Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 3427–3430
Bioorganic & Medicinal Chemistry Letters

Design and synthesis of hydroxy-alkynoic acids and their methyl esters as novel activators of BK channels

Shivaputra Patil,[a,†] Anna N. Bukiya,[b,†] Wei Li,[a] Alejandro M. Dopico[b,*] and Duane Miller[a,*]

[a]*Department of Pharmaceutical Sciences, College of Pharmacy, The University of Tennessee Health Science Center, Memphis, TN 38163, USA*
[b]*Department of Pharmacology, College of Medicine, The University of Tennessee Health Science Center, Memphis, TN 38163, USA*

Received 22 February 2008; revised 25 March 2008; accepted 31 March 2008
Available online 8 April 2008

Abstract—Physiological and pharmacological agents that activate large conductance, voltage-, and calcium-gated potassium (BK) channels located in the smooth muscle are effective vasodilators. Thus, activators of smooth muscle BK channels may be potential therapeutic tools to treat cardiovascular disease associated with vasoconstriction and/or impaired dilation, such as cerebrovascular spasm and constriction. We previously showed that lithocholic acid (LC) and other cholane derivatives activated smooth muscle BK channels and, thus, caused endothelium-independent cerebral artery dilation. However, clinical use of these cholane derivatives could be limited by the actions of these steroids, such as elevation of intracellular calcium and induction of apoptosis. Using LC as template, we designed and synthesized a series of hydroxy-alkynoic acids and corresponding methyl esters, as putative, non-steroid BK channel activators. Indeed, the newly synthesized compounds effectively and reversibly activated rat cerebrovascular myocyte BK channel at concentrations similar to those found effective with LC. Among all the novel compounds tested, C-10 hydroxy-alkynoic acid methyl ester appears to be the most effective activator of vascular myocyte BK channels.

Voltage- and calcium-gated potassium channels of large conductance (BK channels) are the critical determinants of action potential repolarization and, thus, cell excitability.[1] BK channels are activated by both membrane depolarization and micromolar levels of intracellular calcium. BK channel activation leads to K^+ outward currents, which tend to hyperpolarize the membrane potential. Thus, BK currents cause membrane repolarization that opposes depolarization-induced Ca^{2+} influx. In vascular smooth muscle, this BK channel-mediated negative feed-back serves to limit contraction and favor relaxation.[2,3] Given the critical role of smooth muscle tone in vascular reactivity, any agent that effectively activates smooth muscle BK channels is an effective vasodilator. We previously showed that several cholane derivatives, including naturally occurring bile acids and synthetic analogs, effectively activated vascular smooth muscle BK channels.[4] In particular, the naturally occurring monohydroxylated lithocholic acid (5β-cholanic acid 3α-ol) (LC) was the most effective bile acid in increasing the activity of these channels, which reached up to 300% of control values.[4,5] In addition, LC activated cerebrovascular myocyte BK channels at concentrations that evoked endothelium-independent dilation of pressurized, resistance-size cerebral arteries.[5] Moreover, LC-induced vasodilation was prevented by the selective BK channel blockers but not the blockers of voltage-gated potassium channels other than BK.[5] Together, these findings support the notion that LC activation of myocyte BK channels effectively leads to vasodilation.

LC and other bile acids, however, interact with a variety of proteins other than BK channels, including the G-protein-coupled TGR5,[6] vitamin D,[7] ryanodine, and IP_3 receptors,[8] cytosolic steroid-binding proteins, membrane transporters, and transcription factors.[9] The likelihood of widespread side effects in the body determined by bile acid interactions with such a heterogeneous set of proteins might limit the use of LC and analogs as clinically useful vasodilators, in particular when chronic therapy is considered. Given that several of the proteins

Keywords: MaxiK channel; Activators; Smooth muscle; Lithocholic acid; Hydroxy-alkynoic acid.
* Corresponding authors. Tel.: +1 901 448 6026; fax: +1 901 448 3446 (D.M.). ; e-mail: dmiller@utmem.edu
† These authors contribute to the paper equally.

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.03.080

mentioned above appear to recognize the steroid moiety of the bile acid molecule, we decided to design and synthesize LC-based non-steroidal analogs, and probe their effects on arterial smooth muscle BK channels.

