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Synthesis, hydrolysis, and intraocular pressure lowering effects of fadolmidine prodrugs

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Abstract

The objective of this study was to synthesize and evaluate various esters of fadolmidine, a novel alpha$_2$-adrenergic agonist, as potential ophthalmic prodrugs. All studied prodrugs released the parent drug (i.e., fadolmidine) quantitatively via enzymatic hydrolysis in 80% human serum. The pivalyl ester was considered to be the most promising prodrug in this series, due to its good chemical stability (pH 5.0; 37 °C; $t_{1/2} = 310$ days) and optimal lipophilicity ($\log P_{app} = 1.8$; 1-octanol/phosphate buffer, pH 5.0), and was selected for further evaluation of its intraocular pressure (IOP) lowering effects in normotensive rabbits. The pivalyl ester showed increased IOP lowering ability when compared to an equimolar dose of fadolmidine, which was probably due to its increased lipophilicity and subsequent enhanced corneal penetration. The duration of action for the pivalyl ester was also longer than that of fadolmidine.
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1. Introduction

Fadolmidine (**1**) is a novel, full alpha$_2$-adrenoceptor agonist having low nanomolar affinity (Lehtimäki et al., 1999). Fadolmidine, previously known as MPV-2426, was developed for spinal pain therapy, and provides a spatially restricted and effective antinociception with minor side-effects (Eisenach et al., 1999; Xu et al., 2000a,b).

Alpha$_2$-agonists are also known to decrease intraocular pressure (IOP), and the first report of an IOP lowering effect from these therapeutic agents was published in 1966 (Makabe, 1966). That study demonstrated the ocular hypotensive effects of the systemic antihypertensive drug clonidine, the first alpha$_2$-agonist to be marketed for the treatment of glaucoma. Later, the clonidine derivatives apraclonidine (Chandler and DeSantis, 1985) and, more recently, brimonidine (Burke and Potter, 1986; Greenfield et al., 1997) proved to be safer than clonidine by providing

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fewer systemic side-effects, which essentially resulted from their poor ability to cross the blood–brain barrier. $Alpha_2$-agonists depress IOP by decreasing aqueous humor production and by decreasing resistance to aqueous outflow (Coleman, 1999).

Prodrugs are pharmacologically inactive derivatives of drug molecules that, after chemical or enzymatic transformation, release the active drug to exert the therapeutic action (Sinkula and Yalkowsky, 1975; Stella et al., 1985). Prodrugs are designed to overcome various pharmaceutical or biopharmaceutical problems associated with the parent drug. Since the invention of dipivefrine (a prodrug of epinephrine) (Mandell et al., 1978), various prodrugs have been designed to improve the therapeutic properties of ophthalmic drugs (Järvinen and Järvinen, 1996). Generally, ophthalmic prodrugs have been designed to enhance corneal penetration of the parent drug, to improve the efficacy, to reduce systemic side-effects, and/or to prolong the duration of action. In the treatment of glaucoma, reduced systemic side-effects are due to increased corneal absorption, which allows the application of smaller doses.

The aim of the present study was to synthesize ester prodrugs of fadolmidine. The physicochemical properties of the synthesized compounds and their hydrolytic stability in both phosphate buffer (pH 5.0 and 7.4) and in 80% human serum were evaluated. The most promising compound was selected for in vivo evaluation of its ability to lower IOP in normotensive rabbits.

2. Materials and methods

2.1. Chemicals

HPLC grade acetonitrile was purchased from Rathburn (Walkerburn, Scotland), and potassium dihydrogen phosphate from Merck KgaA (Darmstadt, Germany). Fadolmidine was obtained from Orion Pharma (Helsinki, Finland). All other chemicals used in the syntheses were high-purity reagent-grade materials and used without further purification.

2.2. Instrumentation

^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer, operating at 500.1 and 125.8 MHz, respectively, and using tetramethylsilane (TMS) as a reference. Mass spectra were recorded by a LCQ quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA).

