



Synthesis and differential antiproliferative activity of Biginelli compounds against cancer cell lines: Monastrol, oxo-monastrol and oxygenated analogues

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Abstract

The synthesis and differential antiproliferative activity of monastrol (**1a**), oxo-monastrol (**1b**) and eight oxygenated derivatives **3a,b–6a,b** on seven human cancer cell lines are described. For all evaluated cell lines, monastrol (**1a**) was shown to be more active than its oxo-analogue, except for HT-29 cell line, suggesting the importance of the sulfur atom for the antiproliferative activity. Monastrol (**1a**) and the thio-derivatives **3a**, **4a** and **6a** displayed relevant antiproliferative properties with 3,4-methylenedioxy derivative **6a** being approximately more than 30 times more potent than monastrol (**1a**) against colon cancer (HT-29) cell line.

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1. Introduction

In recent years, dihydropyrimidinones and their derivatives have occupied an important place in natural and synthetic organic chemistry mainly due to their wide range of biological activities [1,2], notably as calcium channel blockers [3,4]. Additionally, the structurally related marine alkaloids batzelladine A and B were shown to be the first low molecular weight natural products to inhibit the binding of HIV gp-120 to CD4 cells, so disclosing new vistas towards the development of AIDS therapy [5].

More recently, ethyl 4-(3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate, also known as monastrol (**1a**), was identified as a novel low molecular weight cell-permeable molecule for the development of potentially new anticancer drugs [6]. This compound specifically affects the cell division (mitosis) by a new mechanism, which does not involve the binding to tubulin in contrast with the natural taxanes, vinca alkaloids and epothilones. It has been established that the activity of monastrol is based on the specific and reversible inhibition of the motility of mitotic kinesin Eg5, a motor protein required for bipolar spindle formation during mitosis [7–11].

Moreover, Maliga et al. have demonstrated that monastrol inhibits the motor activity of Eg5 by inhibiting ATP hydrolysis through an allosteric mechanism, whereas the corresponding 4-hydroxyphenyl derivative is a weak Eg5 inhibitor and that (*S*)-monastrol [(*S*)-(**1a**)] is the biologically active enantiomer, indicating a more potent and specific Eg5 inhibitor [12].

Although many reports have been dedicated to elucidate the mechanism of action of monastrol as mitotic inhibitor in the cell cycle [13–15], few examples concerning the anticancer activity [16–19] were reported. Recently, Leizerman and coworkers described the differential effects of monastrol on AGS and HT-29 cell lines in comparison with taxol [20].

Due to these reasons, we decided to investigate firstly the differential antiproliferative activity of monastrol (**1a**) and its oxo-analogue, named oxo-monastrol (**1b**), as well as the thio-analogues **2a–6a** and the corresponding oxo-analogues **2b–6b** (all compounds in the racemic form) on seven human cancer cell lines (Fig. 1).

2. Material and methods

2.1. General

All reagents and solvents were obtained from commercial suppliers and used without further purification except for THF and CH₃CN which were dried from sodium/benzophenone and calcium hydride, respectively, prior to use. Melting points were recorded on Melting Point Electrothermal IA-9000 apparatus and are uncorrected. ¹H NMR and ¹³C NMR spectra were recorded on a Varian VXR 200 spectrometer in DMSO-*d*₆ with all chemical shifts reported in ppm relative to internal TMS (δ 0.0) or with the solvent reference relative to TMS (DMSO-*d*₆, δ 2.50 ppm for ¹H NMR and δ 39.5 ppm for ¹³C NMR). The IR spectra were recorded on a Perkin–Elmer FT/IR-1600 spectrophotometer using KBr optics or neat.

2.2. Typical procedure for the synthesis of the 3,4-dihydropyrimidinones **1a,b–6a,b**

A 50 mL round-bottomed flask was charged with 3.6 mmol of urea or thiourea, 3.0 mmol of aldehyde, 3.0 mmol of ethyl acetoacetate, 0.6 mmol of the specified Lewis