A previous study indicated that the presence of a hydroxyl group at C3 and a high hydrophobic index of the steroid nucleus (formed by A/B/C/D rings) are both important for bile acids to activate vascular myocyte BK channels. Thus, the monohydroxylated LC is the most effective bile acid in activating this channel.[4] In addition, we recently showed that the esterification of the C24 carboxylate caused a significant reduction in LC action on BK channels (A.N. Bukiya et al., unpublished data). Thus, we hypothesized that LC-based activators of BK channels should include a hydroxyl and a carboxyl at each end, as found in LC, with a non-steroidal hydrophobic linker joining these two polar groups. The model for LC-based non-steroidal pharmacophores is presented in Figure 1.

The model shows that the hydrophobic linker was proposed to be made of methylene groups. However, to provide some rigidity to the linker (formed by the hydrophobic steroidal rings in LC nucleus) we introduced a triple bond near the linear compound hydroxyl end. To determine the length of the linker area between LC's two functional groups, we used a computational approach and, thus, obtained a three-dimensional structure of the LC molecule. First, we performed a conformational search for LC structure using the MacroModel in Schrodinger software package and the OPLS_2005 forcefield. Since the only known area in the BK channel complex that contributes to LC-sensing is a protein transmembrane domain,[10] our LC conformational search relied on chloroform as a solvent to mimic the non-polar lipid environment that very likely surrounds LC during BK channel activation. The lowest energy conformation for LC under these conditions renders a bean-shaped molecule (Fig. 2). This is in agreement with the earlier reports based on crystallographic data.[11]

The LC molecule width, defined as the distance between the hydroxyl and the carboxylate groups, is about 12 Å. The height, defined as the distance between the center of

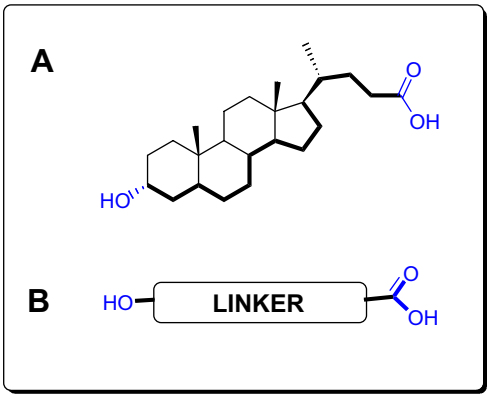


Figure 1. (A) Lithocholic acid and (B) hypothesized pharmacophore of non-steroidal structure.

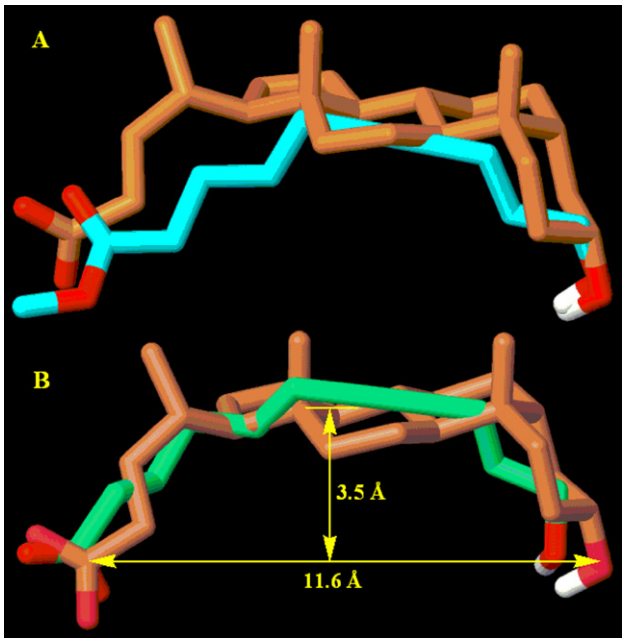


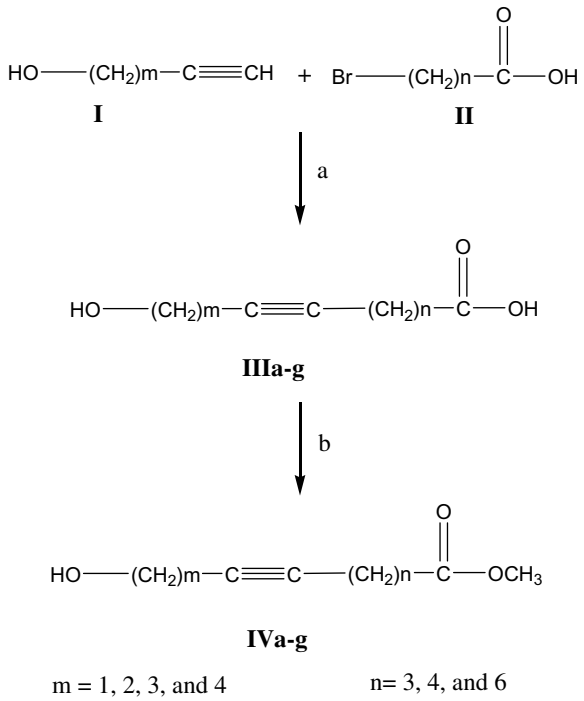
Figure 2. Minimum energy conformation of LC (light and dark orange sticks) and its overlap with C-10 ester (blue sticks) in (A), and C-11 acid (green sticks) in (B). For both panels, oxygen is shown in red, and the hydrogen of hydroxyl-group is in white.

