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Article

Synthesis and Antiproliferative Activity of Novel Limonene Derivatives with a Substituted Thiourea Moiety

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No presente trabalho descrevemos a síntese e a avaliação da atividade antiproliferativa, frente a linhagens de células tumorais humanas, de derivados do *R*-(+)-limoneno (**3**-**18**) contendo uma unidade tiouréia substituída. Os derivados com substituintes arílicos (**3**-**6**) exibiram atividade citostática frente a todas linhagens testadas, com inibição de 50% do crescimento celular (GI_{50}) em concentrações na faixa de 2,5 a 24 μmol L^{-1}. Os compostos **3**, **10**, **12** e **16** foram os mais ativos, com GI_{50} na faixa de 0,41 a 3,0 μmol L^{-1}, frente a diferentes linhagens celulares.

A series of *R*-(+)-limonene derivatives bearing a substituted thiourea moiety (**3-13**) and five S-methyl analogs (**14-18**) were synthesized and evaluated for their *in vitro* antiproliferative activity against human cancer cell lines. Compounds bearing aromatic substituents (**3-6**) exhibit cytotastic activity in the full panel of cell lines tested, with GI_{50} values in the range of 2.5 to 24 μmol L^{-1}. Compounds **3**, **10**, **12** and **16** were the most active with GI_{50} values in the range of 0.41 to 3.0 μmol L^{-1}, against different cell lines.

Keywords: limonene derivatives, thioureas, antiproliferative activity

Introduction

In the last years several approaches have been employed for cancer therapy, and to discover and develop novel therapeutic agents for the treatment of malignancy. In this context, the use of natural products as prototypes has been pointed out as one of the successful approaches to discover novel anticancer drugs.

Monoterpenes are a class of compounds, which occurs naturally in plant, and possess a range of pharmacological properties. Several studies have demonstrated the efficacy of this class of compounds as potential anticancer agents.[1-3] D-Limonene, a monoterpene found in a variety of foods and essential oils, have been shown to exert chemopreventive and chemotherapeutic activities in a variety of carcinogen-induced animal tumor models.[4-7] Dietary administration of D-limonene causes complete regression of N-nitrosourea (NMU)-induced and 7,12-dimethylbenzyl[a]anthracene (DMBA)-induced mammary carcinomas with minimal toxicity.[8,9]

As a result of its chemopreventive and chemotherapeutic potential, limonene is under clinical trials. Phase I and pharmacokinetic study in patients with advanced cancer confirm its low toxicity and support D-limonene as prototype of a novel class of chemotherapeutic drugs.[10]

The potential use of limonene as an anticancer agent led us to focus our attention on the synthesis and antiproliferative activity evaluation of new limonene derivatives, as part of our research program in this area. We present herein the synthesis and the results of the antiproliferative activity evaluation of a series of limonene derivatives (**3**-**13**) bearing a substituted thiourea moiety, and of five their S-methyl analogues (**14**-**18**).

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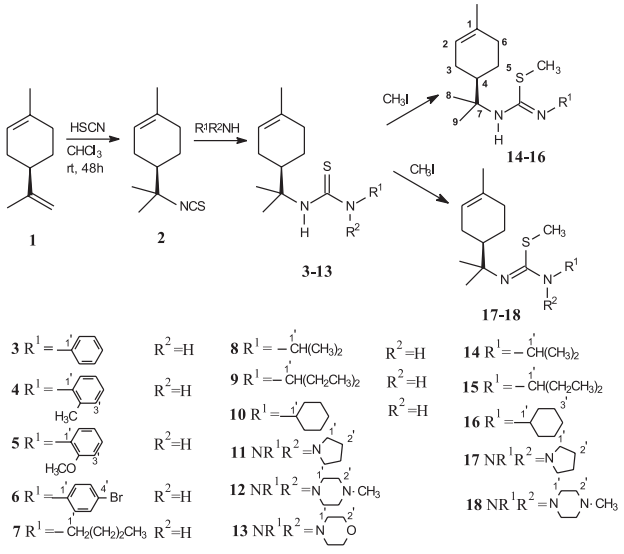
Results and Discussion

