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Haloenol pyranones and morpholinones as antineoplastic agents of prostate cancer

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Abstract

Haloenol pyran-2-ones and morpholin-2-ones were synthesized and evaluated as inhibitors of cell growth in two different prostate human cancer cell lines (PC-3 and LNCaP). Analogs derived from L- and D-phenylglycine were found to be the most effective antagonists of LNCaP and PC-3 cell growth. Additional studies reveal that the inhibitors induced G2/M arrest and the (*S*)-enantiomer of the phenylglycine-based derivatives was a more potent inhibitor of cytosolic iPLA₂β.

Keywords

Haloenol; Pyranones; Morpholinones; Phospholipase A₂; Prostate cancer

Haloenol pyranones¹ are mechanism-based inhibitors of serine proteases due to their ability to alkylate enzyme active sites following ring hydrolysis and unmasking of a reactive α-haloketone functionality (Figure 1). To date, the most evaluated of these inhibitors is bromoenol lactone, (*E*)-6- (bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (**4**) or BEL. Interestingly, the popularity of BEL stems not as a deactivator of serine proteases, but rather for its ability to inhibit Ca²⁺-independent phospholipases A₂ (iPLA₂), which are responsible for the catabolism of membrane glycerophospholipids. Over the last 20 years, BEL has enabled researchers to probe the role of iPLA₂ in pathologies involving oxidative stress and inflammation including cardiovascular², Alzheimer's³ and Parkinson's diseases⁴, diabetes mellitus⁵, and more recently, carcinogenesis^{6,7}.

Mammalian cells possess multiple isoforms of iPLA₂⁸. The most studied are cytosolic iPLA₂β, (Group VIA-1 and A-2 PLA₂) and the membrane localized iPLA₂γ (Group VIB PLA₂), which together govern the release of fatty acids arachidonic acid and 2-lysophospholipids from membrane phospholipids. For many years, phospholipid remodeling^{8,9} was thought to be the only function of these enzymes; however, beginning in the 1990's researchers began finding evidence that iPLA₂ participates in cell signaling¹⁰, proliferation¹¹, and death^{4,12}. It was established that the products arising from the breakdown of phospholipids functioned as signaling molecules for promoting cell growth and that the enzymes responsible for generating the lipids (i.e. PLA₂) are in greater abundance in carcinoma cells¹³.

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The effects of iPLA₂ γ and iPLA₂ β on cell signaling and proliferation have recently been studied by enantiomer-based inhibition^{6,14} strategies using (*R*)- and (*S*)-BEL, respectively (Figure 2). The mechanisms involved in their selectivity are currently under study although it was demonstrated that LNCaP and PC-3 prostate cancer cells display moderate increases in chemosensitivities to racemic BEL compared to the individual enantiomers⁶. These results suggest that the (*R*)- and (*S*)-conformers could be acting in a synergistic manner as cell growth inhibitors. The studies further established that enantiomers of haloenol pyranones may be used to selectively and pharmacologically inhibit iPLA₂ γ , iPLA₂ β , and possibly other enzymes involved in critical cell processes. In this Letter, we report on the antineoplastic activities of haloenol pyran-2-one analogs of BEL against prostate cancer. In addition, the evaluation of novel haloenol morpholin-2-ones constructed from L- and D-amino acids and their inhibitory effects on the cell cycle and iPLA₂ activity are described.

To evaluate whether analogs of BEL could have similar inhibitory effects on iPLA₂ and prostate cancer growth, we set forth to synthesize various haloenol pyran-2-ones from α -substituted and unsubstituted acetylenic acids. Standard *E*-specific haloenol lactonization procedures^{1,15} with N-halosuccinimides (X = Br, I) were used to generate the pyranone analogs (Scheme 1). In the case of the phenyl analog **4**, the acid precursor **3** required preparation from phenylacetic acid and 4-bromobut-1-yne using classical enolate chemistry¹⁸. Subsequent attempts to separate the (*R*)- and (*S*)-enantiomers of lactone **4** by chiral HPLC were unsuccessful, which led to us to consider the use of chiral pool amino acids to construct novel iPLA₂ inhibitors containing a *E*-haloenol morpholin-2-one framework (Figure 2).

