Cytotoxic evaluation of substituted azetopyrroloazepinones

Roberto Martínez,¹ * Gustavo Ávila-Zárraga,² Ma. Teresa Ramírez,¹ and Araceli Pérez¹

*Instituto de Química,¹ Facultad de Química,² Universidad Nacional Autónoma de México.
Circuito Exterior, Ciudad Universitaria, 04510 Coyoacán, México, D. F.*

E-mail: robmar@servidor.unam.mx

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Abstract

Azetopyrroloazepinones **1a-g** were synthesized in six steps from 5,5-dimethyl-1,3-cyclohexanedione as starting material in good yields. These compounds were tested on five tumoral cell lines with the aim of elucidating the relationship between the substituents attached to the 3-phenyl ring and their cytotoxic activity. The results are inconclusive in regard to the relationship between the physicochemical properties of the substituents on the 3-phenyl group of azeto-pyrroloazepinones **1a-g** and the inhibition of the growth in tumor cell lines. However, the results show a clear link between the presence of halogens on position 3- or 4- and the cytotoxicity of these compounds.

Keywords: Azeto-pyrroloazepinones, cancer cell lines, cytotoxic activity

Introduction

Cancer is one of the main causes of death in the world despite considerable progress in the understanding of its biology and pharmacology. The traditional therapeutic strategies for the treatment of this disease are surgery, radiotherapy, immunotherapy and chemotherapy. For the time being, 50% of the patients diagnosed with cancer are cured either through one of these methods or by a combination of them. For some types of disseminated cancers, chemotherapy is the only effective therapy because it distributes anticancer drugs through the circulatory system.¹ We are currently engaged in a program aimed at synthesizing novel heterocyclic compounds that inhibit the growth of cancer cells.² One of these compounds is azetopyrroloazepinone **1a**, which showed in vitro cytotoxic activity against PC-3 (prostate) and U251 (CNS) cancer cells lines.³ The mechanism of action of antiproliferative activity of compound **1a** is still unknown, therefore, further exploration of the azetopyrroloazepinone pharmacophore is advisable. The aim of the present study was both to explore the role of the substituents attached to the 3-phenyl ring of compounds **1a-g** on the inhibition of cancer cell growth, and to identify an optimal candidate

among currently available compounds. It was also intended to ascertain potential directions for synthetic lead-optimization studies.⁴

Compounds **1b-e** were prepared in order to study the tendency of halogens to present activity. In addition, compounds **1f** (R= CH₃) and **1g** (R= OCH₃) were included in the study to investigate the electron donating effects of -CH₃ and -OCH₃ groups.

Results and Discussion

Chemistry

For this study, we synthesized a set of derivatives as previously described (Scheme 1).⁵

Scheme 1. (a) $\text{NH}_2\text{OH}\cdot\text{HCl}$, AcONa , H_2O , EtOH , 2 h, reflux; (b) PPA, 90-120 °C, 3 h; (c) Lawesson's reagent, toluene, 1-2 h; (d) (i) CH_3I , CH_2Cl_2 , rt, 1 h; (ii) $\text{NaHCO}_3(\text{aq})$ (e) $\text{PhOCH}_2\text{COCl}$, NEt_3 , C_6H_6 , reflux, 8 h.

This synthetic approach starts from the Beckmann rearrangement of tetrahydro-4-indolone-oximes **3a-g**, easily prepared from tetrahydroindol-4-ones **2a-g**, to the corresponding azepinones **4a-g**, which are transformed to methylsulfanyl-hexahydropyrrolo-azepines. These imines react with phenoxyacetyl chloride (Staudinger reaction) to give the 3-aryl-2, 5, 5-trimethyl-9a-(methylsulfanyl)-9-phenoxy-4, 5, 6, 8, 9, 9a-hexahydro-3*H*-azeto[1,2-] pyrrolo[3,2-*c*]azepin-8-ones **1a-g**. While the Beckmann reaction occurred in a regioselective way, the Staudinger reaction proceeded with high stereoselectivity. All the compounds were purified either by recrystallization in hexane or by silica gel column chromatography.

Antiproliferative activity

Azeto-pyrroloazepinones **1a-g** were evaluated in vitro for their ability to inhibit the growth of PC-3 prostate, U251 central nervous system, K562 leukemia, HCT-15 colon and MCF7 breast cancer cells. The percentage of inhibition of the growth of the five tumoral cell lines after treatment with each compound at a concentration of 100 μM is given in Table 1 and the IC_{50} values (μM) on Table 2.