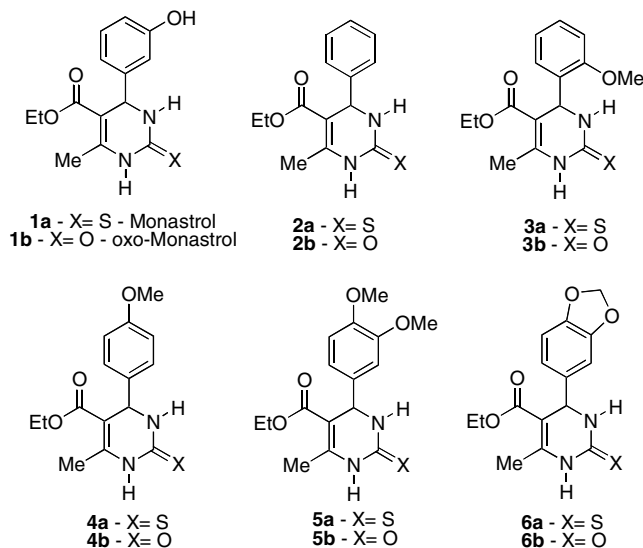


Fig. 1. Monastrol (**1a**), oxo-Monastrol (**1b**), thio-analogues (**2a–6a**) and oxo-analogues (**2b–6b**).

Table 1

Synthesis of dihydropyrimidin-2(1*H*)-ones **1–6** under different Lewis acids catalyst

Entry	Solvent ^a	Time (h)	Compound	Yield (%)	Reference
1	CH ₃ CN	4	1a	81	[40]
2	CH ₃ CN	4	1b	86	[44]
3	THF	4	1a	65	
4	THF	4	1b	75	
5	CH ₃ CN	6	2a	90	[43]
6	CH ₃ CN	6	2b	97	[41]
7	CH ₃ CN	6	3a	65	
8	CH ₃ CN	6	3b	79	[42]
9	CH ₃ CN	6	4a	73	[35]
10	CH ₃ CN	6	4b	88	[44]
11	CH ₃ CN	6	5a	89	[32]
12	CH ₃ CN	6	5b	95	[31]
13	CH ₃ CN	6	6a	65	[45]
14	CH ₃ CN	6	6b	90	[31]

^a Previously dried.

acid and 4.0 mL of the appropriate solvent (Table 1). The mixture was heated under reflux and magnetic stirring for 4–8 h. The reaction mixture was cooled to room temperature and 10 mL of cold water was added and the mixture was additionally stirred for 15–20 min. The resulting solid formed was filtered under suction, washed with cold ethanol (3 mL) and recrystallized from hot ethanol (for **1b**, **2a,b–6a,b**) or purified by silica gel column chromatography (hexanes-ethyl acetate 75:25, for **1a**) to afford the desired products.

2.2.1. Ethyl 4-(2-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (**3a**)

Solid, m.p. 199–201 °C

¹H NMR (200 MHz, DMSO-*d*₆): δ 10.20 (s, 1H); 9.21 (bs, 1H); 7.28–6.86 (m, 4H); 5.49 (d, *J* = 3.3 Hz, 1H); 3.94 (q, *J* = 6.9 Hz, 2H); 3.79 (s, 3H); 2.30 (s, 3H); 1.04 (t, *J* = 6.9 Hz).

¹³C NMR (50 MHz, DMSO-*d*₆): δ 174.0, 165.0, 156.5, 144.9, 130.5, 129.0, 127.6, 120.1, 111.2, 99.3, 59.3, 55.4, 49.4, 17.0, 14.0.

IR (neat): 3346, 3257, 3161, 2945, 1707, 1649, 1615, 1577, 1489 cm⁻¹.

2.3. Bioassays

Human tumour cell lines of different histological origins were used: UACC62 (melanoma), MCF-7 (breast), OVCAR03 (ovarian), PC0 3 (prostate), HT-29 (colon), 786-0 (renal) and NCI-ADR (breast expressing phenotype multiple drugs resistance) which were kindly provided by Frederick Cancer Research & Development Center—National Cancer Institute—Frederick, MA, USA.

Stock cultures were grown in a medium containing 5 mL of RPMI 1640 (Gibco-BRL, Life Technologies) and supplemented with 5% of fetal bovine serum.

Gentamicine (50 µg/mL) was added to the experimental cultures. Cells distributed in 96-well plates (100 µL cells/well) were exposed to various concentrations of 3,4-dihydropyrimidin 2(1*H*)-ones **1–6** in DMSO (0.25, 2.5, 25 and 250 µg/mL) at 37 °C, 5% of CO₂ in air for 48 h.