L- and D-phenylglycine (Phg), L-phenylalanine (Pha), and glycine (Gly) were chosen as base materials to perform the asymmetrical synthesis of morpholinone analogs (Scheme 2). Protected *tert*-butyl esters forms of the amino acids were first prepared from *tert*-butyl acetate¹⁶ then converted to the corresponding N,N-propargyl α -amino esters **11–13**¹⁷. Following deprotection of the carboxylic acid, bromo- and iodoenol morpholin-2-one analogs **14–17** were generated in 6–23% yield under the conditions described for pyranones **2** and **4**.

The synthesis of additional L-Phg-based analogs was also attempted from the monopropargyl intermediate **18** (Scheme 3). Benzylation of the secondary amine followed by acid deprotection and cyclization gave the corresponding N-benzyl bromoenol morpholin-2-one **20**. Efforts to prepare the unsubstituted analog **21** were unsuccessful however, which was attributed to chemical instability of the N-protio ring system.

Minimum inhibitory concentrations (IC₅₀s) were determined by MTT staining for the haloenol pyranones (**2**, **4**) and morpholinones (**14–17**, **20**) against LNCaP cells, and the more resistant PC-3 human prostate cancer cell line. With racemic BEL as a haloenol standard, IC₅₀ measurements were taken at 24, 48, and 72 h (Table 1). BEL was found to inhibit growth in a time-dependent manner at 5–13 μ M and 14–34 μ M of LNCaP and PC-3, respectively, over 72 h which corroborated previous findings⁶. Activity comparison of BEL to pyranones **2** revealed that the unsubstituted analogs were equally efficacious inhibitors at 5–10 and 14–32 μ M for the corresponding cell lines. For the α -substituted phenyl analog **4**, slightly enhanced activities were observed with IC₅₀s ranging from 6–27 μ M against PC-3.

The morpholinones analogs similarly demonstrated antineoplastic activity with IC₅₀s reaching 3 μ M for the Phg-based derivatives **14** (Table 1). The inhibitors also appeared to be more rapid-acting antagonists of prostate cancer growth compared to BEL and its phenyl pyranone analog **4**. Moreover, activity comparison of the enantiomers revealed that (*R*)-**14b** was a more effective inhibitor than (*S*)-**14a** particularly against PC-3 cells (IC₅₀ 3–8 μ M).

As a compound derived from the unnatural D-form of Phg, the augmented activity of (*R*)-**14b** was attributed in part to higher proteolytic susceptibility (e.g. chymotrypsin) than the L-Phg-based (*S*)-**14a** may have in the cell.

Other haloenol morpholinones were found to have weaker inhibitory activities including the L-Pha- and Gly-derived analogs **16** and **17**, respectively. Surprisingly, chemosensitivity for the iodoenol derivative **15** was also considerably lower than its bromoenol counterparts **14**. Conversely, the N-benzyl L-Phg-based analog **20** proved to be the most potent antagonist in the study (IC₅₀ 1–4 μM). The compound demonstrated rapid and sustained inhibitory effects on cell proliferation for both LNCaP and PC-3 cells over the 72 h evaluation period.

To determine if growth inhibition was due to cytostatic or cytotoxic effects by the antagonists, cell viability was assessed by phase-contrast microscopy¹⁸. Comparisons of morphology were made by visual inspection of LNCaP cells following 72 h treatment with *rac*-BEL, *rac*-**4**, (*S*)-**14a**, and (*R*)-**14b** (Figure 3). Exposure to 5 μM of BEL and its monophenyl analog **4** induced little to no morphological changes in cell shape, differentiation, and death compared to the vehicle (DMSO) control. For the morpholinone analogs **14**, apoptosis and/or necrosis was evident at the same concentrations particularly for the (*R*)-enantiomer. It was concluded from these microscopic images that prostate cancer cells had greater chemosensitivity to haloenol morpholinones than to the analogous haloenol pyranones which corroborated the IC₅₀ data.

The inhibitory effects by *rac*-BEL, pyranone **4**, and morpholinones **14** were additionally assessed by monitoring changes in the cell cycle by flow cytometry with propidium iodide^{6b} (Figure 3). Moderate increases of LNCaP cell counts in the G1 phase were observed following 24 h treatment with 5 and 10 μM of the test compounds. It is believed that the elevated G1 levels led to the decrease in S and G2/M phase cell percentages and the effects were greatest for **14a** and **14b** which induced complete cell cycle arrest at 10 μM. Likewise, on comparison to cultures treated with the 5 μM of the inhibitors, the increase of cells residing in S phase may have been due to the lack of cells entering the G2/M phase. These results further suggest that the cytotoxic effects of morpholinone-based analogs may be the result of DNA hypoploidy, which is associated with DNA fragmentation and apoptosis. Examples of agents that block mitosis by inhibiting chromosome replication include DNA alkylating agents (e.g. nitrogen mustards) and antagonists of glutathione S-transferase (e.g. α-chloroacetamides¹⁹), which protect cells from oxidative DNA damage.