Synthesis

The synthetic route for the preparation of limonene derivatives is presented in Scheme 1. The limonene isothiocyanate (**2**) was obtained from the reaction of the limonene (**1**) with HSCN, in chloroform, according to a previously reported procedure.[11,12] Treatment of **2** with different amines (primary, heterocyclic and aromatic) afforded compounds **3**-**13**. For primary and heterocyclic amines the reactions were carried out at 25 °C for 15 h, by using 2 equivalents of the amine in chloroform as solvent, and the title thioureas were obtained in 67 – 97% yield. However, limited yields (20-30%) were observed for the reaction of **2** with aromatic amines such as aniline, *o*-toluidine, *o*-anisidine and *p*-bromoaniline. In these cases, reactions were performed with a large excess of amine, at 100 °C for 24 h. The products (**3**-**13**) were characterized by analysis of their spectroscopic data. The presence of the C=S group was evidenced by the IR absorption bands at 1523-1566 and 1250-1410 cm^{-1}, together with the signal at δ 177.3 – 181.8 in the ^{13}C NMR spectra. The monoterpene moiety was characterized by the signals at δ_H/δ_C 5.30-5.40 (1H, brs, H-2)/119.7-120.5 (C-2), 1.30-1.49 (3H, s, H-8)/24.1-24.9 (C-8), 1.34-1.50 (3H, s, H-9)/24.2-25.4 (C-9) and 1.61-1.73 (3H, s, H-10)/23.0-23.5 (C-10) in the $^1H/^{13}C$ NMR spectra. The NMR data were also consistent for the substituents attached to the nitrogen atom N-1, as showed in the Experimental Section. The EI-mass spectra showed peaks at *m/z* 58 and at *m/z* ($M^{+\bullet}$ - 135) as main fragments.



Scheme 1.

The proposed mechanisms for the fragmentation are shown in Figure 1.

The S-methylated derivatives (**14**-**18**) were prepared from the reaction of the corresponding thioureas with methyl iodide at 0 °C, in chloroform, for 24 h in quantitative yields. The ^1H NMR spectra of the S-methylthioureas showed signal at δ 2.78 – 2.85 corresponding to the SCH_3 group. The formation of S-methythiourea was also evidenced by the presence of the signals at δ 16.9 – 18.5 ($S\text{-}CH_3$) and δ 143.7 – 169.2 (C=N) in the ^{13}C NMR spectra, besides of the absorption at 1600-1690 cm^{-1} (C=N), in the IR spectra.

Antiproliferative activity

The results of the antiproliferative assays are showed in Tables 1 and 2. The response parameter GI_{50} (Table 1) refers to the drug concentration that produce a 50% reduction of cellular growth when compared to untreated control cells. Table 2 includes data for the compounds that reached TGI and LC_{50} values. The TGI and LC_{50} parameters refer, respectively, to the drug concentration for total growth inhibition, and that for killing 50% of the cells.

As shown in Table 1, the compounds bearing aromatic substituents (**3**-**6**) exhibit cytotastic activity against all cancer cell lines tested, with GI_{50} values in the range of 2.5 to 24 μmol L^{-1}. Analysis of TGI and LC_{50} data presented in Table 2 show that compounds **3**-**5** had higher potency for total growth inhibition, and for killing 50% of the cells. From this series, compound **3** was the most active, with GI_{50} and TGI values of 2.5 μmol L^{-1} and 22.5 μmol L^{-1}, respectively, against breast resistant NCI/ADR cancer cell line. On the other hand, compounds **7**-**18** of the aliphatic series exhibit different profiles and dependence on the nature of the substituent on the nitrogen atom. Compared to aromatic series, the specificity was increased with the most of the compounds

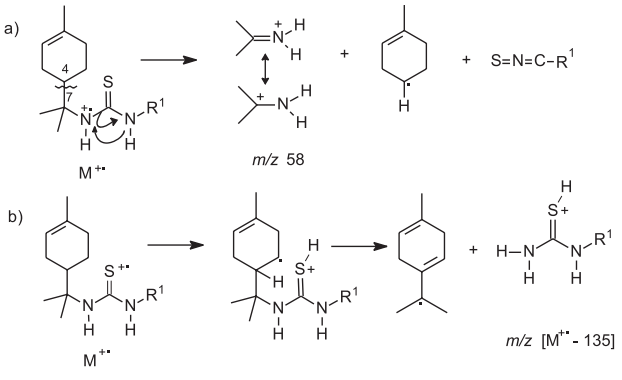


Figure 1. Proposed mechanism for formation of the fragments a) *m/z* 58 and b) *m/z* ($M^{+\bullet}$ - 135).