The final concentration of DMSO did not affect the cell viability. Then, a 50% of trichloroacetic acid solution was added and after incubation for 30 min at 4 °C, washing and drying, the cell proliferation was determined by spectrophotometric quantification (540 nm) of the cellular protein content using sulforhodamine B assay described by Skehan et al. [21].

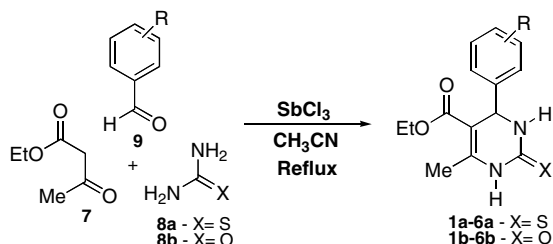
3. Results and discussion

3.1. Chemistry

The original Biginelli's reaction is a three-component reaction between ethyl acetoacetate (**7**), urea (**8a**) or thiourea (**8b**) and an aldehyde (**9**), under Brønsted acidic catalysis that affords 3,4-dihydropyrimidin 2(1*H*)-ones [22]. However, this reaction suffers from the harsh conditions, long reaction times and frequently low yields.

Although there are many methods for the preparation of dihydropyrimidinones [1,23,24], we are particularly interested in those based on a multicomponent process which allows to rapidly accessing a large number of derivatives based on a short and simple protocol. Chiral versions of multi-step [25] or multicomponent [26–29] synthesis of dihydropyrimidinones were recently reported.

The use of Lewis acids as catalyst has been extensively explored [30–36] and recently we have demonstrated that the 3,4-dihydropyrimidin-2(1*H*)-ones can be easily synthesized by the multicomponent cyclocondensation of ethyl acetoacetate (**7**), urea (**8a**) and aldehydes **9** under SnCl₂ · 2H₂O [37] and In(OTf)₃ [38] catalysis.



Scheme 1. Synthesis of dihydropyrimidin-2(1H)-ones by the multicomponent Biginelli's reaction.

In the same line of previous works, we report in this paper the use of SbCl_3 as a new Lewis acid catalyst to promote the Biginelli's reaction to prepare monastrol (**1a**) and oxo-monastrol (**1b**), as well as, the analogues **2a,b-6a,b** (Scheme 1).

Although high yields have been achieved carrying out the reactions in a protic solvent such as EtOH, we preferred the use of anhydrous CH_3CN or THF to avoid a possible decomposition of the catalyst.

In general, the yields of the oxo-derivatives **1b-6b** (75–97%) were higher than for the thio-derivatives **1a-6a** (65–89%), as shown in Table 1.

The structural assignments for compounds **1a,b, 2a,b, 3b, 4a,b, 5a,b** and **6a,b** were based on comparison with literature data while the spectral data for compound **3a** are shown for the first time.

For our preparative purposes, these conditions were employed as the standard experimental protocol for the preparation of compounds **1a,b-6a,b** in good to excellent yields securing sufficient amounts of dihydropyrimidin-2(1H)-ones for biological tests.

3.2. Biological activities

3.2.1. Antiproliferative effect

Initially, we evaluated the antiproliferative activities of monastrol (**1a**) and oxo-monastrol (**1b**) against the following human cancer cell lines: MCF-7 (breast), NCI-ADR (breast expressing the resistance phenotype for Adriamycin), UACC.62 (melanoma), 786-0 (kidney), OVCAR03 (ovarian), PCO.3 (prostate), and HT-29 (colon), which were grown in vitro [39]. Chemotherapeutic doxorubicin (DOX) was used as the positive control [21].

Monastrol (**1a**) displayed significant cytostatic activity (growth inhibition of the initial cell number after 48 h) and moderate to relevant cytotoxic activity (reduction of the initial cell number after 48 h) for UACC.62 and 786-0 cell lines at 250 $\mu\text{g/mL}$ (Fig. 2A) while **1b** has shown only cytostatic activities for all cell lines, as depicted in Fig. 2B.

The percentage values for the growth inhibition of the cell proliferation at four different concentrations are shown in the Table 2. At 0.25 $\mu\text{g/mL}$ and 2.5 $\mu\text{g/mL}$, the cell growth inhibition was low for both compounds **1a** and **1b** becoming more important at 25 $\mu\text{g/mL}$ of **1a**, particularly for PCO.3 (Table 2, entry 3), MCF-7 (entry 6), 786-0 (entry 2), UACC.62 (entry 1), and NCI-ADR (entry 4). At that same concentration, oxo-monastrol (**1b**) presents much lower cytostatic activity for all cell lines, except for OVCAR03 (Table 2, entry 7).

