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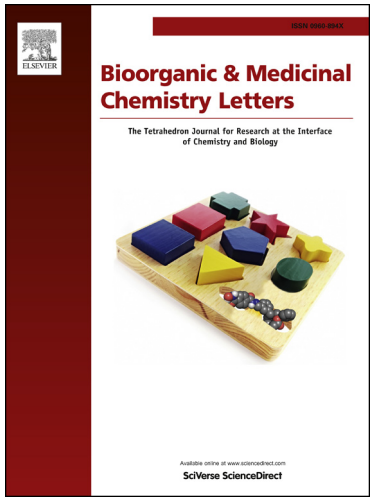

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Factors influencing the cytotoxicity of α-methylene-γ-hydroxy esters against pancreatic cancer

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ABSTRACT

A systematic study to identify the factors influencing the cytotoxicity of α-methylene-γ-hydroxy esters against three pancreatic cancer cell lines (Panc-1, MIA-PaCa-2, and BxPC-3) has established that, in addition to Michael acceptor abilities, the possibility to lactonize to α methylene-γbutyrolactones is as important. The substitution pattern and the number of carbons between the hydroxy and ester moieties also influence the bio-activity.

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Pancreatic adenocarcinoma is the fourth leading cause of cancer-related deaths in the U.S.[1] The late detection of this deadly disease results in only 10% of patients being candidates for surgery, the leading therapeutic approach. The low five year survival rate (7.2%) has remained relatively unchanged over the past few decades despite 5-flourouracil, gemcitabine and oxaliplatin-based regimens, all FDA-approved with therapeutic effects.[2] Accordingly, new chemotherapeutic treatment options, natural and synthetic, are actively being pursued and agents directed at single or multiple targets are being sought urgently.

αMethylene-γbutyrolactones (AMGBLs, Fig. 1. **I**), a key functionality found in a large number of natural products, exhibit a wide range of biological activities including anticancer, anti-inflammatory, antibacterial, anticoagulant, antiviral, and antifungal properties.[3] Four decades ago, molecules bearing these moieties were investigated for their anticancer properties, which were attributed to their Michael-acceptor capabilities.[4] Concerns over the lack of selectivity of the biological Michael reactions and the associated safety issues stalled further investigations with these molecules. The benefits of parthenolide (PT, Fig. 1, **II**), isolated from feverfew (*Tanacetum parthenium*), as a folk medicine and its potential as an anti-cancer agent[5] has contributed to a renewed interest in AMGBLs and related compounds in medicinal chemistry.

We have been investigating synthetic AMGBLs as potential inhibitors of nuclear factor-kappaB (NF-κB)[6] with the goal of arresting the spread of pancreatic cancer.[7] We had reported that substitution at the β and γpositions of AMGBLs with aryl groups is important for increased cytotoxicity. During the course of our investigations, we had noticed that the precursor *α*-methylene-*γ*-hydroxy esters [AMGHEs, Fig. 1, **IV**(**b**)] possessed activity similar to those of the corresponding lactones.[7c,8]

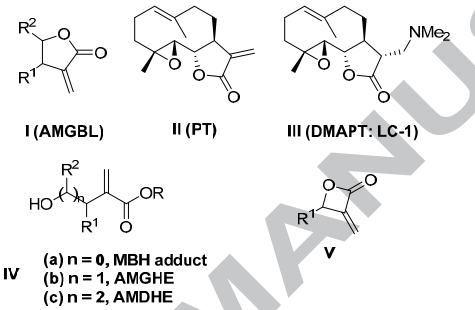

Figure 1. Potential Anti-cancer Agents

We became interested in understanding the origins of the cytotoxicity of AMGHEs. The ease of synthesis of the hydroxy esters and their increased solubility provided the necessary impetus for our study. In addition to the substituents at the β and γpositions, our attention also focused on the hydroxyl and ester moieties to delineate the link between the structure and activity. We suspected that the γhydroxy ester might act as a pro-drug undergoing lactonization in vivo to the methylene lactones, similar to the conversion of the aminolactones to the parent AMGBLs, eg. dimethylaminoparthenolide (DMAPT, Fig. 1, **III**).[9] Our study involving synthesis and bio-assays has revealed that, in addition to Michael acceptor abilities, the possibility to lactonize to αmethylene-γ butyrolactones is a necessary condition for AMGHEs to be cytotoxic. The details follow.