Table 1. GI_{50} values (in μmol L^{-1} concentrations) of the limonene derivatives (**3**-**18**)

Compounds	R^1	R^2	Cancer cell lines								
			Melanoma UACC-62	Breast MCF7	Lung NCI-460	Leukemia K-562	Ovarian OVCAR	Prostate PCO-3	Colon HT29	Renal 786-0	Breast resistant NCI/ADR
Thioureas	Aromatic										
3	Ph	H	13.9	18.4	17.3	nt	16.6	15.6	24.0	23.2	2.5
4	2-Me-Ph	H	12.0	21.0	18.3	18.7	17.2	19.8	9.3	16.1	12.4
5	2-OMe-Ph	H	11.8	13.6	15.0	10.5	9.5	11.0	18.2	12.7	8.0
6	4-Br-Ph	H	nt	21.5	19.9	20.1	16.7	16.5	14.6	20.9	19.0
	Aliphatic										
7	n-Butyl	H	>100	>100	>100	39.5	98.1	>100	60.6	92.4	54.1
8	i-Propyl	H	31.8	70.7	29.5	18.9	28.3	>100	30.7	32.6	13.8
9	i-Pentyl	H	97.7	46.5	54.3	6.6	56.2	62.6	17.5	92.1	32.7
10	Cyclohexyl	H	nt	37.3	33.9	16.3	3.0	12.5	nt	26.5	6.1
11	Pyrrolidyl		53.3	26.1	34.5	23.6	44.3	43.0	36.8	59.6	28.5
12	1-N-Methylpiperazyl		>100	>100	>100	3.0	>100	>100	>100	>100	88.0
13	Morpholyl		>100	>100	>100	>100	>100	>100	>100	>100	>100
S-Methylthioureas											
14	i-Propyl		83.8	>100	>100	25.4	>100	>100	81.8	>100	85.3
15	i-Pentyl		28.9	16.0	17.6	88.7	91.3	16.2	16.7	14.8	10.3
16	Cyclohexyl		60.7	60.7	58.9	0.41	39.1	62.5	16.7	75.6	75.6
17	Pyrrolidyl		90.0	>100	>100	>100	60.9	>100	>100	>100	91.3
18	1-N-Methylpiperazyl		>100	>100	>100	76.7	>100	>100	>100	>100	>100
Doxorubicin			2.36	8.84	1.21	3.04	9.67	7.88	2.73	1.76	>100

nt = not tested.

Table 2. TGI values and LC_{50} (values in parenthesis), in μmol L^{-1} concentrations

Compounds	R^1	R^2	Cancer cell lines								
			Melanoma UACC-62	Breast MCF7	Lung NCI-460	Leukemia K-562	Ovarian OVCAR	Prostate PCO-3	Colon HT29	Renal 786-0	Breast resistant NCI/ADR
Thioureas											
3	Ph	H	34.6	55.5	86.6	>100	>100	58.9	>100	86.6	22.5 (76.6)
4	2-Me-Ph	H	29.7 (80.9)	86.9	47.2	>100	49.5	>100	>100	29.8 (65.0)	30.0 (65.0)
5	2-OMe-Ph	H	39.2	64.6	39.5	>100	32.0	58.0	>100	26.8 (57.7)	26.9
6	4-Br-Ph	H	>100	>100	71.0	>100	72.0	>100	95.0	71.0	71.0
9	i-Pentyl	H	>100	>100	>100	95.0	>100	>100	>100	>100	>100
S-Methylthioureas											
15	i-Pentyl		>100	68.3	87.8	>100	>100	>100	87.8	58.0	70.6
16	Cyclohexyl		>100	>100	>100	82.5	>100	>100	93.0	>100	>100
Doxorubicin			64.0		6.86	>100	>100	92.1		>100	

of the aliphatic series exhibiting smaller GI_{50} values for leukemic K-562 cell line. From the thiourea aliphatic series, compounds **9** and **12** showed potent activity and particular selectivity against leukemic K-562 cell line with GI_{50} values of 6.6 and 3.0 μmol L^{-1}, respectively. The replacement of the 1-N-methyl group of the compound **12** for an oxygen results in the inactive compound **13**. The thiourea **10** exhibits potent antiproliferative activity against ovarian OVCAR and breast resistant NCI/ADR with GI_{50} 3.0 and 6.1 μmol L^{-1}, respectively. S-methylation of thioureas bearing aliphatic substituents results in changes in the antiproliferative activity profiles. Conversion of the thiourea **10** to the S-methylthiourea analogue **16**, led to the most potent compound, with GI_{50} value of 0.41 μmol L^{-1} and high selectivity against leukemia K-562 cell lines.

Conclusions

In this paper we report the synthesis and cytotoxic evaluation of a series of new limonene derivatives containing a substituted thiourea moiety. The results show the potentiality of some compounds, particularly **3**, **10**, **12** and **16**, as inhibitors of tumor cells proliferation. Some compounds bearing aromatic substituents were also able to kill 50% of the breast resistant NIC/ADR (compounds

3 and **4**), melanoma UACC-62 (compound **4**), and renal 786-0 (compounds **4** and **5**) cancer cell lines.