However, at concentration of 250 $\mu\text{g/mL}$, monastrol (**1a**) presented relevant and moderate cytotoxic activity for UACC.62 (entry 1) and 780-0 (entry 2) cell lines, respectively,

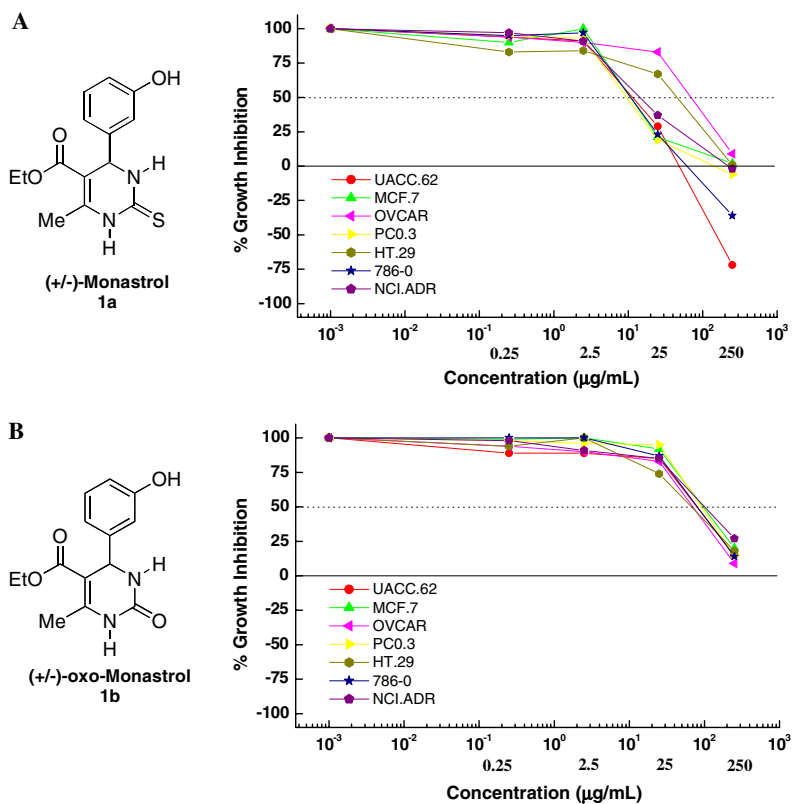


Fig. 2. Effect of monastrol (A) and oxo-monastrol (B) on the growth of different cancer cells lines. Positive values in relation to y axis correspond to cytostatic activity while negative values refer to cytotoxic activity. Data were obtained from a representative experiment done in triplicate.

Table 2

Comparison of antiproliferative activity of monastrol (**1a**) and oxo-monastrol (**1b**) against cancer cell lines as percentage of growth inhibition

Entry	Cell lines	Monastrol (1a) [$\mu\text{g/mL}$]				oxo-Monastrol (1b) [$\mu\text{g/mL}$]			
		0.25	2.5	25	250	0.25	2.5	25	250
1	UACC.62	6	9	71	-72	0	11	15	85
2	786-0	0	3	77	-36	0	0	13	86
3	PCO.3	5	7	81	-6	2	4	5	85
4	NCI ADR	3	9	63	-2	2	9	15	73
5	HT-29	7	16	33	99	0	0	26	82
6	MCF-7	0	0	79	98	0	0	8	80
7	OVCAR03	6	10	17	91	6	10	17	91

while at the same concentration oxo-monastrol (**1b**) exhibited only cytostatic activity for all cancer cell lines investigated.

Since the difference in viability on UACC.62 cells at 250 $\mu\text{g/mL}$ (Table 2, entry 1) was higher for monastrol (**1a**), we took microscopic images of the UACC.62 cells treated with