Lastly, *rac*-BEL, pyranone **4**, and morpholinones **14** were evaluated for their ability to inhibit iPLA₂β from rat kidney. Cytosolic fractions were treated for 0.5 h with 0–100 μM of the compounds prior to inoculation with the arachidonoyl thiophosphatidylcholine, a hydrolysable thioester-containing probe of PLA₂ activity^{6b}. Both *rac*-BEL and its phenyl-substituted analog **4** demonstrated nearly identical efficacy to inhibit the enzyme in a concentration-dependent manner (Figure 4). Inhibitory activity was also noted for (*S*)-**14a** but to a lesser degree compared to pyranone-based antagonists. Little to no effects on iPLA₂β activity was observed for (*R*)-**14b** which correlates to earlier findings^{6,14} that the (*S*)-enantiomer of BEL selectively inhibits cytosolic iPLA₂β while (*R*)-BEL possesses higher affinity for microsomal iPLA₂γ.

In summary, haloenol pyran-2-ones were found to be efficacious inhibitors of prostate carcinoma cell growth and iPLA₂β activity however, as with BEL, a definitive correlation could not be made. Novel haloenol morpholin-2-ones constructed asymmetrically from chiral amino acids were also discovered to be antagonists of cell proliferation. Differences in the effects on the cell cycle and iPLA₂β activity suggested that the morpholinone analogs **14** may have a greater capacity to directly or indirectly cause DNA damage. Glutathione S-

transferase which has a role in protecting DNA from oxidative damage is known to be inhibited by haloenol lactones²⁰ and could be a primary or secondary target for the Phg-based derivatives. Finally, during the course of these studies it became apparent that the chemical instability of the haloenol pyranones and morpholinones would likely preclude them from being viable drug candidates for prostate cancer. Their use as research tools in the study of tumorigenesis and validation of new therapeutic targets may be of great value though to the drug discovery community.