2.3. HPLC determinations

HPLC determinations were measured on a Merck LaChrom HPLC system, consisting of L-7250 programmable autosampler, L-7100 HPLC pump, L-7400 UV-detector, D-7000 interface module, and D-7000 HPLC system manager (all from Hitachi Ltd., Tokyo, Japan). The injection loop was 100 μL stainless steel and injection volumes of 20–50 μL were typically used. Separations were performed on a Purospher RP-C18e column (125 mm × 4 mm i.d., 5 μm) (Merck KGaA, Darmstadt, Germany). The gradient eluent system consisted of two eluents (A and B), which were delivered at a flow-rate of 1.2 mL/min. Eluent A was a 20 mM potassium dihydrogen phosphate buffer, and the pH was adjusted to 7.0 with 1 M aqueous sodium hydroxide solution. Eluent B was 80% acetonitrile in water. The gradient was from 30 to 85% B in 9 min and back to 30% B in 1 min. The total run time was 16 min. The analytes were detected by monitoring UV-absorbance at 206 nm. The retention times of fadolmidine, **2**, **3**, and **4** were 3.5, 5.1, 7.9, and 8.8 min, respectively.

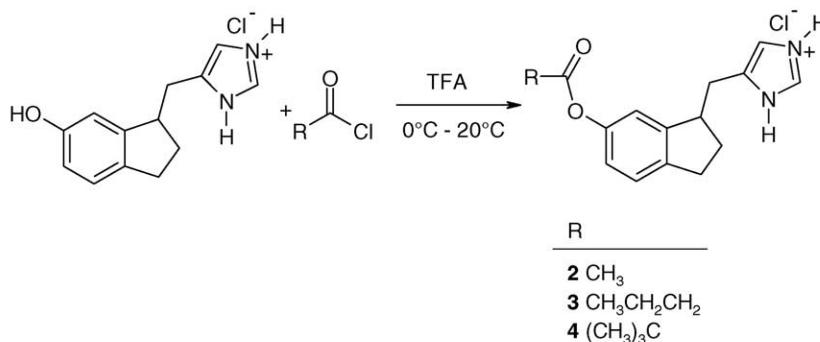
2.4. General procedure for the synthesis of fadolmidine prodrugs

*2.4.1. Fadolmidine (4-(6-hydroxy-indan-1-ylmethyl)-3H-imidazol-1-ium chloride) (**1**)*

Fadolmidine (100 mg, 0.399 mmol) was dissolved in 1 mL of trifluoroacetic acid (TFA), and carboxylic acid chloride (0.510 mmol) was added (Scheme 1). The mixture was stirred at room temperature for 24 h. TFA was evaporated and the residue was dissolved in water and made basic with 2 M NH_3 (aq.). The aqueous phase was extracted with dichloromethane (DCM). The combined extracts were dried (Na_2SO_4), filtered, and evaporated in vacuo. The residue was dissolved in diethyl ether and the solution was saturated with dry HCl gas. The white precipitate was filtered and dried under vacuum.

*2.4.2. 4-(6-Acetoxy-indan-1-ylmethyl)-3H-imidazol-1-ium chloride (**2**)*

Yield 105 mg (82%) of a white hygroscopic solid. ^1H NMR ($CDCl_3$): δ 1.72 (m, 1H), 2.18–2.26 (m, 4H), 2.72–2.87 (m, 3H), 3.17 (dd, 1H), 3.56 (qui, 1H), 6.85



Scheme 1.

(m, 2H), 6.92 (s, 1H), 7.16 (d, 1H), 8.61 (s, 1H), 14.44 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 21.11, 29.60, 30.46, 31.68, 43.93, 115.72, 117.00, 120.29, 125.33, 132.32, 132.40, 141.37, 146.32, 149.39, 170.11. HPLC–MS (EI): $m/z = 257.2$ $((M + H)^+–Cl^-)$.

2.4.3. 4-(6-Butyryloxy-indan-1-ylmethyl)-3H-imidazol-1-ium chloride (3)

Yield 90 mg (77%) of a white hygroscopic solid. ^{1}H NMR ($CDCl_3$): δ 1.02 (t, 3H), 1.70–1.79 (m, 3H), 2.18–2.24 (m, 1H), 2.52 (t, 2H), 2.73–2.86 (m, 3H), 3.18 (dd, 1H), 3.53 (qui, 1H), 6.84 (dd, 1H), 6.87 (s, 1H), 6.92 (s, 1H), 8.66 (s, 1H), 11.28 (s (broad), 2H). ^{13}C NMR ($CDCl_3$): δ 13.66, 18.45, 29.68, 30.52, 31.68, 36.24, 43.98, 115.79, 117.05, 120.36, 125.36, 132.32, 132.59, 141.32, 146.36, 149.51, 172.85. HPLC–MS (EI): $m/z = 285.2$ $((M + H)^+–Cl^-)$.