Experimental

IR spectra were recorded on KBr pellets in a Bomem model MB-100 spectrophotometer. Mass spectra were measured on Shimadzu GC/MS, QP 2000A, at 70 eV. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury Plus 300 MHz in $CDCl_3$ and TMS as internal reference. Column chromatography was performed on silica gel Merck 230-400 mesh ASTM.

*General procedure for thioureas **3** – **13***

To a solution of limonene isothiocyanate **2** (1 mmol) in $CHCl_3$ (10 mL) was added drop-wise the amine (2 mmol).[11,12] The solution was kept at room temperature for 15 hours and then the solvent was removed under reduced pressure. For aromatic amines, reactions were performed with a large excess of amine, without solvent, at 100 °C for 24 h. The residue was purified by column chromatography using hexane and a mixture of hexane-ethyl acetate in increasing polarity as solvent.

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(phenyl)]thiourea (**3**).* Yield: 30%; IR ν_{max}/cm^{-1}: 3387 (N-H), 3156 (C-H arom), 1590 (C=C arom), 1540 and 1298 (C=S); EI-MS *m/z* (rel. int.): 288 ($M^{+\bullet}$, 5), 153 (100, $M^{+\bullet}$-135, $[H_2NCSNHC_6H_5]^+$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.18 and 1.62 (1H each, m, H-5), 1.40 (3H, s, H-8), 1.45 (3H, s, H-9), 1.61 (3H, s, H-10), 1.84 and 1.90 (4H, m, H-3 and H-6), 2.59 (1H, m, H-4), 5.36 (1H, brs, H-2), 7.20 (2H, d, *J* 7.5 Hz, H-2' and H-6'), 7.29 (1H, t, *J* 7.5 Hz, H-4'), 7.43 (2H, t, *J* 7.5 Hz, H-3' and H-5'); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10), 24.2 (C-9), 24.3 (C-5), 24.5 (C-8), 26.7 (C-3), 31.2 (C-6), 41.1 (C-4), 59.5 (C-7), 120.5 (C-2), 125.2 (C-2' and C-6'), 127.1 (C-4'), 130.2 (C-3' and C-5'), 134.3 (C-1), 136.8 (C-1'), 179.5 (C=S).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(2-methylphenyl)]thiourea (**4**).* Yield: 30%. IR ν_{max}/cm^{-1}: 3367 (N-H), 3173 (C-H arom), 1643 (C=C arom), 1621 (C=C), 1533 and 1251 (C=S); EI-MS *m/z* (rel. int.): 302 ($M^{+\bullet}$, 10), 167 (100, $M^{+\bullet}$ - 135, $[H_2NCSNH$-2-methylphenyl$]^+$), 91 (45), 58 (70), 41 (46); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.39 (3H, s, H-8), 1.45 (3H, s, H-9), 1.61 (3H, s, H-10), 1.75 (2H, m, H-5), 1.93 (4H, m, H-3 and H-6), 2.29 (3H, s, CH_3-Ar), 2.48 (1H, m, H-4), 5.30 (1H, brs, H-2), 7.20 (1H, m, H-6'), 7.25 (1H, m, H-5'), 7.28 (1H, m, H-4'), 7.31 (1H, m, H-3'); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.1 ($C\underline{H}_3$-Ar), 23.5 (C-10), 24.1 (C-8), 24.2 (C-5), 24.4 (C-9), 26.6 (C-3), 31.2 (C-6), 41.4 (C-4), 59.2 (C-7), 120.5 (C-2), 127.7 (C-6'), 127.8 (C-5'), 127.9 (C-2'), 128.7 (C-4'), 131.9 (C-3'), 134.3 (C-1), 136.0 (C-1'), 179.9 (C=S).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(2-methoxyphenyl)]thiourea (**5**).* Yield: 31%; IR ν_{max}/cm^{-1}: 3370 (N-H), 3048 (C-H arom), 1599 and 1535 (C=C), 1460 and 1250 (C=S); EI-MS *m/z* (rel. int.): 318 ($M^{+\bullet}$, 7), 183 (27, $M^{+\bullet}$ - 135, $[H_2NCSNH$-2-methoxyphenyl$]^+$); 123 (93, $[H_2N$-2-methoxyphenyl$]^{+\cdot}$), 93 (27), 58 (100), 41 (43); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.42 (3H, s, H-8), 1.48 (3H, s, H-9), 1.63 (3H, s, H-10), 1.77 (2H, m, H-5), 1.90 (4H, m, H-3 and H-6), 2.00 (1H, m, H-4), 3.84 (3H, s, $OC\underline{H}_3$), 5.36 (1H, brs, H-2), 6.92 (1H, m, H-3'), 6.94 (1H, d, *J* 7.8 Hz, H-6'), 6.98 (1H, t, *J 7.8* Hz, H-5'), 7.20 (1H, t, *J* 7.8 Hz, H4'); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.4 (C-10), 24.3 (C-5), 24.6 (C-8), 24.6 (C-9), 26.7 (C-3), 31.2 (C-6), 41.7 (C-4), 55.8 (OCH_3), 59.3 (C-7), 110.3 (C-4'),120.5 (C-2), 121.0 (C- 5' and C-6'), 124.5 (C-3'), 134.1 (C-1'), 134.2 (C-1), 154.6 (C-2'), 179.3 (C=S).