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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 iPLA$_2\beta$.

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Haloenol pyranones[1] 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 (Fig. 1). To date, the most evaluated of these inhibitors is *b*romo*e*nol *l*actone, (*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 Ca^{2+}-independent phospholipases A_2 (iPLA$_2$), which are responsible for the catabolism of membrane glycerophospholipids. Over the last 20 years, BEL has enabled researchers to probe the role of iPLA$_2$ in pathologies involving oxidative stress and inflammation including cardiovascular,[2] Alzheimer's[3] and Parkinson's diseases,[4] diabetes mellitus,[5] and more recently, carcinogenesis.[6,7]

Mammalian cells possess multiple isoforms of iPLA$_2$.[8] The most studied are cytosolic iPLA$_2\beta$, (Group VIA-1 and A-2 PLA$_2$) and the membrane localized iPLA$_2\gamma$ (Group VIB PLA$_2$), which together govern the release of fatty acids 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 1990s researchers began finding evidence that iPLA$_2$ participates in cell signaling,[10] proliferation,[11] 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., PLA$_2$) are in greater abundance in carcinoma cells.[13]

The effects of iPLA$_2\gamma$ and iPLA$_2\beta$ on cell signaling and proliferation have recently been studied by enantiomer-based inhibition[6,14] strategies using (*R*)- and (*S*)-BEL, respectively (Fig. 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 enantiomers.[6] These results suggest that the (*R*)- and (*S*)-conformers could be acting in a

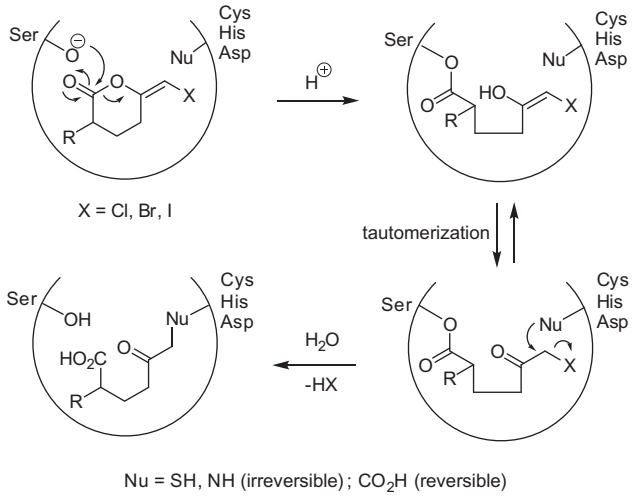

Figure 1. Mechanism of serinase inhibition by haloenol pyran-2-ones.

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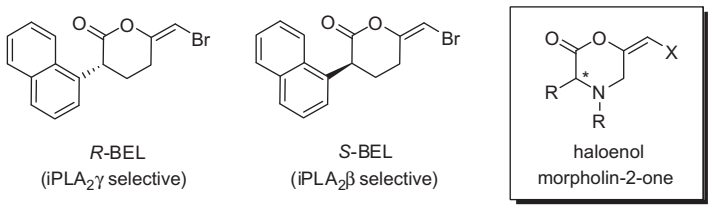
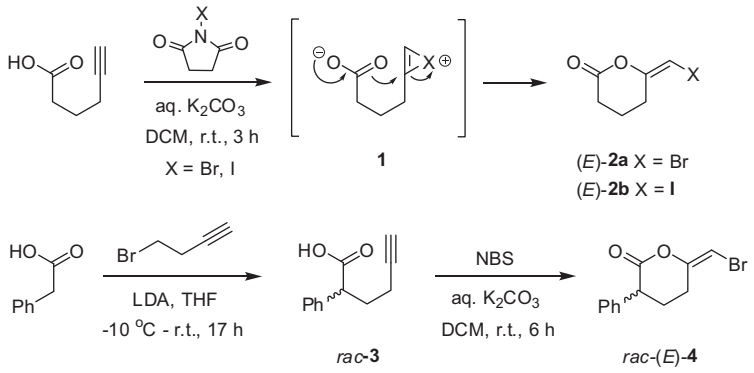


Figure 2. Chemical structures of BEL and haloenol morpholinones.



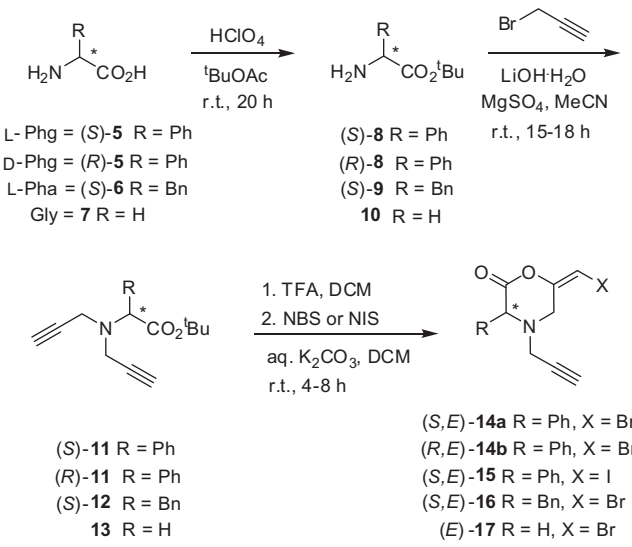
Scheme 1. Synthesis of unsubstituted and monophenyl BEL analogs.

synergistic manner as cell growth inhibitors. The studies further established that enantiomers of haloenol pyranones may be used to selectively and pharmacologically inhibit iPLA$_2\gamma$, iPLA$_2\beta$, 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 iPLA$_2$ activity are described.

