate **8** was also highly unstable, with a half-life of only 52 min. At neutral pH,  $\alpha$ -amino acid esters have been reported to undergo general base catalyzed hydrolysis, assisted by complexing to buffer components,<sup>27</sup> and this behavior proved to be operative for these analogous oxime esters as well. This aqueous instability could have skewed solubility results at neutral pH, as the common byproduct of hydrolysis was the highly insoluble **3** in each case. To address the chemical stability issue, we generated a series of analogues with altered water-solubilizing groups. Restricting a  $\beta$ - or  $\gamma$ -amino nitrogen inside a nipecotate or isonipecotate moiety, respectively, led to much higher stability, with half-lives ranging from 1.5 to 4 days, while increasing solubility. Tertiary substitution on *N,N*-dimethylglycine ester **10** led to a half-life of only 6.2 h, but including the  $\alpha$ -nitrogen in a piperazine ring as in **13** increased the half-life to nearly 3 days. Analogues with an alkyl C–O bond, such as **5**, were indefinitely stable but had very low biological activity (*vide infra*).

Having resolved the chemical stability issue in aqueous media, we next evaluated the pharmacokinetic profile of these compounds after im injection in rats. Again, we looked at both **1** and **7** as analogous representatives of the C-3 and C-20 oxime families. Interestingly, as shown in Figure 4, both compounds generated measurable concentrations of only the corresponding oximes without evidence of either the parent prodrug or PROG. Also, the C-20 oxime **7** generated a significantly higher



**Figure 4.** Plasma levels of free oxime in rats after im injection of parent compounds.

$C_{\text{max}}$  and AUC than the relative C-3 oxime **1**. The more chemically stable oxime ester at C-20, **13**, produced levels of free oxime that were similar to those from **1**, thus suggesting that other properly designed C-20 oxime esters could potentially generate similar concentration profiles.

We hypothesize that the observed difference in pharmacokinetic profiles between iv and im administration may be due to differences in the metabolic stability of the free oximes relative to their relative oxime esters. We have shown that both **7** and **1** are readily cleaved in both rat and human plasma (see Supporting Information), which explains the appearance of the free oxime after either mode of administration. By contrast, the oxime esters behaved differently than the free oximes when exposed to rat S9 liver fractions. Oxime ester **7** generated measurable quantities of the free oxime **3**, as well as PROG. However, when **3** was exposed directly to S9 fractions, it was relatively stable and no measurable quantities of PROG were generated. We therefore hypothesize that the oxime esters are metabolized in the liver to give PROG directly, without the involvement of the free oxime as an intermediate in the process. It appears that when the esters are administered iv, a substantial fraction reaches the liver before being hydrolyzed, resulting in the production of PROG. However, when the agents are administered im, they are hydrolyzed to their corresponding oximes before they reach general circulation, thereby avoiding conversion to PROG in the liver.

Given that our intended route of administration for these compounds is im, it became critical to determine whether free oximes **2** and **3** are intrinsically active. Accordingly, we administered the C-3 oxime **2** iv at 4 mg/kg and showed that it was cleared from plasma with a half-life of 2.7 h *without the formation of measurable concentrations of PROG* (see Supporting Information for data). We then demonstrated that when **2** was administered iv at 4 mg/kg to rats 1 h after a controlled cortical impact, it reduced brain edema by 60% (Figure 3B). We therefore conclude that oxime **2** is intrinsically active. Experiments to determine the levels of the intrinsic neuroprotective activity of **3** are currently in progress.

In conclusion, we have developed a set of water-soluble analogues of PROG with substantial efficacy in animal models of TBI, which compare favorably to native PROG. These analogues possess intrinsic neuroprotective activity and are well suited for field administration via im injection. A set of these analogues has been optimized to maximize their aqueous solubility and stability. We are now in the process of characterizing key compounds from this series with a view toward identifying a clinical candidate and developing it as a field-ready treatment for TBI. These new agents should be rapidly and easily administered in the field to reduce the time

between injury and treatment to improve overall patient outcomes.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Synthetic experimental details, analytical data of compounds, and biological assay protocols. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Author Contributions

The manuscript was written through contributions of D.B.G., D.G.S., M.A.L., M.G.N., and D.C.L. All authors have given approval to the final version of the manuscript.

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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

PROG, progesterone; TBI, traumatic brain injury; EDCI, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; DMAP, 4-dimethylaminopyridine; TFA, trifluoroacetic acid

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