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(4-bromophenyl)]thiourea (**6**).* Yield: 20%; IR ν_{max}/cm^{-1}: 3281 (N-H), 3103 (C-H), 1578 and 1530 (C=C, arom.), 1488 and 1364 (C=S); EI-MS *m/z* (rel. int.): 366 ($M^{+\bullet}$, 7), 215 (23), 58 (100); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.24 and 1.68 (1H each, m, H-5'), 1.42 (3H, s, H-8), 1.48 (3H, s, H-9), 1.63 (3H, s, H-10), 1.68 and 1.97 (1H each, m, H-3), 1.96 (2H, m, H-6), 2.59 (1H, m, H-4), 5.36 (1H, brs, H-2), 7.09 (2H, d, *J* 8.7 Hz, H-2' and H-6'), 7.54 (2H, d, *J* 8.7 Hz, H-3' and H-5'); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.5 (C-10), 24.4 (C-5), 24.6 (C-8), 24.6 (C-9), 26.7 (C-3), 31.2 (C-6), 40.9 (C-4), 59.7 (C-7), 120.4 (C-2), 120.6 (C-4'), 126.8 (C2' and C-6'), 133.4 (C3' and C-5'), 134.4 (C-1), 136.0 (C-1'), 179.4 (C=S).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(butyl)]thiourea (**7**).* Yield: 97%; IR ν_{max}/cm^{-1}: 3269 (N-H), 1543 and 1342 (C=S); EI-MS *m/z* (rel. int.): 268 ($M^{+\bullet}$, 10), 133 (100, $M^{+\bullet}$ - 135, $[H_2NCSNH$-butyl$]^+$), 58 (68), 41 (60); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.95 (3H, t, *J* 7.2 Hz, H-4'); 1.35 (3H, s, H-8), 1.39 (3H, s, H-9), 1.41 (2H, m, H-3'), 1.60 (2H, m, H-6), 1.64 (3H, s, H-10), 1.82 (2H, m, H-5), 1,99 (3H, m, H-3 and H-4), 2.01 (2H, m, H-2'), 3.54 (2H, q, *J* 6.0 Hz, H-1'), 5.40 (1H, brs, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 13.7 (C-4'), 20.1 (C-3'), 23.2 (C-10), 24.2 (C-5), 24.8 (C-8), 25.0 (C-9), 26.5 (C-3), 30.9 (C-2'), 31.2 (C-6), 58.3 (C-7), 42.2 (C-4), 45.1 (C-1'), 120.0 (C-2), 133.9 (C-1), 180.8 (C=S).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(isopropyl)]thiourea (**8**).* Yield: 84%; IR ν_{max}/cm^{-1}: 3272 (N-H), 1538 and 1325 (C=S); EI-MS *m/z* (rel. int.): 254 ($M^{+\bullet}$, 7), 119 (100, $M^{+\bullet}$ - 135, [H_2NCSNH-isopropyl]$^+$), 58 (65), 41 (60); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.23 (6H, d, *J* 6.0 Hz, NHCH(C$\underline{H}_3$)$_2$), 1.35 (3H, s, H-8), 1.40 (3H, s, H-9), 1.65 (3H, s, H-10), 1.78 (2H, m, H-5), 1.99 (5H, m, H-3, H-4 and H-6), 4.40 (1H, m, H-1'), 5.40 (1H, brs, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 22.6 (NHCH(C$\underline{H}_3$)$_2$), 23.2 (C-10), 24.2 (C-5), 24.9 (C-8), 25.2 (C-9), 26.6 (C-3), 30.9 (C-6), 42.5 (C-4), 47.2 (C-1'), 58.3 (C-7), 120.0 (C-2), 134.0 (C-1), 179.8 (C=S).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(isopentyl)]thiourea (**9**).* Yield: 80%; IR ν_{max}/cm^{-1}: 3258 (N-H), 1643 (C=C), 1537 and 1322 (C=S); (KBr); EI-MS *m/z* (rel. int.): 282 ($M^{+\bullet}$, 10), 147 (100, $M^{+\bullet}$ - 135, [H_2NCSNH-isopentyl]$^+$), 58 (70); 41 (65); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.93 (6H, t, *J* 7.5 Hz, NHCH(CH$_2$C$\underline{H}_3$)$_2$), 1.30 and 1.83 (1H each, m, H-5), 1.34 (3H, s, H-8), 1.38 (3H, s, H-9), 1.51 (4H, m, NHCH(C$\underline{H}_2$CH$_3$)$_2$), 1.61(2H, m, H-3), 1.65 (3H, s, H-10), 1.99 (3H, m, H-4 and H-6), 4.23 (1H, m, H-1'), 5.36 (1H, brs, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 10.0 (NHCH(CH$_2$C$\underline{H}_3$)$_2$), 23.3 (C-10), 24.3 (C-5), 25.1 (C-8), 25.4 (C-9), 26.6 (C-3), 27.0 (NHCH(C$\underline{H}_2$CH$_3$)$_2$), 31.0 (C-6), 42.8 (C-4), 58.2 (C-7), 58.3 (C-1'), 119.7 (C-2), 134.1 (C-1),180.7 (C=S).