Table 1. % Inhibition of growth of compounds **1a-g** to the five cancer cell lines

Compound	R	PC-3 (prostate)	U-251 (CNS)	K562 (leukemia)	HCT-15 (colon)	MCF-7 (breast)
1a	4-I	64.71	68.04	37.90	35.74	46.10
1b	4-Br	90.43	99.98	40.59	-29.33	73.20
1c	4-Cl	78.9	49.44	77.64	-45.59	45.59
1d	3-Br	61.88	33.29	65.76	44.67	57.03
1e	3-Cl	76.07	43.73	59.34	-169.0	21.78
1f	4-CH ₃	54.58	18.33	46.05	40.10	38.51
1g	4-OCH ₃	68.81	34.06	64.77	52.71	55.39

Compounds **1a-g** were examined to analyze the importance of the relative position of the substituents attached to the 3-phenyl ring of these compounds and their cytotoxic activity (Table 2). The inhibition resulted by **1a** lead to the conclusion that electronegativity and/or size of halogens could be implicated in the cytotoxic activity. To test this idea, compounds **1b** and **1c** were synthesized. The series of 4-halogen derivatives showed that activity followed the order **1c**(Cl)>**1b**(Br)>**1a**(I). To complete the series of halogen compounds, **1d**(3-Br) and **1e**(3-Cl) were obtained and they showed a greater activity than the 4-halogens derivatives.

These results indicated an apparent relationship between both the electronegativity and the size of the halogen *versus* the cytotoxic activity of the 4-halogen derivatives. However, this statement is not clear in the case of 3-halogen derivatives. On other hand, compound **1f** (4-CH₃) was the most selective displaying a relatively good inhibition in the PC-3 cell line. In contrast, compound **1g** (4-OCH₃) was the less selective. An analysis of the cytotoxic activity by cell line type indicated that all compounds **1a-g** are active on the PC-3 cell line, derivative **1d** being four times more active than the leading compound **1a**. In contrast, on U-251 cell line, all compounds **1b-g** tested showed a complete lack of activity. Compound **1g** (4-OCH₃) is the only one that induces cytotoxic activity on the HCT-15 cell line. In the case of K-562 and MFC-7 cell lines the compounds **1a-g** did not show any tendency.

Table 2. The IC₅₀ values (μM) of compounds **1a-g** to the five cancer cell lines

Compound	R	PC-3 (prostate)	U-251 (CNS)	K-562 (leukemia)	HCT-15 (colon)	MCF-7 (breast)
1a	4-I	87.0±8.6	40.0±3.6	>100	>100	>100
1b	4-Br	69.58±1.1	>100	>100	>100	35.29±1.1
1c	4-Cl	45.74±1.1	>100	50.85±1.1	>100	>100
1d	3-Br	20.79±1.0	>100	20.46±1.4	>100	51.64±1.2
1e	3-Cl	37.46±1.5	>100	20.70±1.8	>100	>100
1f	4-CH ₃	26.89±1.1	>100	>100	>100	>100
1g	4-OCH ₃	29.44±1.0	>100	21.13±1.3	50.93±1.1	64.46±1.0
Doxorubicine		0.32±0.02	0.09±0.02	0.28±0.01	0.23±0.01	0.4±0.01

Conclusions

The data presented here are inconclusive in regard to the relationship between electronegativity and size of the halogens substituents on the 3-phenyl group of azeto-pyrroloazepinones **1a-g** and the inhibition of the growth in tumor cell lines. However, the results show a clear link between the presence of halogens on position 3- or 4- and the cytotoxicity of these compounds. Likewise, this study reveals the interesting finding that the compounds that presented cytotoxic activity were primarily those containing halogens mainly on position 3- or a CH₃- / -OCH₃ group on position.4-.

Experimental Section

General Procedures. All reactions were performed in oven-dried glassware under a positive pressure of nitrogen. Reaction mixtures and chromatography fractions were concentrated by

using a rotary evaporator (*ca.* 20 °C/ 20 Torr). For column chromatography, the Merck silica gel 60 F254 was employed. Commercial grade reagents were used without further purification except when indicated. Benzene and toluene were distilled from a sodium/ benzophenone mixture immediately prior to use, and CH₂Cl₂ from P₂O₅.

1-(R-Phenyl)-2,6,6-trimethyl-4,5,6,7-tetrahydroindol-4-ones (2a-g). Compounds **2a-g** have been prepared following a reported procedure: **2c**, mp 171-173 °C (lit.⁶ 170-172 °C); **2f**, mp 170-171 °C (lit.⁶ 170-172 °C); **2g**, mp 143-145 °C (lit.⁶ 142-144 °C). The spectral data where in agreement with the reported data.⁶ Spectral data for the unknown tetrahydroindol-4-ones **2a**, **2b**, **2d** and **2e** are described below.