the two functional groups and the center of the three methyl groups, is about 4 Å (Fig. 2). Considering that the average length of a single C–C bond in the lipidic microenvironment ≈1.52 Å, the optimal length of linear analogs to adopt the LC bean-shaped conformation would require from 9 to 12 C. Remarkably, after we overlapped as closely as possible the designed acid and ester structures with LC in its minimum energy conformation, linear analog structures with 10 or 11 C separating the hydroxyl and carbonyl groups showed best fit. Examples for the C-10 ester and C-11 acid are shown in Figure 2A and B, respectively.

To evaluate our computational predictions, we synthesized a set of C-8–C-16 hydroxy-alkynoic acids and their corresponding methyl esters.

The final products were achieved in two steps as shown in Scheme 1. In the first step, bromo-acid (1 mmol) was condensed with alkynol (1 mmol) using sodium hydride (5 mmol) in anhydrous tetrahydrofuran (30 ml) to obtain the hydroxy-alkynoic acids (**IIIa**–**g**).[12,13] These hydroxy-alkynoic acids (1 mmol) (**IIIa**–**g**) were refluxed under methanol (50 ml) in the presence of a catalytic amount of sulfuric acid to obtain the corresponding methyl esters (**IVa**–**g**). All esters were purified by column chromatography (9:1 ethyl acetate/hexanes; phosphomolybdic acid reagent 20 wt% in ethanol was used to visualize the compounds after heating). Structures of all compounds were confirmed by mass and nuclear magnetic resonance spectroscopic data.

To evaluate the efficacy of these newly synthesized compounds in potentiating myocyte BK channel currents, we applied standard patch-clamp, voltage-clamp tech-



Scheme 1. Reagents and conditions: (a) NaH, THF, rt; (b) MeOH, H_2SO_4(Catalytic), reflux.

niques to individual myocytes freshly isolated from rat small cerebral arteries (130–170 μM external diameter), following the methods previously described.[5,14] BK channel unitary currents were continuously recorded from excised, inside–out (I/O) membrane patches, with the membrane voltage set to values ranging from −40 to −20 mV, and free $[Ca^{2+}] = 3$ μM. These conditions are found in the vicinity of the native cerebrovascular BK channel during myocyte contraction.[15,16] Stock solutions for compounds of interest were prepared in DMSO/EtOH 1:9 (v/v) vehicle and further diluted in bath solution (for composition, see Ref. 14) to render a final concentration of hydroxyl-alkynoic acid/ester of 150 μM. Each compound diluted in bath solution was applied to the intracellular side of the patch, using vehicle-containing bath solution as perfusion control. BK channel steady-state activity (NPo)[17] was obtained from all-points amplitude histograms before (control), during, and after (washout) compound application, for no less than 30 s of continuous recording under each condition.

We found that C-10 ester was the most effective BK channel activator among the hydroxy-alkynoic methyl ester series, increasing channel NPo up to two times of control values. C-9 methyl ester caused moderate activation, C-11 and C-12 were poor activators, and C-8, C-14, and C-16 showed no effect on BK channel activity (Fig. 3). Thus, data indicate that a length of 9 carbons in the linker area is optimal for alkynoic methyl esters to activate BK channels.