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(cyclohexyl)]thiourea (**10**).* Yield: 80%; IR ν_{max}/cm^{-1}: 3258 (N-H); 1540 and 1338 (C=S); EI-MS *m/z* (rel. int.): 294 ($M^{+\bullet}$, 10), 58 (100), 41 (60); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.37 (3H, s, H-9), 1.34 (3H, s, H-8), 1.60 (m, H-4'), 1.65 (3H, s, H-10), 1.67 (4H, m, H-3'and H-5'),1.80 (2H, m, H-6), 1.81 (4H, m, H-3 and H-5), 1,93 (1H, m, H-4), 2.02 (4H, m, H-2'and H-6'), 4.12 (1H, m, H-1'), 5.36 (1H, brs, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.3 (C-10), 24.2 (C-5), 24.7 (C-3'and C-5'), 24.9 (C-8), 25.2 (C-9), 25.4 (C-4'), 26.6 (C-3), 31.0 (C-6), 32.9 (C-2'and C-6'), 42.7 (C-4), 53.9 (C-1'), 58.3 (C-7), 120.0 (C-2), 134.0 (C-1), 179.7 (C=S).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(pyrrolidyl)]thiourea (**11**).* Yield: 67%; IR ν_{max}/cm^{-1}: 3395 (N-H), 1643 (C=C), 1531 and 1347 (C=S); EI-MS *m/z* (rel. int.): 266 ($M^{+\bullet}$); 233 (28); 131 (100, $M^{+\bullet}$ - 135; [H_2NCSNH-pyrrolidyl]$^+$), 114 (92); 93 (30); 58 (38); 41 (58); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.25 and 1.78 (1H each, m, H-5), 1.49 (3H, s, H-8), 1.55 (3H, s, H-9), 1.64 (3H, s, H-10), 1.77 and 2.04 (1H each, m, H-3), 1.98 (2H, m, H-6), 1.98 (4H, m, H-2'and H-3'), 2.70 (1H, tdd, *J* 12.0, 2.4 and 2.1, H-4), 3.55 (4H, m, H-1' and H-4'), 5.37 (1H, brs, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.3 (C-10), 24.1 (C-5), 24.6 (C-8), 24.9 (C-9), 25.5 (C-2'and C-3'), 26.6 (C-3), 31.1 (C-6), 40.8 (C-4), 49.2 (4H, m, C-1' and C-4'), 58.9 (C-7), 120.6 (C-2), 134.0 (C-1), 177.3 (C=S).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(N-methylpiperazyl)]thiourea (**12**).* Yield: 90%; IR ν_{max}/cm^{-1}: 3438 (N-H), 1629 (C=C), 1566 and 1410 (C=S); EI-MS *m/z* (rel. int.): 295 ($M^{+\bullet}$); 195 (20), 121 (34), 93 (64), 58 (100), 41 (97); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.46 (3H, s, H-8), 1.53 (3H, s, H-9), 1.63 (3H, s, H-10), 1.73 (2H, m, H-5), 1.81 and 1.96 (1H each, m, H-3), 1.96 (2H , m, H-6), 2.30 (3H, s, NC$\underline{H}_3$), 2.43 (4H, t, *J* 5.1 Hz, H-2'and H-3'), 2.81 (1H, m, H-4), 3.78 (4H, t, *J* 5.1 Hz, H-1'and H-4'), 5.36 (1H, brs, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.0 (C-10), 23.8 (C-5), 24.2 (C-9), 24.3 (C-8), 26.2 (C-3), 30.7 (C-6), 39.9 (C-4), 45.4 (N$\underline{C}$H$_3$), 46.8 (C-1' and C-4'), 54.2 (C-2' and C-3'), 58.9 (C-7), 120.3 (C-2), 133.5 (C-1), 180.8 (C=S).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(morpholyl)]thiourea (**13**).* Yield: 88%; IR ν_{max}/cm^{-1}: 3408 (N-H), 1523 and 1347 (C=S); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, s, H-8), 1.54 (3H, s, H-9), 1.65 (2H, m, H-5), 1.73 (3H, s, H-10), 2.02 (4H, m, H-3 and H-6), 2.75 (1H, m, H-4), 3.73 (8H, m, H-1', H-2', H-3'and H-4'), 5.36 (1H, brs, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.3 (C-10), 24.3 (C-5), 24.6 (C-8), 24.7 (C-9), 26.5 (C-3), 30.7 (C-6), 44.5 (C-4), 47.4 (C-1'and C-4'), 59.5 (C-7), 66.3 (C-2'and C-3'), 119.7 (C-2), 134.1 (C-1), 181.8 (C=S).