1-(4-Iodophenyl)-2,6,6-trimethyl-4,5,6,7-tetrahydroindol-4-one (2a). mp 172-173 °C; IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1648; ¹H-NMR (CDCl₃, 200 MHz) δ 1.06(H-9,9'), 2.04(H-8), 2.35(H-5), 2.38(H-7), 6.36(H-3), 6.9-7.87(Ar-H); MS (EI) *m/z* (relative intensity) 379 (M⁺, 100); HRMS (EI) Calcd for C₁₇H₁₈NOI 379.0433, Found 379.0454.

1-(4-Bromophenyl)-2,6,6-trimethyl-4,5,6,7-tetrahydroindol-4-one (2b). mp 171-173 °C; IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1648; ¹H-NMR (CDCl₃, 200 MHz) δ 1.05(H-9,9'), 2.04(H-8), 2.35(H-5), 2.37(H-7), 6.37(H-3), 7.08-7.67(Ar-H); MS (EI) *m/z* (relative intensity) 331 (M⁺, 100), 333(M⁺+2, 100); HRMS (EI) Calcd for C₁₇H₁₈NOBr 331.0572, Found 331.0576.

1-(3-Bromophenyl)-2,6,6-trimethyl-4,5,6,7-tetrahydroindol-4-one (2d). mp 134-135 °C; IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1649; ¹H-NMR (CDCl₃, 200 MHz) δ 1.07(H-9,9'), 2.05(H-8), 2.35(H-5), 2.39(H-7), 6.36(H-3), 7.09-7.48(Ar-H); MS (EI) *m/z* (relative intensity) 331 (M⁺, 100), 333(M⁺+2, 100); HRMS (EI) Calcd for C₁₇H₁₈NOBr 331.0572, Found 331.0574.

1-(3-Chlorophenyl)-2,6,6-trimethyl-4,5,6,7-tetrahydroindol-4-one (2e). mp 129-131 °C; IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1649; ¹H-NMR (CDCl₃, 200 MHz) δ 1.07(H-9,9'), 2.05(H-8), 2.36(H-5), 2.39(H-7), 6.36(H-3), 7.14-7.65(Ar-H); MS (EI) *m/z* (relative intensity) 287 (M⁺, 100), 289 (M⁺+2, 33.5); HRMS (EI) Calcd for C₁₇H₁₈NOCl 287.1077, Found 287.1079

1-(R-Phenyl)-2,6,6-trimethyl-4,5,6,7-tetrahydroindol-4-one oximes (3a-g). Oximes **3a-g** have been prepared following a reported procedure: **3b**, mp 209-210 °C (lit.⁷ 208-210 °C); **3e**, mp 175-177 °C (lit.⁷ 175-178 °C); **3f**, mp 220-222 °C (lit.⁷ 220-223 °C); **3g**, mp 213-215 °C (lit.⁷ 212-213 °C). The spectral data where in agreement with the reported data.⁷ Spectral data for the unknown mixture (*syn/anti*) tetrahydroindol-4-one oximes **3a**, **3c** and **3d** are described below.

1-(4-Iodophenyl)-2,6,6-trimethyl-4,5,6,7-tetrahydroindol-4-one oximes (*syn/anti*) (3a). mp 210-212 °C, IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 3589; ¹H-NMR (CDCl₃, 200 MHz) δ 0.96-0.92(H-9,9'), 2.05(H-8), 2.58-2.25(H-5), 2.37(H-7), 6.82-6.23(H-3), 6.9-7.87(Ar-H); MS (EI) *m/z* (relative intensity) 394 (M⁺, 100);, HRMS (EI) Calcd for C₁₇H₁₉N₂OI 394.0542, Found 394.0554

1-(4-Chlorophenyl)-2,6,6-trimethyl-4,5,6,7-tetrahydroindol-4-one oximes (*syn/anti*) (3c). mp 185-187 °C, IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 3588; ¹H-NMR (CDCl₃, 200 MHz) δ 0.93-0.90(H-9,9'), 2.06(H-8), 2.58-2.27(H-5), 2.28(H-7), 6.82-6.25(H-3), 7.11-7.48(Ar-H); MS (EI) *m/z* (relative intensity) 302 (M⁺, 100), 304 (M⁺+2, 33.8); HRMS (EI) Calcd for C₁₇H₁₉N₂OCl 302.1186, Found 302.1190.