On the basis of the reported Michael acceptor ability of AMGBLs,[4] our initial focus was on similar properties of AMGHEs. A study was begun with methyl αmethylene-βγdiphenylbutanoate (**1a**), prepared using known protocols.[10] When assayed against the three selected pancreatic cell lines (Panc-1, MIA PaCa-2, and BxPC-3, in a triplicate growth assay[11], **1a** revealed cytotoxicity similar to that of the corresponding AMGBL (*cis*-αmethylene-βγdiphenylbutyrolactone, **2a**). This prompted us to examine Morita-Baylis-Hillman (MBH) adducts [Fig. 1, **IV(a)**][12] against the same cells since they also possessed, in addition to the hydroxyl group, a conjugated methylene and ester moieties, the critical units responsible for the Michael addition. Were this the only/key condition necessary for cytotoxicity, the MBH adduct should be suppressing cell growth, similar to **1a**. Our rationale was that if the MBH adduct fail to show any activity, it could be attributed to the additional carbon in **IV(b)** (Fig. 1) that will constitute the backbone of the γlactone. A mining of the literature data revealed that MBH adducts have been examined against several cancer cell lines,[13] although not against pancreatic cancer, with IC_{50} values in the low μM range. Also, silylation of the hydroxyl group had no deleterious effect on the activity. Similarly, we were interested in examining the bio-activity of *α*-methylene-β-propiolactones (Fig 1, **V**) and comparing them to the AMGBLs. To the best of our knowledge, *α*-methylene-β-propiolactones have never been tested against cancer cells.

Accordingly, the MBH adduct **3a** prepared (**Scheme 1**) from benzaldehyde and methyl acrylate was examined against the three pancreatic cancer cell lines. There was no effect of this molecule on the cell growth even at 10 μM concentration.[14] Substituting the 4-position of the phenyl ring in **3a** with an electron-donating methoxy group (**3b**) or an electron-withdrawing cyano (**3c**) or nitro (**3d**) group had no perceivable effect. However, a 4-trifluoromethyl group (**3e**) suppressed growth somewhat, particularly that of the MIA PaCa cells. This prompted the preparation and testing of other fluorinated MBH adducts **3f-j** [(4-F-Ph)-, (3-F-Ph)-, (2-F-Ph)-, C_6F_5-, and (2-CF_3-Ph)-] respectively. However, none of them showed any improvement on the activity (see the table in supporting information for the summary of the results of MBH adducts).

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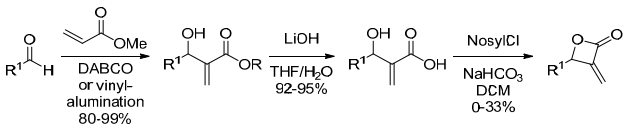
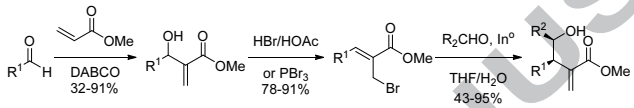


Scheme 1. Synthesis of MBH Adducts and Derivatives

The MBH adducts were saponified with LiOH under mild conditions. However, the lactonization of the βhydroxy acids was successful only for phenyl substituents bearing the 4-CN or 4-NO_2 groups.[15] Both of these αmethylene-βpropiolactones (**4c** and **4d**) were ineffective in suppressing the growth of any of the three pancreatic cells at 10 μM concentration. The failure of the MBH adducts and the corresponding βlactones in inhibiting pancreatic cancer cell growth, despite mildly inhibiting several other cancer cell lines, suggests that a Michael acceptor moiety alone is insufficient to suppress cell growth and other factors in the molecule influence the cytotoxic activity of AMGHEs. The extra carbon in AMGHE appeared critical for cytotoxicity.