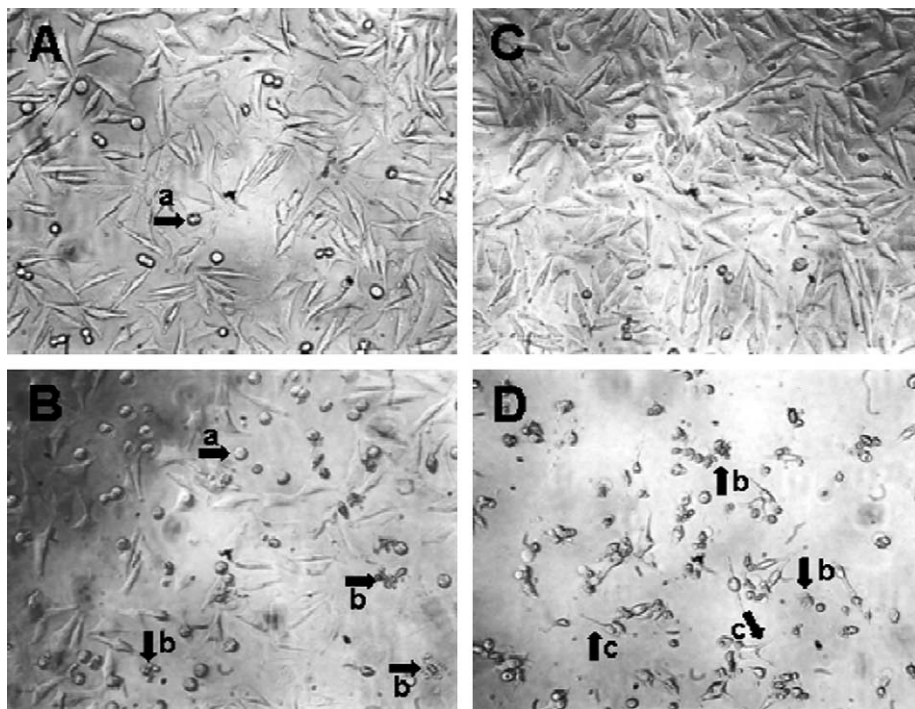


Fig. 3. Morphological characteristics of UACC.62 cells visualized with a phase-contrast microscope. (A) Monastrol-treated cells (25 $\mu\text{g}/\text{mL}$) showing many detached cells after 6 h. (B) Monastrol-treated cells (250 $\mu\text{g}/\text{mL}$) with apoptotic bodies and blisters after 6 h. (C) Non-confluent untreated cells displaying normal morphology. (D) Monastrol-treated cells (250 $\mu\text{g}/\text{mL}$) exhibiting apoptotic bodies after 12 h.

monastrol (**1a**) at that concentration after 6 h and 12 h in order to carry out morphological analysis of the cells (Fig. 3). After 6 h, treatment of melanoma cells (UACC.62) with 25 $\mu\text{g}/\text{mL}$ of monastrol (**1a**) (Fig. 3A) triggered the appearance of many detached cells (**a**).

After treatment with 250 $\mu\text{g}/\text{mL}$ of monastrol (**1a**) for 6 h, the cells exhibited small vesicles called apoptotic bodies and blisters which often appear towards the end of the apoptotic process (Fig. 3B). In contrast, untreated UACC.62 cells exhibited typical patterns and a smooth flattened morphology (Fig. 3C).

After 12 h at 250 $\mu\text{g}/\text{mL}$ (Fig. 3D), apoptotic bodies (**b**) and elongated lamellipodia (**c**) were observed. In both treatments above (Fig. 3B and D), we have observed significant reduction in UACC.62 cells density and evidence of cell death, following the apoptotic process [40].

On the other hand, treatment of UACC.62 cells with oxo-monastrol (**1b**) (25 or 250 $\mu\text{g}/\text{mL}$) did not alter the cell morphology even after 12 h of treatment. The chemotherapeutic doxorubicin (DOX) was used as positive control. These results indicated the direct influence of the sulfur atom on the cytotoxic activity of monastrol (**1a**) as its substitution by an oxygen atom in oxo-monastrol (**1b**) led to the loss of the cytotoxic activity probably due to the soft nature of the sulfur atom which makes monastrol (**1a**) a better nucleophile than oxo-monastrol (**1b**).