2.4.4. 4-(6-Pivalyloxy-indan-1-ylmethyl)-3H-imidazol-1-ium chloride (4)

Yield 74 mg (56%) of a white solid. Mp 176–177 °C. ^{1}H NMR ($CDCl_3$): δ 1.33 (s, 9H), 1.69–1.78 (m, 1H), 2.16–2.25 (m, 1H), 2.74–2.88 (m, 3H), 3.23 (dd, 1H), 3.57 (qui, 1H), 6.83 (dd, 1H), 6.89 (s, 1H), 6.93 (s, 1H), 7.16 (d, 1H), 8.85 (s, 1H), 14.38 (s, 1H), 14.47 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 27.17, 29.64, 30.55, 31.57, 39.07, 43.95, 115.69, 116.96, 120.24, 125.33, 132.12, 132.66, 141.20, 146.30, 149.30, 177.87. HPLC–MS (EI): $m/z = 297.7$ $(M^+–2H^+–Cl^-)$.

2.5. Enzymatic hydrolysis of prodrugs in human serum

An appropriate amount of the test compound (initial concentrations were 0.2–0.5 mM) was dissolved in one volume (e.q. 1 mL) of phosphate buffer (0.16 M, $\mu = 0.5$, pH 7.4) at 37 °C. Four volumes (e.q. 4 mL) of pre-heated human serum were added and the solutions were mixed in a water bath at 37 °C. At suitable time intervals, 300 μL aliquots were withdrawn and deproteinated with 600 μL of acetonitrile. After mixing and centrifugation, 600 μL of the resulting supernatant was evaporated to dryness under a stream of air at 40 °C. The residue was redissolved in 300 μL of mobile phase and analyzed by HPLC. Pseudo-first-order half-lives ($t_{1/2}$) were calculated from the linear slopes of semilogarithmic plots of remaining compound over time.

2.6. Chemical stability of prodrugs in aqueous solutions

An appropriate amount of the test compound (initial concentrations were 0.1–0.4 mM) was dissolved in pre-heated phosphate buffer (0.16 M, $\mu = 0.5$, pH 7.4 or 5.0). The solution was placed in a water bath at 37 °C and aliquots were taken at appropriate time intervals, then analyzed by HPLC to determine the degradation rate of the compound. Pseudo-first-order half-lives ($t_{1/2}$) were calculated from the linear slopes of semilogarithmic plots of remaining compound over time.

2.7. Apparent partition coefficients

The apparent partition coefficients (P_{app}) were evaluated from the distribution of test compounds between 1-octanol and phosphate buffer (0.16 M, $\mu = 0.5$, pH 5.0), using the shake-flask method.

2.8. IOP studies in rabbits

2.8.1. Animals

The experimental animals used were normotensive Dutch Belted pigmented rabbits (National Laboratory Centre, University of Kuopio) of either gender (2.3–4.0 kg, $n = 5$). The rabbits were housed singly in cages under standard laboratory conditions at 12-h dark:12-h light, $20.0 \pm 0.5\,^{\circ}\mathrm{C}$, and 55–75% relative humidity. Water and food were given ad libitum except during the tests. This research adhered to the "Principles of Laboratory Animal Care".

2.8.2. Preparation of eye drops

Fadolmidine and **4** were each dissolved in 20 mM sodium phosphate buffer (pH 5.0). Solution pH was then measured and adjusted if necessary to pH 5.0, and made isotonic with sodium chloride. The final concentration of fadolmidine or **4** in the solution was determined by HPLC.