To evaluate whether analogs of BEL could have similar inhibitory effects on iPLA$_2$ 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 chemistry.[1g] Subsequent attempts to separate the (*R*)- and (*S*)-enantiomers of lactone **4** by chiral HPLC were unsuccessful, which led us to consider the use of chiral pool amino acids to construct novel iPLA$_2$ inhibitors containing a *E*-haloenol morpholin-2-one framework (Fig. 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 acetate[16] then converted to the 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**.

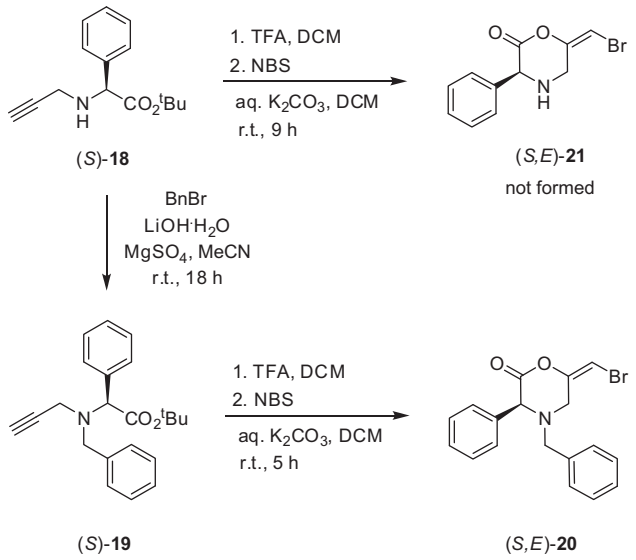
The synthesis of additional L-Phg-based analogs was also attempted from the monopropargyl intermediate **18** (Scheme 3). Benzylation of the secondary amine followed by acid deprotection and cyclization gave the corresponding *N*-benzyl bromoenol morpholin-2-one **20**. Efforts to prepare the unsubstituted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



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

Minimum inhibitory concentrations (IC$_{50}$s) 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, IC$_{50}$ 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 and 14–34 μM in LNCaP and PC-3 cells, respectively, over 72 h, which corroborated previous findings.[6] Activity comparison of BEL to pyranones **2** revealed that the unsubstituted analogs were equally efficacious inhibitors at 5–10 and 14–32 μM for the corresponding cell lines. For the α-substituted phenyl analog **4**, slightly enhanced activities were observed with IC$_{50}$s ranging from 6 to 27 μM against PC-3 cells.

The morpholinones analogs similarly demonstrated antineoplastic activity with IC$_{50}$s reaching 3 μM for the Phg-based



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

Table 1
IC_{50}s (μM) against human prostate cancers after 24, 48, and 72 h exposure to haloenol inhibitors[a]

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

[a] Data represent the calculated IC_{50} using data assessed from 3–5 experiments ran in duplicate using separate passages of cells assessing alteration in MTT staining.

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 (IC_{50} 3–8 μM). 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) that 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 (IC_{50} 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 microscopy.[18] 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** (Fig. 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 IC_{50} data.

The inhibitory effects by *rac*-BEL, pyranone **4**, and morpholinones **14** were additionally assessed my monitoring changes in the cell cycle by flow cytometry with propidium iodide[6b] (Fig. 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-chloroacetamides[19]), which protect cells from oxidative DNA damage.

Lastly, *rac*-BEL, pyranone **4**, and morpholinones **14** were evaluated for their ability to inhibit $iPLA_2\beta$ from rat kidney. Cytosolic fractions were treated for 0.5 h with 0–100 μM of the compounds prior to inoculation with the arachidonoyl thio-phosphatidylcholine, a hydrolysable thioester-containing probe of PLA_2 activity.[6b]