Our attention was now diverted to the structure-activity of AMGHEs and a library of substituted methyl 4-hydroxy-2-methylenebutanoates were synthesized from methyl acrylate and two different aldehydes (R^1 and R^2 = H, aliphatic, aromatic, or a tethered aromatic) according to published protocols (**Scheme 2**)[10] The synthesized AMGHEs (**1a-m**), as well as their biological activities at 10 μM concentrations are summarized in Table 1. The assays of PT and **2a** were repeated (Table 1, entries 1 and 2) and selected assays (entries 7, 9, 13, and 17) from our earlier work[7a] are included for comparison.



Scheme 2. Synthesis of substituted α-methylene-γ-hydroxyesters

The current study of the activity of AMGHEs revealed several parallels with that of our reported study on AMGBLs.[7a] AMGHEs without substitution at the γposition (**1b-d**) resulted in poor activity (entries 4-6). The cytotoxicity is poor if substituents at the both β and γpoisitions are aliphatic groups (**1f-g**, entries 10-11). Among the disubstituted hydroxy esters, a phenyl group at either the β- or γ-position revealed better activity, especially against MIA PaCa-2 and BxPC-3 (**1a, 1h-l**, entries 12, 14-16**,** 18). Except for lactones **2d** and **2h**, the hydroxy esters are slightly better than the lactones at suppressing cell growth (**1a/2a**, **1d-e/2d-e**, **1h/2h**, and **1k/2k**, entries 2-3, 6-9, 12-13, and 16-17). Lactones **2e** and **2k** (entries 9 and 17) are almost completely inactive while the corresponding hydroxy esters, **1e** and **1k** (entries 8 and 16), show relatively good activity. Interestingly, an aliphatic tether could be inserted with no significant loss of activity (**1m**, entry 19). Substituted aromatics were not pursued in this study with the hydroxy esters since we expected their activity to be similar to those of the corresponding lactones.

The similarity in the activity of both the open and cyclic esters supported our assumption that AMGHEs might be undergoing cyclization *in vivo* before acting on the cells. Suppressing the cyclization was then targeted to substantiate this hypothesis. The literature had reported that protecting the hydroxyl group of the MBH adducts with a silyl group did not affect the bio-activity.[13] The γ-hydroxyl group of the AMGHE **1a** was protected as a methyl and TBS-ether (**1a'** and **1a"**, respectively). Methylation was carried out in the presence of silver oxide[16] and TBS-protection was achieved with excess TBSCl and imidazole. Surprisingly, the cytotoxic activity of **1a** was completely blocked in ethers **1a'** and **1a"** (**Table 2**, entries 1 and 2), backing our hypothesis that cyclization to AMGBL might be a necessary condition for bio-activity. Additional support was sought by modifying the ester moiety. Rather than carrying out the synthesis with different acrylates to prepare the corresponding AMGHEs, we resorted to saponification of the **1a"**, followed by Steiglich esterification[17] with the corresponding alcohols and removal of the silyl group. Thus, the ethyl, benzyl, and phenyl esters (**5-7**) were prepared and examined. There is little effect of the ester moiety in suppressing cell growth (**Table 2**, entries 3-5).

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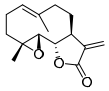
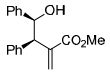
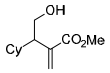
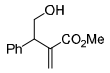
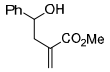
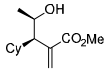
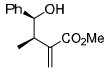
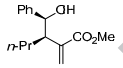
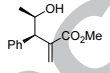
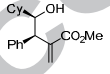
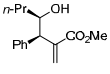
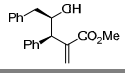
Table 1.
Cytotoxic activity of α-methylene-γ-hydroxy esters against Panc-1, MIA PaCa-2, and BxPC-3 cell lines.

			% Cell Growth		
Entry	Structure	(#)	Panc-1	MIA PaCa-2	BxPC-3
1		**PT**	22	11	12
2		**2a**	12	9	3
3		**1a**	14	9	2
4		**1b**	120	124	126
5		**1c**	164	98	86
6		**1d**	102	29	60
7		**2d**[a]	31	14	40
8		**1e**	67	14	1
9		**2e**[a]	135	106	136
10		**1f**	110	147	122
11		**1g**	145	77	120
12		**1h**	91	32	49
13		**2h**[a]	62	14	41
14		**1i**	104	32	38
15		**1j**	96	24	27
16		**1k**	83	29	23
17		**2k**[a]	104	81	95
18		**1l**	33	16	8
19		**1m**	14	16	6

a) Results from ref 7a.