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References and notes

- (a) Rai R, Katzenellenbogen JA. *J Med Chem.* 1992; 35:4150. [PubMed: 1433218] (b) Reed PE, Katzenellenbogen JA. *J Biol Chem.* 1991; 266:13. [PubMed: 1985887] (c) Sofia MJ, Katzenellenbogen JA. *J Med Chem.* 1986; 29:230. [PubMed: 3512826] (d) Daniels SB, Katzenellenbogen JA. *Biochemistry.* 1986; 25:1436. [PubMed: 3964685] (e) Daniels SB, Cooney E, Sofia MJ, Chakravarty PK, Katzenellenbogen JA. *J Biol Chem.* 1983; 258:15046. [PubMed: 6654902] (f) Chakravarty PK, Krafft GA, Katzenellenbogen JA. *J Biol Chem.* 1982; 257:610. [PubMed: 7054169] (g) Krafft GA, Katzenellenbogen JA. *J Am Chem Soc.* 1981; 103:5459. (h) Rando RR. *Science.* 1974; 185:320. [PubMed: 4209261]
- McHowat J, Creer MH. *Curr Med Chem Cardiovasc Hematol Agents.* 2004; 2:209. [PubMed: 15320787]
- (a) Sun GY, Xu J, Jensen MD, Yu S, Wood WG, González FA, Simonyi A, Sun AY, Weisman GA. *Mol Neurobiol.* 2005; 31:27. [PubMed: 15953810] (b) Hoozemans JJM, Veerhuis R, Janssen I, Rozemuller AJM, Eikelenboom P. *Exp Gerontol.* 2001; 36:559. [PubMed: 11250126]
- (a) Farooqui AA, Horrocks LA. *Neuroscientist.* 2006; 12:245. [PubMed: 16684969] (b) Farooqui AA, Ong WY, Horrocks LA. *Pharmacol Rev.* 2006; 58:591. [PubMed: 16968951]
- (a) Song K, Zhang X, Zhao CY, Ang NT, Ma ZMA. *Mol Endocrinol.* 2005; 19:504. [PubMed: 15471944] (b) Ramanadham S, Song HW, Hsu FF, Zhang S, Crankshaw M, Grant GA, Newgard CB, Bao SZ, Ma ZM, Turk J. *Biochemistry.* 2003; 42:13929. [PubMed: 14636061] (c) Ma Z, Wang X, Nowatzke W, Ramanadham S, Turk J. *J Biol Chem.* 1999; 274:9607. [PubMed: 10092647] (d) Ma Z, Ramanadham S, Hu Z, Turk J. *Biochim Biophys Acta.* 1998; 1391:384. [PubMed: 9555100]
- (a) Sun B, Zhang XL, Yonz C, Cummings BS. *Biochem Pharmacol.* 2010; 79:1727. [PubMed: 20171194] (b) Sun B, Zhang X, Talathi S, Cummings BS. *J Pharmacol Exp Ther.* 2008; 326:59. [PubMed: 18441250]
- (a) Song Y, Wilkins P, Hu WH, Murthy KS, Chen J, Lee Z, Oyesanya R, Wu JH, Barbour SE, Fang XJ. *Biochem J.* 2007; 406:427. [PubMed: 17555408] (b) Zhang XH, Zhao C, Seleznev K, Song K, Manfredi JJ, Ma ZA. *J Cell Sci.* 2006; 119:1005. [PubMed: 16492706] (c) Zhang L, Peterson BL, Cummings BS. *Biochem Pharmacol.* 2005; 70:1697. [PubMed: 16226224]
- (a) Cummings BC. *Biochem Pharmacol.* 2007; 74:949. [PubMed: 17531957] (b) Schaloske RH, Dennis EA. *Biochim Biophys Acta.* 2006; 1761:1246. [PubMed: 16973413] (c) Balsinde J, Perez R, Balboa MA. *Biochim Biophys Acta.* 2006; 1761:1344. [PubMed: 16962822] (d) Casas J, Gijon MA, Vigo AG, Crespo MS, Balsinde J, Balboa MA. *J Biol Chem.* 2006; 281:6106. [PubMed: 16407173] (e) Balboa MA, Balsinde J, Dennis EA. *Biochem Biophys Res Commun.* 2000; 267:145. [PubMed: 10623589]
- (a) Balsinde J, Balboa MA, Dennis EA. *J Biol Chem.* 1997; 272:29317. [PubMed: 9361012] (b) Balsinde J, Dennis EA. *J Biol Chem.* 1997; 272:16069. [PubMed: 9195897] (c) Balsinde J, Dennis EA. *J Biol Chem.* 1996; 271:31937. [PubMed: 8943239]
- (a) Hooks SB, Cummings BS. *Biochem Pharmacol.* 2008; 76:1059. [PubMed: 18775417] (b) Xu J, Weng YI, Simonyi A, Krugh BW, Liao Z, Weisman GA, Sun GY. *J Neurochem.* 2002; 83:259. [PubMed: 12423237] (c) Balsinde J, Balboa MA, Li WH, Llopis J, Dennis EA. *J Immunol.* 2000; 164:5398. [PubMed: 10799904] (d) Bonventre JV. *J Lipid Mediat Cell Signal.* 1997; 17:71.