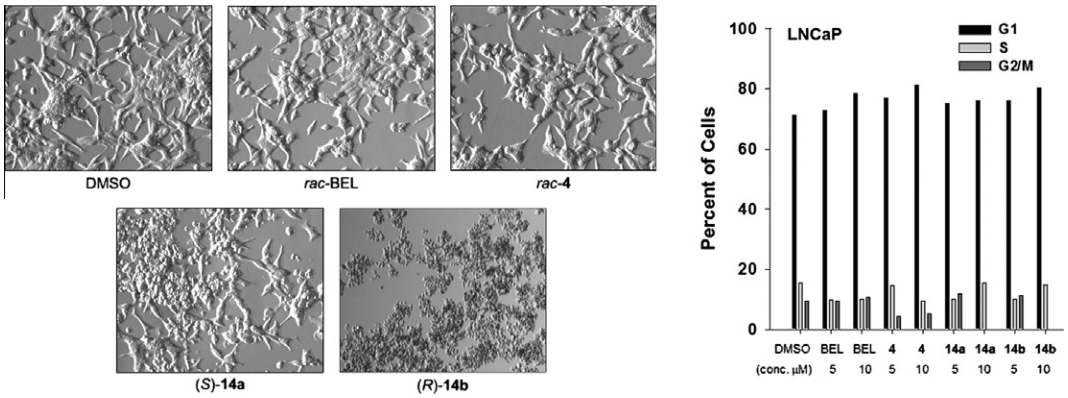


Figure 3. Changes in morphology (left-40× magnification) and cell cycle (right) of LNCaP cells following treatment with *rac*-BEL, *rac*-**4**, (*S*)-**14a**, and (*R*)-**14b**.

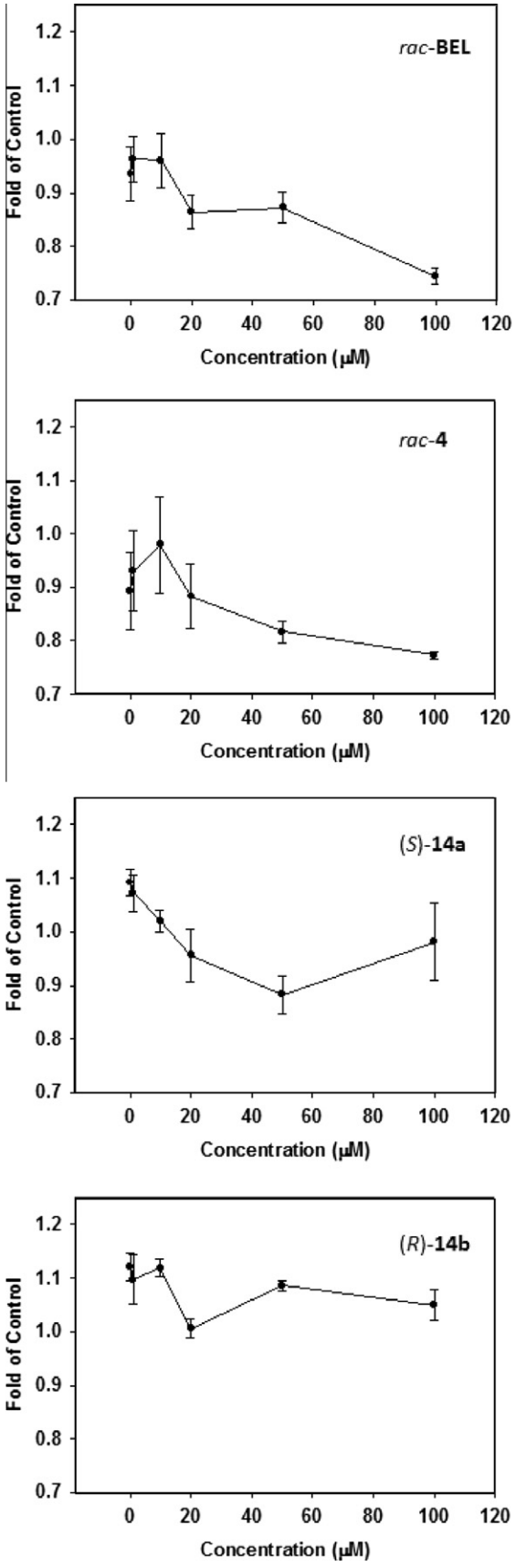


Figure 4. Inhibitory effects of *rac*-BEL, *rac*-**4**, (*S*)-**14a**, and (*R*)-**14b** on iPLA$_2\beta$ activity in rat kidney cytosol in the presence of 4 mM EGTA. Data are represented as the mean ± the S.E.M. of at least 3 separate experiments.

Both *rac*-BEL and its phenyl-substituted analog **4** demonstrated nearly identical efficacy to inhibit the enzyme in a concentration-dependent manner (Fig. 4). Inhibitory activity was also noted for (*S*)-**14a** but to a lesser degree compared to pyranone-based antagonists. Little to no effects on iPLA$_2\beta$ activity was observed for (*R*)-**14b,** which correlates to earlier findings[6,14] that the (*S*)-enantiomer of BEL selectively inhibits cytosolic iPLA$_2\beta$ while (*R*)-BEL possesses higher affinity for microsomal iPLA$_2\gamma$.

In summary, haloenol pyran-2-ones were found to be efficacious inhibitors of prostate carcinoma cell growth and iPLA$_2\beta$ 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 iPLA$_2\beta$ 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 lactones[20] 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.

Acknowledgments

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