In support of our hypothesis that the AMGHEs are acting as prodrugs, converting themselves to AMGBLs in vivo, we could predict that the corresponding αmethylene-γhydroxy ketone to be inactive, in spite of its increased Michael acceptor properties.[18] Accordingly, we prepared 5-hydroxy-3-methylene-4,5-diphenylpentan-2-one (**8**) using a procedure similar to the one in Scheme 2,

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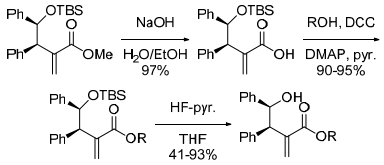
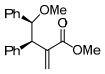
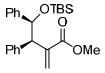
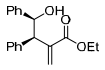
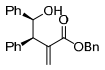
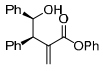
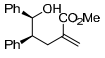
starting with methyl vinyl ketone in place of methyl acrylate. Expectedly, the hydroxy ketone **8** showed no cytotoxicity (**Table 2**, entry 6), confirming that 1,4-addition alone is insufficient for suppressing cell growth.

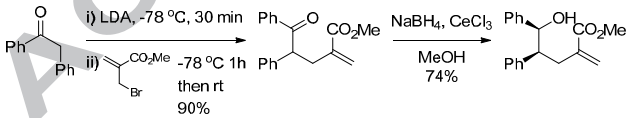


Scheme 3. Synthesis of various esters

Table 2.
Anti-pancreatic cancer activities of α-methylene carbonyls against Panc-1, MIA-PaCa-2, and BxPC-3 cell lines.

			% Cell Growth		
Entry	Structure	(#)	Panc-1	MIA PaCa-2	BxPC-3
1		**1a'**	123	102	94
2		**1a"**	135	110	117
3		**5**	5	13	3
4		**6**	6	12	2
5		**7**	9	8	1
6		**8**	105	53	104
7		**9**	124	59	129
8		**10**	105	87	117

The effectiveness of AMGHEs might be attributed to the binding of the α-methylene-γ-lactones, formed *in vivo*, to the active site. The failure of α-methylene-β-hydroxy esters (MBH adducts) to suppress growth compared to the corresponding γ-hydroxy esters persuaded us to compare the δ-hydroxy esters as well. A representative δ-hydroxy ester, methyl 5-hydroxy-2-methylene-4,5-diphenylpentanoate (**9**, Table 2, entry 7) was synthesized from benzyl phenyl ketone via allylation followed by Luche reduction (**Scheme 4**). Cell proliferation assay revealed poor activity. The δ-hydroxy ester should be kinetically very slow to lactonize as compared to the γ-hydroxy ester, which might be responsible for the poor activity. We had recently demonstrated this to be the case when a δ-hydroxy ester did not undergo aminolactonization, contrary to γ-hydroxy esters.[7c]



Scheme 4. Synthesis of δ-hydroxy ester

In our earlier report, we had shown that, compared to AMGBLs, an α-ethylidene-γ-hydroxy lactone is inactive for growth suppression of pancreactic cancer cells.[7a] This was attributed to decreased Michael acceptor capabilities. In this study, we prepared a benzylidene-γ-lactone (*cis*-α-benzylidene-β,γ-diphenylbutanoate, **10**) and examined its activity.[19] The benzylidene moiety is slightly electronically activated, but is sterically hindered, so it is not surprising that it is inactive (**Table 2**, entry 8).

In conclusion, we have demonstrated that the biological activity of α-methylene hydroxy esters depends on several factors. In addition to Michael acceptor properties, the ability to cyclize to the corresponding lactone appears to be crucial for the activity.

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Additionally, the substitution pattern and the number of carbons are both critical to obtain good activity. The hydroxy esters are easier to prepare and offer improved solubility over the lactones. Further work to better understand the activity of these compounds, and to optimize the structures is ongoing.

Acknowledgments

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Factors influencing the cytotoxicity of α-methylene-γ-hydroxy esters against pancreatic cancer

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