Table 3

Comparison of antiproliferative activity of monastrol analogues **2–6** at 250 µg/mL against cancer cell lines as percentage of growth inhibition

Entry	DHPMs	UACC.62	786-0	PCO.3	NCI ADR	HT-29	MCF-7	OVCAR03
1	2a	90	48	44	31	28	23	42
2	2b	72	33	58	53	8	47	61
3	3a	–78	90	95	–17	78	–31	–29
4	3b	90	74	31	–2	89	72	–50
5	4a	–87	–74	–28	–21	–42	–73	–37
6	4b	67	30	17	30	30	2	24
7	5a	–1	52	–57	89	55	50	60
8	5b	57	16	30	16	8	27	22
9	6a	–85	–64	–12	–24	–38	–43	–3
10	6b	73	43	75	71	52	61	84

To gain additional information on the influence of oxygenated substituents in the aromatic ring of the dihydropyrimidin-2(*1H*)-ones, we evaluated the antiproliferative activities of compounds **2a,b–6a,b** under the same conditions described above.

The antiproliferative activities of compounds **2a,b–6a,b** at 250 µg/mL are depicted in Table 3.

In fact, monastrol (**1a** see Table 2) was more potent than its deoxy derivative (**2a**) for all cell lines evaluated, showing that the hydroxyl group in the aromatic ring is essential for the cytotoxic activity.

However, the presence of the hydroxyl group seems not to be the best option, as can be observed for the derivative compounds **3a**, **4a** and **6a** (2-methoxy, 4-methoxy and 3,4-methylenedioxy, entries 3,5 and 9, respectively) which were more potent than monastrol (**1a**) for all investigated cell lines, except for the compound **3a** on the 786-0, PCO.3 and HT-29 cell lines. Particularly relevant were the cytotoxic activities of these compounds on UACC.62 cell line (78–87%).

3.2.2. The IC_{50} values

All the other compounds listed in Table 3 did not show significant cytotoxic activity, therefore, IC_{50} values were determined only for compounds **1a**, **3a**, **4a** and **6a** using a

Table 4

Comparison of IC_{50}^a values of monastrol (**1a**), oxygenated derivatives **3a**, **4a** and **6a** and doxorubicin(DOX)^b against cancer cell lines

Compound	IC_{50} (µg/mL) ^a -cancer cell line						
	UACC.62	786-0	PCO.3	NCI-ADR	HT-29	MCF-7	OVCAR03
1a	49.9	25.0	12.3	19.6	>100	12.9	33.5
3a	ND ^c	ND ^c	ND ^c	73.3	ND ^c	ND ^c	>100
4a	95.1	>100	59.6	67.9	>100	ND ^c	67.2
6a	6.0	2.0	21.4	37.2	2.5	1.9	6.6
DOX	3.24	0.4	18.0	52.7	3.4	4.9	5.0

^a IC_{50} values (concentration that elicits 50% inhibition) were determined from non-linear regression analysis by GraphPad Prism software ($r^2 > 0.9$).

^b Doxorubicin (DOX) was employed as positive control.

^c Value not determined.

non-linear regression analysis by GraphPad Prism software, using doxorubicin as a positive control (see Table 4).

The IC₅₀ values for derivatives **3a** (UACC.62, 786-0, PCO.3, HT-29 and MCF-7 cell lines) and **4a** (MCF-7 cell line) could not be determined by this method due to the linear behavior observed for the concentration versus growth inhibition curve.

As shown in Table 4, 3,4-methylenedioxy derivative **6a** was more potent than monastrol (**1a**) in all cases, except for PCO.3 and NCI ADR cell lines, with a remarkable difference in cytotoxic activity being observed for the HT-29 cancer cell line, with derivative **6a** being approximately more than 30 times more potent than monastrol (**1a**).

4. Conclusion

The synthetic approach described here for the synthesis of monastrol (**1a**), oxo-monastrol (**1b**) and analogues **2ab–6ab** by SbCl₃ as Lewis acid promoter of Biginelli's reaction represents a one-step synthesis of these biologically active heterocycles amenable to provide these compounds in sufficient amounts for the assays.

Monastrol (**1a**) displayed antiproliferative activity against the cancer cell lines tested at 250 µg/mL, particularly against the UACC.62 cell line, while oxo-monastrol (**1b**) did not show any cytotoxic activity in the same concentration.

The morphological analysis of the UACC.62 cells treated with monastrol (**1a**) at 250 µg/mL using phase-contrast microscopy showed significant changes in the cell morphology, which were not observed when oxo-monastrol (**1b**) was employed in the same concentration.

In addition, we have identified the DHPM analogue **6a** as a more potent cytotoxic agent than monastrol (**1a**) against melanoma (UACC.62), kidney (786-0), breast (MCF-7), ovarian (OVCAR03) and, particularly, colon (HT-29) cancer cell lines.

Acknowledgments

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