*General procedure for S-methylthioureas **14-18***

To a solution of thiourea (1 mmol) in $CHCl_3$ (10 mL) was added methyl iodide (5 mmol) at 0 °C. The mixture was kept at 0 °C for 24 h and then the solvent and excess of methyl iodide were removed under reduced pressure to give the salt of the corresponding S-methylthiourea in quantitative yield for all compounds.

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(isopropyl)]-S-methylthiourea (**14**).* IR ν_{max}/cm^{-1}: 3193 (N-H), 1600 (C=N); EI-MS *m/z* (rel. int.): 268 (7, $M^{+\bullet}$), 220 (68, $M^{+\bullet}$ – CH_3SH), 205 (57), 128 (100), 127 (60), 93 (77), 58 (91), 43 (96), 41 (85); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.49 (6H, d, *J* 6.3 Hz, NCH(C$\underline{H}_3$)$_2$), 1.50 (3H, s, H-8), 1.58 (3H, s, H-9), 1.65 (3H, s, H-10), 1.76 (2H, m, H-5), 1.93 (4H, m, H-3 and H-6), 2.27 (1H, m, H-4), 2.85 (3H, s, SC$\underline{H}_3$), 4.30 (1H, m, H-1'), 5.35 (1H, brs, H-

2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.4 (S<u>C</u>H$_3$), 22.7 (NCH(<u>C</u>H$_3$)$_2$), 23.3 (C-10), 24.4 (C-5), 25.1 (C-8), 25.7 (C-9), 26.7 (C-3), 30.8 (C-6), 42.7 (C-4), 51.2 (C-1'), 57.4 (C-7), 120.1 (C-2), 134.1 (C-1), 143.7 (C=N).

*N-[1-(4R)-(4-isoproyl-1-methylcyclohexenyl)]-N'-[2-(isopentyl)]-S-methylthiourea (**15**).* IR ν_{max}/cm^{-1}: 3347 (N-H), 1690 (C=N); EI-MS *m/z* (rel. int.): 248 (40, M$^{+\bullet}$ – CH_3SH), 233 (50), 219 (55), 128 (81), 127 (49), 93 (61), 58 (74); 43 (100), 41 (86); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.99 (6H, t, *J* 7.3 Hz, NCH(CH$_2$C<u>H</u>$_3$)$_2$), 1.33 (1H, m, H-5), 1.55 (3H, s, H-8), 1.58 (3H, s, H-9), 1.65 (3H, s, H-10), 1.77 (1H, m, H-5'), 1.79 (4H, m, NCH(C<u>H</u>$_2$CH$_3$)$_2$), 1.94 (2H, m, H-3), 2.01 (2H, m, H-6), 2.26 (1H, m, H-4), 2.78 (3H, s, SC<u>H</u>$_3$), 3.78 (1H, m, H-1'), 5.37 (1H, brs, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 10.9 (NCH(CH$_2$<u>C</u>H$_3$)$_2$), 18.4 (S<u>C</u>H$_3$), 23.3 (C-10), 24.4 (C-5), 25.1 (C-8), 25.6 (C-9), 26.7 (C-3), 27.5 (NCH(<u>C</u>H$_2$CH$_3$)$_2$), 30.8 (C-6), 42.8 (C-4), 61.5 (C-7), 63.4 (C-1'), 119.4 (C-2),134.4 (C-1), 169.2 (C=N).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(cyclohexyl)]-S-methyl-thiourea (**16**).* IR ν_{max}/cm^{-1}: 3186 (N-H), 1685 (C=N), 1591 (C=C); EI-MS *m/z* (rel. int.): 308 (M$^{+\bullet}$), 261 (31, M$^{+\bullet}$ – $CH_3S^{\cdot}$), 128 (63), 127 (39), 93 (53), 58 (55), 55 (54), 43 (57), 41 (100); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.34 and 1.81 (1H each, m, H-6), 1.55 (3H, s, H-8), 1.57 (3H, s, H-9), 1.62 (2H, m, H-4'), 1.65 (3H, s, H-10), 1.81 (2H, m, H-5), 1.81 (4H, m, H-3'and H-5'), 1.93 (4H, m, H-2'and H-6'), 1.93 (2H, m, H-3), 2.25 (1H, m, H-4), 2.83 (3H, s, SC<u>H</u>$_3$), 3.88 (1H, m, H-1'), 5.37 (1H, brs, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.5 (S<u>C</u>H$_3$), 23.3 (C-10), 24.4 (C-4'), 24.6 (C-5), 24.8 (C-3' and C-5'), 25.6 (C-8 and C-9), 26.7 (C-3), 30.7 (C-6), 32.6 (C-2'and C-6'), 42.7 (C-4), 56.8 (C-1'), 63.4 (C-7), 119.4 (C-2), 134.3 (C-1), 168.1 (C=N).