2.8.3. Intraocular pressure measurements

To perform an IOP test, the rabbit was placed in a plastic restraining box, located in a quiet room. A single drop (25 μL) of the test solution was instilled unilaterally into the left eye on the upper corneoscleral limbus. During instillation, the upper eyelid was gently pulled away from the globe. IOP was measured using a BioRad (Cambridge, MA) Digilab Modular One Pneumatonometer. Before each measurement, one or two drops of oxybuprocaine (0.06%) were applied to the cornea to eliminate discomfort. The upper and lower eyelids were then gently retracted, and the applanation sensor was brought into contact with the center of the cornea. For each determination, at least two readings were taken from each treated (ipsilateral) and untreated (contralateral) eyes, and the mean of these readings was used. IOP of the rabbits was measured at 2, 1, and 0 h before, and then at 0.5, 1, 2, 3, 4, and 5 h after eyedrop administration. IOP at the time of eyedrop administration (0 h) was used as the baseline value. All studies were set up using a masked and randomized crossover design. At least 72 h wash-out time was allowed for each rabbit between experiments.

2.8.4. Analysis of the data

Results are presented as a change in IOP (mmHg), mean ± standard error (S.E.). A one-factor analysis of variance (ANOVA) for repeated measurements was used to test the statistical significance of differences between groups. Any significance in the difference of the means was tested using Fisher's Protected Least Significant Difference (PLSD) at the 95% confidence level.

3. Results and discussion

3.1. Hydrolytic behavior of fadolmidine prodrugs

All synthesized esters of fadolmidine released the parent drug quantitatively via enzymatic hydrolysis in 80% human serum (pH 7.4) (Fig. 1). The hydrolysis of **4** was over 90-fold slower than that of **2** and **3** (Table 1), which is in accordance with studies that show straight chain esters to be more rapidly hydrolyzed than those having branched chains, due to the steric hindrance effect of branched chain esters (Geraldine and Jordan, 1997; Iley et al., 1997). The slower release of fadolmidine from **4** suggests that it may provide a longer duration of alpha$_2$-adrenergic action than fadolmidine, if it is retained for a longer time than fadolmidine at the site of action.

Ophthalmic drugs are generally administered topically as aqueous solutions, and therefore, the stability of the ocular prodrugs against spontaneous chemical hydrolysis is of major importance. The chemical stability of the prodrugs investigated in the present study was evaluated in phosphate buffer at pH 5.0 and 7.4 at $37\,^{\circ}\mathrm{C}$. At pH 7.4, compounds **2** and **3** had half-lives of 27 and 60 h, respectively, and at pH 5.0 their stability

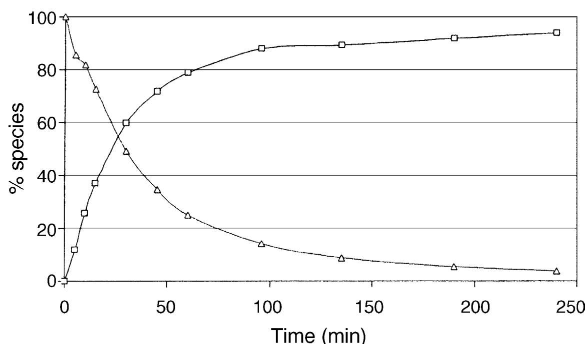


Fig. 1. The formation of fadolmidine (□) upon hydrolysis of **4** (△) in 80% human serum at $37\,^{\circ}\mathrm{C}$.

Table 1
Apparent partition coefficients (P_{app}) and hydrolysis half-lives (in 80% human serum, and 0.16 M phosphate buffer) of the studied compounds

Compound	$\log P_{app}$ (mean ± S.D., $N=3$) pH 5.0	Chemical hydrolysis in phosphate buffer				Enzymatic hydrolysis	
		pH 5.0		pH 7.4		80% serum	
		$t_{1/2}$ (days)	k_{obs} (min^{-1})	$t_{1/2}$	k_{obs} (min^{-1})	$t_{1/2}$	k_{obs} (min^{-1})
1	0.01 ± 0.02	–	–	–	–	–	–
2	0.13 ± 0.00	23	2.1×10^{-5}	27 h	4.4×10^{-4}	<20 s	>2
3	1.25 ± 0.01	49	9.9×10^{-6}	60 h	1.9×10^{-4}	<20 s	>2
4	1.75 ± 0.01	310	1.5×10^{-6}	31 days	1.6×10^{-5}	30 min	2.3×10^{-2}

was 20-fold higher (Table 1). The chemical stability of **4** was superior, compared to that of **2** and **3**. The half-lives of **4** at pH 5.0 and 7.4 were 310 and 31 days, respectively.