When compared with their methyl esters, hydroxy-alkynoic acids appear to have a milder dependence on chain length for BK channel activation. However, a maximum activation was still observed, in this case with the C-11 and then the C-12 members of the series. Our results

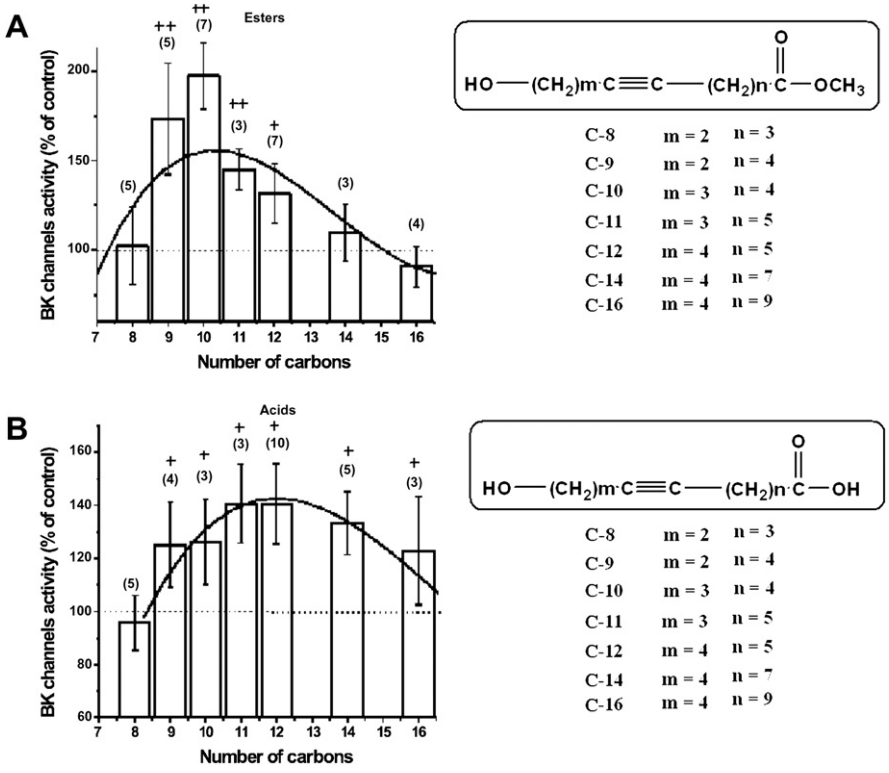


Figure 3. Increase in BK channels activity (% of control level) in the presence of C-8–C-16 hydroxy-alkynoic acids (B), and corresponding methyl esters (A). Data are expressed as mean ± SEM; number of patches for each data point is given in parentheses. +, Significantly different from control, $p < 0.05$; ++, $p < 0.01$.

confirm a computational prediction: molecular superposition of LC with C-10 ester and C11 acid reveals that both linear analogs fit the LC dimensions and easily adopt the bean shape of this steroid (Fig. 2).

In summary, using a steroidal BK channel activator as a template (LC), we carried out the synthesis of non-steroidal compounds (hydroxy-alkynoic acids and their corresponding methyl esters) and found that they, indeed, can activate BK channels. Overall, their length-dependence of channel activation confirms the computational predictions of favored structural overlapping between LC and the most effective among the non-steroidal analogs (the C-10 methyl ester). Our findings provide a basis for the future rationale design of novel activators of smooth muscle BK channels.

Acknowledgment

This work was supported by the NIH Grants HL77424 (A.M.D.).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.03.080.