- [PubMed: 9302656] (e) Ramanadham S, Wolf MJ, Li B, Bohrer A, Turk J. *Biochim Biophys Acta*. 1997; 1344:153. [PubMed: 9030192]
11. (a) Hassan S, Carraway RE. *Regul Pept*. 2006; 133:105. [PubMed: 16330112] (b) Saavedra G, Zhang W, Peterson B, Cummings BS. *J Pharmacol Exp Ther*. 2006; 318:1211. [PubMed: 16763094] (c) Bao S, Bohrer A, Ramanadham S, Jin W, Zhang S, Turk J. *J Biol Chem*. 2006; 281:187. [PubMed: 16286468] (d) Longo WE, Grossmann EM, Erickson B, Panesar N, Mazuski JE, Kaminski DL. *J Surg Res*. 1999; 84:51. [PubMed: 10334889] (e) Teslenko V, Rogers M, Lefkowitz JB. *Biochim Biophys Acta*. 1997; 1344:189. [PubMed: 9030195]
12. (a) Cummings BS, McHowat J, Schnellmann RG. *J Pharmacol Exp Ther*. 2000; 294:793. [PubMed: 10945826] (b) Atsumi G, Tajima M, Hadano A, Nakatani Y, Murakami M, Kudo I. *J Biol Chem*. 1998; 273:13870. [PubMed: 9593733]
13. (a) Dong Q, Patel M, Scott KF, Graham GG, Russell PJ, Sved P. *Cancer Lett*. 2006; 240:9. [PubMed: 16182442] (b) Jiang J, Neubauer BL, Graff JR, Chedid M, Thomas JE, Roehm NW, Zhang S, Eckert GJ, Koch MO, Eble JN, Cheng L. *Am J Pathol*. 2002; 160:667. [PubMed: 11839587] (c) Graff JR, Konicek BW, Deddens JA, Chedid M, Hurst BM, Colligan B, Neubauer BL, Carter HW, Carter JH. *Clin Cancer Res*. 2001; 7:3857. [PubMed: 11751475] (d) Yamashita S, Yamashita J, Ogawa M. *Br J Cancer*. 1994; 69:1166. [PubMed: 8198986] (e) Yamashita S, Ogawa M, Sakamoto K, Abe T, Arakawa H, Yamashita J. *Clin Chim Acta*. 1994; 228:91. [PubMed: 7988039] (f) Yamashita S, Yamashita J, Sakamoto K, Inada K, Nakashima Y, Murata K, Saishoji T, Nomura K, Ogawa M. *Cancer*. 1993; 71:3058. [PubMed: 8490834]
14. (a) Kinsey GR, Cummings BS, Beckett CS, Saavedra G, Zhang W, McHowat J, Schnellmann RG. *Biochem Biophys Res Commun*. 2005; 327:287. [PubMed: 15629460] (b) Jenkins CM, Han X, Mancuso DJ, Gross RW. *J Biol Chem*. 2002; 277:32807. [PubMed: 12089145]
15. Dai W, Katzenellenbogen JA. *J Org Chem*. 1991; 56:6893.
16. Chen H, Feng Y, Xu Z, Ye T. *Tetrahedron*. 2005; 61:11132.
17. Cho JH, Kim BM. *Tetrahedron Lett*. 2002; 43:1273.
18. Cummings BS, Schnellmann RG. *J Pharm Exp Therap*. 2002; 302:8.
19. Tsuboi K, Bachovchin DA, Speers AE, Spicer TP, Fernandez-Vega V, Hodder P, Rosen H, Cravatt BF. *J Am Chem Soc*. 2011; 133:16605. [PubMed: 21899313]
20. Wu Z, Minhas GS, Wen D, Jiang H, Chen K, Zimniak P, Zheng J. *J Med Chem*. 2004; 47:3282. [PubMed: 15163208]

Figure 1.
Mechanism of serinase inhibition by haloenol pyran-2-ones.

Figure 2.
Chemical structures of BEL and haloenol morpholinones.

Figure 3. Changes in morphology (left-40X magnification) and cell cycle (right) of LNCaP cells following treatment with *rac*-BEL, *rac*-4, (*S*)-14a, and (*R*)-14b.

Figure 4. Inhibitory effects of *rac*-BEL, *rac*-4, (*S*)-14a, and (*R*)-14b on iPLA₂β activity in rat kidney cytosol in the presence of 4 mM EGTA. Data are represented as the mean ± the S.E.M. of at least 3 separate experiments.

Scheme 1.

Synthesis of unsubstituted and monophenyl BEL analogs.

Scheme 2.

Synthesis of N-propargyl bromoenol morpholin-2-ones **14–17**.

Scheme 3.
Synthesis of N-benzyl bromoenol morpholin-2-one **20**.

Table 1

IC₅₀s (μM) against human prostate cancers after 24, 48, and 72 h exposure to haloenol inhibitors^a

compd	LNCaP			PC-3		
	24	48	72	24	48	72
<i>rac</i> -BEL	13	5	9	34	26	14
2a	10	5	5	19	23	14
2b	9	5	7	32	15	16
<i>rac</i> - 4	31	5	4	27	10	6
(<i>S</i>)- 14a	8	3	3	15	13	5
(<i>R</i>)- 14b	6	6	3	8	6	3
(<i>S</i>)- 15	26	23	20	21	21	25
(<i>S</i>)- 16	41	26	32	33	57	39
17	25	29	28	13	10	7
(<i>S</i>)- 20	3	4	3	4	1	4

^aData represent the calculated IC₅₀ using data assessed 3–5 experiments ran in duplicate using separate passages of cells assessing alteration in MTT staining.