3.2. Lipophilicity of fadolmidine prodrugs

Acetylation ($\log P_{app} = 0.13$) of the phenolic OH-group did not have a substantial effect on the lipophilicity of fadolmidine ($\log P_{app} = 0.01$), while butyryl and pivalyl subtitutions increased $\log P_{app}$ to 1.25 and 1.75, respectively (Table 1). Apparent partition coefficients were determined between 1-octanol and phosphate buffer at pH 5.0.

The optimum $\log P_{app}$ for corneal drug penetration appears to be in the range of 2–3 (Schoenwald and Ward, 1978; Schoenwald and Huang, 1983), and therefore, **4** was selected for further IOP studies in normotensive rabbits. Also, the better chemical stability and slower enzymatic degradation of **4** contributed to its selection for further in vivo evaluation.

3.3. IOP responses in rabbits

The biological activity of **4** was evaluated by determining its ability to decrease IOP in normotensive rabbits. The pH selection (5.0) of the eyedrop solution was based on chemical stability of **4**. Changes in IOP after the topical unilateral administration of three different doses (0.1, 1.0, and 3.4 μg) of **4** are shown in Table 2. Doses of 1.0 and 3.4 μg both decreased IOP in treated eyes at 1–5 h after treatment. The maximum IOP decreases were −4.6 ± 1.4 and −6.5 ± 1.1 mmHg at 2 h after administration of 1.0 and 3.4 μg of **4**, respectively,

Table 2
Intraocular pressure changes (mean mmHg ± S.E., $n=5$) in normotensive rabbits at predetermined times (h) after administering unilaterally 25 μL of fadolmidine pivalyl ester (**4**) solutions (pH 5.0), or fadolmidine solutions (pH 5.0)

Dose	Time (h)						
	0	0.5	1	2	3	4	5
Treated eye							
Buffer pH 5.0 (vehicle)	0.0 ± 0.0	0.1 ± 0.4	−0.7 ± 1.1	0.9 ± 0.6	1.4 ± 1.2	−0.4 ± 0.4	2.0 ± 0.4
0.1 μg of **4**	0.0 ± 0.0	1.7 ± 0.9	0.2 ± 1.3	0.8 ± 1.2	0.6 ± 0.6	1.8 ± 0.5	2.0 ± 0.9
1.0 μg of **4**	0.0 ± 0.0	−1.0 ± 0.3	−3.6 ± 0.7	−4.6 ± 1.4*	−4.3 ± 1.1*	−2.0 ± 1.4	−0.9 ± 1.8*
3.4 μg of **4**	0.0 ± 0.0	−0.9 ± 0.9	−2.9 ± 0.5	−6.5 ± 1.1*	−5.2 ± 1.7*	−3.2 ± 0.9	−1.4 ± 0.7*
2.5 μg of fadolmidine (equivalent to 3.4 μg of **4**)	0.0 ± 0.0	0.1 ± 1.0	−2.6 ± 1.1	−3.0 ± 0.7*	−2.4 ± 0.5*	−0.4 ± 1.2	−0.1 ± 0.8
Untreated eye							
Buffer pH 5.0 (vehicle)	0.0 ± 0.0	−0.6 ± 0.4	−1.7 ± 0.6	0.4 ± 0.4	0.3 ± 0.6	−0.6 ± 0.6	0.3 ± 1.2
0.1 μg of **4**	0.0 ± 0.0	1.4 ± 0.1	0.9 ± 0.5	1.3 ± 0.2	1.3 ± 0.7	1.0 ± 0.9	2.4 ± 1.3
1.0 μg of **4**	0.0 ± 0.0	0.0 ± 1.4	−2.1 ± 1.2	−2.0 ± 1.8	−0.9 ± 2.4	−1.4 ± 0.7	−0.8 ± 2.1
3.4 μg of **4**	0.0 ± 0.0	0.4 ± 0.7	−2.7 ± 0.8	−1.3 ± 0.6	−2.8 ± 0.8	−0.8 ± 0.7	−0.8 ± 1.0
2.5 μg of fadolmidine (equivalent to 3.4 μg of **4**)	0.0 ± 0.0	0.3 ± 1.0	−1.3 ± 0.7	−1.6 ± 0.9	−1.7 ± 0.6	0.4 ± 1.1	0.4 ± 1.1

* Significantly different from the buffer (vehicle) at the 95% confidence level (ANOVA, Fisher's PLSD test).

while less significant changes in IOP were observed in the untreated (contralateral) eyes. The smallest dose of **4** (0.1 μg) did not result in a significant IOP decrease in either the treated or untreated eye.