References and notes

1. Orio, P.; Rojas, P.; Ferreira, G.; Latorre, R. *News Physiol. Sci.* **2002**, *17*, 156.
2. Brayden, J. E.; Nelson, M. T. *Science* **1992**, *256*, 532.
3. Jaggar, J. H.; Wellman, G. C.; Heppner, T. J.; Porter, V. A.; Perez, G. J.; Gollasch, M.; Kleppisch, T.; Rubart, M.; Stevenson, A. S.; Lederer, W. J.; Knot, H. J.; Bonev, A. D.; Nelson, M. T. *Acta Physiol. Scand.* **1998**, *164*, 577.
4. Dopico, A. M.; Walsh, J. V., Jr.; Singer, J. J. *J. Gen. Physiol.* **2002**, *119*, 251.
5. Bukiya, A. N.; Liu, J.; Toro, L.; Dopico, A. M. *Mol. Pharmacol.* **2007**, *72*, 359.
6. Maruyama, T.; Miyamoto, Y.; Nakamura, T.; Tamai, Y.; Okada, H.; Sugiyama, E.; Nakamura, T.; Itadani, H.; Tanaka, K. *Biochem. Biophys. Res. Commun.* **2002**, *298*, 714.
7. Makishima, M.; Lu, T. T.; Xie, W.; Whitfield, G. K.; Domoto, H.; Evans, R. M.; Haussler, M. R.; Mangelsdorf, D. J. *Science* **2002**, *296*, 1313.
8. Gerasimenko, J. V.; Flowerdew, S. E.; Voronina, S. G.; Sukhomlin, T. K.; Tepikin, A. V.; Petersen, O. H.; Gerasimenko, O. V. *J. Biol. Chem.* **2006**, *281*, 40154.
9. Modica, S.; Moschetta, A. *FEBS Lett.* **2006**, *580*, 5492.
10. Bukiya, A. N.; Vaithianathan, T.; Toro, L.; Dopico, A. M. *FEBS Lett.*, in press.
11. Carey, M. C. Physical–chemical properties of bile acids and their salts. In *Sterols and Bile Acids*; Danielsson, H., Sjovall, J., Eds., 1985, p 345.
12. Cobbledick, R. E.; Einstein, F. W. *Acta Cryst.* **1980**, *B36*, 287.
13. General procedure for the preparation of hydroxy-alkynoic acids (**IIIa**– **g**) and corresponding esters (**IVa**– **g**): NaH (2.56 mmol) was added to a mixture of alkynols (0.66 mmol) and bromo-acids (0.512 mmol) in anhydrous THF (in case C-14 and C-16 used anhydrous DMF), and the reaction mixture was stirred at room temperature for nearly 24 h. Reaction mixture was cooled and dilute hydrochloric acid was added to the reaction mixture till acidic. The mixture was extracted with diethyl ether. The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, and solvents were evaporated under reduced pressure. Residue was taken to the next step without further purification. The crude residue from the above reaction was refluxed nearly 2 h under methanol (50 ml) in the presence of a catalytic amount of sulfuric acid. Solvents were reduced to half under reduced pressure and extracted with ethyl acetate. The organic layer was dried and evaporated to dryness. The crude residue was purified 9:1 ethyl acetate/hexanes (pure methyl esters were re-hydrolyzed using NaOH to get pure acids).
14. Basilar and middle cerebral arteries were dissected out from each rat brain under a stereo-zoom microscope and placed into ice-cold 'dissociation medium' (DM) (mM): 0.16 $CaCl_2$, 0.49 EDTA, 10 Hepes, 5 KCl, 0.5 KH_2PO_4, 2 $MgCl_2$, 110 NaCl, 0.5 NaH_2PO_4, 10 $NaHCO_3$, 0.02 phenol red, 10 taurine, 10 glucose. Arteries were cut into 1–2 mm long rings (~30 rings/experiment). Rings were put in 3 ml DM containing 0.03% papain, 0.05% bovine serum albumin (BSA), and 0.004% dithiothreitol (DTT) for 15 min at 37 °C in a polypropylene centrifuge tube, and then incubated in a shaking water bath at 37 °C and 60 oscillations/min for 15 min. Then the supernatant was discarded, and the pellet resuspended in 3 ml of DM containing 0.06% soybean trypsin inhibitor. Finally, the tissue was pipetted using a series of borosilicate Pasteur pipettes having fire-polished, diminishing internal diameter tips. The procedure rendered a cell suspension containing relaxed, individual myocytes ($\geqslant$5 myocytes/field using a 40× objective) that could be easily identified under microscope. The cell suspension was stored in ice-cold DM containing 0.06% BSA, and the cells were used for patch-clamping up to 4 h after isolation. For electrophysiological experiments, both bath and electrode solutions contained (mM) 135 KCl, 5 EGTA, 1 $MgCl_2$, 15 Hepes, 10 glucose, pH 7.35. Free Ca^{2+} in solution was adjusted to 3 μM by adding $CaCl_2$. Nominal free Ca^{2+} was validated experimentally using Ca^{2+}-selective electrodes. Patch-recording electrodes were prepared before each experiment and fire-polished to give resistances of 5–9 MΩ when filled with solution. An agar bridge with Cl^- as the main anion was used as ground electrode. After excision from the cell, the membrane patch was exposed to a stream of bath solution containing each agent at final concentration. Solutions were applied onto the patches using an automated pressurized drug-delivery system via a micropipette tip with an internal diameter of 100 μm. Experiments were carried out at room temperature (21 °C). Currents were recorded using an EPC8 amplifier at 1 kHz using a low-pass, eight-pole Bessel filter 902LPF. Data were digitized at 5 kHz using a Digidata 1320A A/D converter and analyzed with pCLAMP 8.0.
15. Knot, H. J.; Nelson, M. *J. Physiol.* **1998**, *508*, 199.
16. Perez, G. J.; Bonev, A. D.; Nelson, M. T. *Am. J. Physiol. Cell Physiol.* **2001**, *281*, C1769.
17. Dopico, A. M.; Lemos, J. R.; Treistman, S. N. *Mol. Pharmacol.* **1996**, *49*, 40.