*N-[1-(4R)-(4-isopropyl-1-methylcyclohexenyl)]-N'-[2-(pyrrolidyl)]-S-methyl-thiourea (**17**).* IR ν_{max}/cm^{-1}: 1677 (C=N), 1584 (C=C); EI-MS *m/z* (rel. int.): 280 (M$^{+\bullet}$),142 (52), 128 (93), 127 (69), 84 (44), 70 (42), 57 (42), 43 (100), 42 (87), 41 (40); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.36 and 1.82 (1H each, m, H-5), 1.60 (3H, , H-9), 1.62 (3H, s, H-8), 1.65 (3H, s, H-10), 1.82 (m, H-5), 1.96 (2H, m, H-3), 2.01 (2H, m, H-6), 2.18 (4H, m, H-2'and H-3'), 2.35 (1H, m, H-4), 2.70 (3H, s, SC<u>H</u>$_3$), 4.04 (4H, m, H-1'and H-4'), 5.37 (1H, brs, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 17.6 (S<u>C</u>H$_3$), 23.5 (C-10), 24.6 (C-5), 25.4 (C-2'and C-3'), 26.2 (C-8), 26.6 (C-9), 27.1 (C-3), 31.1 (C-6), 43.3 (C-4), 53.5 (C-1'and C-4'), 64.1 (C-7),119.9 (C-2), 134.4 (C-1), 164.2 (C=N).

N-*[1-(4R)-(4-isoproyl-1-methylcyclohexenyl)]-N'-[2-(N-methypiperazyl)]-S-methyl-thiourea (**18**).* IR ν_{max}/cm^{-1}: 1675 (C=N), 1610 (C=C); EI-MS *m/z* (rel. int.): 304 (M$^{+\bullet}$), 195 (43), 136 (55), 121 (28), 93 (100), 81 (53), 58(16), 44 (56), 41 (75); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.27 (3H, s, H-8), 1.28 (3H, s, H-9), 1.65 (3H, s, H-10), 1.76 (2H, m, H-5), 1.80 and 1.97 (1H each, m, H-3), 1.97 (3H, m, H-4 and H-6), 2.34 (3H, s, NC<u>H</u>$_3$), 2.73 (3H, s, SC<u>H</u>$_3$), 3.59 (4H, m, H-1'and H-4'), 3.70 (4H, m, H-2'and H-3'), 5.37 (1H, brs, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 16.9 (S<u>C</u>H$_3$), 23.4 (C-10), 24.3 (C-5), 24.6 (C-8), 24.8 (C-9), 26.9 (C-3), 31.3 (C-6), 42.9 (C-1'and C-4'), 46.2 (C-4), 58.8 (C-7), 61.5 (C-2'and C-3'), 52.2 (N<u>C</u>H$_3$), 121.1 (C-2), 133.9 (C-1), 151.1 (C=N).

Antiproliferative assays

Synthesized compounds were evaluated *in vitro* against a nine-cell panel lines consisting of melanoma UACC-62, breast MCF7, lung NCI-460, leukemia K-562, ovarian OVCAR, prostate PCO-3, colon HT29, renal 786-0 and breast resistant NCI/ADR according NCI standard protocol.[17] Doxorubicin was used as positive control. Assays were performed in a 96-well plate using four concentrations at 10-fold dilutions (0.25 mg mL^{-1} to 250 mg mL^{-1}) for each test compound. The anticancer activity was deduced from dose-response curves and three dose response parameters (GI_{50}, TGI and LC_{50}) were calculated.

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