The IOP decrease observed in the untreated eye after unilateral topical doses of **4** was most probably due to systemic transfer of the drug to alpha$_2$-adrenoceptors in the contralateral eye. The IOP decrease in contralateral eyes might also be mediated by receptors located in the CNS, or other systemic sites.

In a subsequent experiment, the IOP lowering effect of **4** was compared to an equivalent dose of fadolmidine (Fig. 2). The pivalyl ester **4** produced a significantly stronger IOP decrease in the treated eye at 2 h after treatment, compared to fadolmidine. The maximum IOP reduction of both fadolmidine and **4** in the treated eye occurred at 2 h after treatment, but the IOP lowering effect of **4** was observed to be slightly prolonged when compared to fadolmidine. The maximum effect of **4** was 2.2-fold compared to fadolmidine. The higher decrease in IOP observed with **4**, when compared to fadolmidine, was most probably due to its increased ocular absorption because of its higher lipophilicity. The IOP effects of **4** and fadolmidine were quantitatively similar in the untreated eyes (Fig. 2).

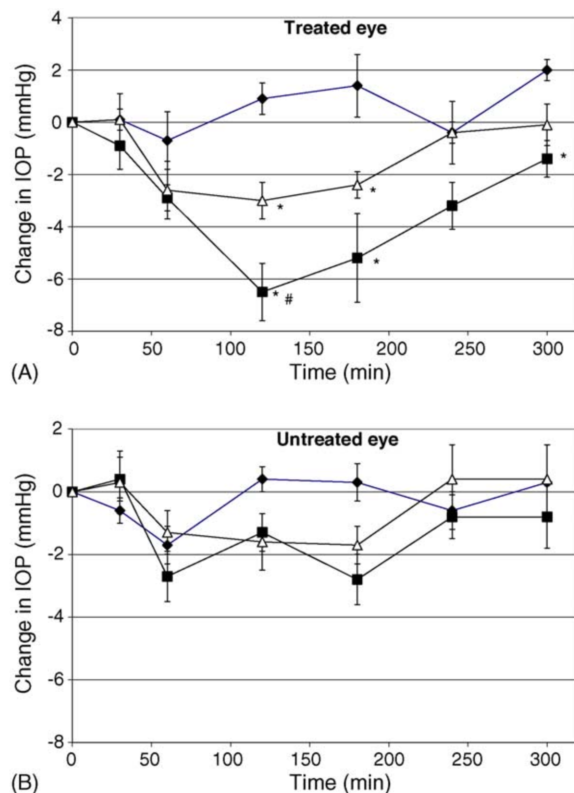


Fig. 2. IOP changes in treated (A) and untreated (contralateral) (B) eyes of normotensive pigmented rabbits after unilateral ocular administration (25 μL) of 3.4 μg of **4** (■), 2.5 μg fadolmidine (△) (equimolar to 3.4 μg of **4**), or vehicle, 20 mM phosphate buffer, pH 5.0 (♦), mean ± S.E. ($N = 5$). Symbols indicate data that are significantly different from values for the vehicle (*), or from fadolmidine (#), at the 95% confidence level (ANOVA, Fisher's PLSD test).

In conclusion, the present study demonstrates that esters of fadolmidine can serve as prodrugs because they release the parent compound via enzymatic hydrolysis. The pivalyl ester **4** was the most chemically stable prodrug, and provided a significant enhancement of lipophilicity and IOP lowering effect in rabbits when compared to fadolmidine. The fadolmidine prodrugs evaluated in the present study may also provide therapeutic advantages in spinal pain therapy, for which fadolmidine was originally developed, and this deserves evaluation in further studies.

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