1-(3-Bromophenyl)-2,6,6-trimethyl-4,5,6,7-tetrahydroindol-4-one oximes (*syn/anti*) (3d). mp 174-175 °C, IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 3251; ¹H-NMR (CDCl₃, 200 MHz) δ 1.03-1.04(H-9,9'), 2.07(H-8), 2.61-2.29(H-5), 2.36(H-7), 6.82-6.33(H-3), 7.12-7.62(Ar-H); MS (EI) m/z (relative intensity) 346 (M⁺, 100), 348 (M⁺+2, 100); HRMS (EI) Calcd for C₁₇H₁₉N₂OBr 346.0681, Found 346.0684

6H-1-(R-Phenyl)-2,7,7-trimethyl-4,5,7,8-tetrahydropyrrolo[3,2-c]azepin-4-ones (4a-g)

The azepinones **4a-g** have been prepared following a reported procedure: **4b**, mp 247-249 °C (lit.⁷ 247-248 °C); **4e**, mp 170-171 °C (lit.⁷ 169-170 °C); **4f**, mp 248-250 °C (lit.⁷ 250-251 °C); **4g**, mp 229-231 °C (lit.⁷ 230-231 °C); The spectral data were in agreement with the reported data.⁷ Spectral data for the unknown azepinones **4a**, **4c**, and **4d** are described below.

6H-1-(4-Iodophenyl)-2,7,7-trimethyl-4,5,7,8-tetrahydropyrrolo[3,2-c]azepin-4-one (4a). mp 264-265 °C; IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1631; ¹H-NMR (CDCl₃, 200 MHz) δ 0.96 (H-10-10'), 1.96 (H-9), 2.31 (H-8), 2.98 (H-6), 6.30 (exchangeable with D₂O, N-H), 6.46 (H-3), 6.91-7.85 (Ar-H); MS (EI) m/z (relative intensity) 394 (M⁺, 100); HRMS (EI) Calcd for C₁₇H₁₉N₂OI 394.0542, Found 394.0550.

6H-1-(4-Chlorophenyl)-2,7,7-trimethyl-4,5,7,8-tetrahydropyrrolo[3,2-c]azepin-4-one (4c). mp 247-249 °C; IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1635; ¹H-NMR (CDCl₃, 200 MHz) δ 0.96 (H-10-10'), 1.96 (H-9), 2.33 (H-8), 3.0 (H-6), 6.02 (exchangeable with D₂O, N-H), 6.46 (H-3), 7.09-7.51 (Ar-H); MS (EI) m/z (relative intensity) 302 (M⁺, 100), 304 (M⁺+2, 34); HRMS (EI) Calcd for C₁₇H₁₉N₂OCl 302.1186, Found 302.1194

6H-1-(3-Bromophenyl)-2,7,7-trimethyl-4,5,7,8-tetrahydropyrrolo[3,2-c]azepin-4-one (4d). mp 178-180 °C; IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1630; ¹H-NMR (CDCl₃, 200 MHz) δ 0.97 (H-10-10'), 1.97 (H-9), 2.32 (H-8), 3.02 (H-6), 6.22 (exchangeable with D₂O, N-H), 6.46 (H-3), 7.11-7.64 (Ar-H); MS (EI) m/z (relative intensity) 346 (M⁺, 100), 348 (M⁺+2, 100); HRMS (EI) Calcd for C₁₇H₁₉N₂OBr 346.0681, Found 346.0681

6H-1-(R-Phenyl)-2,7,7-trimethyl-4,5,7,8-tetrahydropyrrolo[3,2-c]azepin-4-thiones(4a'-g'). Compounds **4a'-g'** have been prepared following a reported procedure: **4b'**, mp 268-270 °C (lit.⁵ 267-269 °C); **4c'**, mp 284-286 °C (lit.⁵ 283-285 °C); **4f'**, mp 271-273 °C (lit.⁵ 272-274 °C); **4g'**, mp 281-283 °C (lit.⁵ 280-282 °C). The spectral data were in agreement with the reported data.⁵ Spectral data for the unknown tetrahydroindol-4-thiones **4a'**, **4d'** and **4e'** are described below

6H-1-(4-Iodophenyl)-2,7,7-trimethyl-4,5,7,8-tetrahydropyrrolo[3,2-c]azepin-4-thione (4a'). mp 250-255 °C; IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1179; ¹H-NMR (CDCl₃, 200 MHz) δ 0.95 (H-10, 10'), 1.94 (H-9), 2.26 (H-8), 3.09 (H-6), 6.65 (H-3), 6.89-7.84 (Ar-H), 8.91 (exchangeable with D₂O, N-H); MS (EI) m/z (relative intensity) 410 (M⁺, 100); HRMS (EI) Calcd for C₁₇H₁₉N₂IS 410.0314, Found 410.0319.

6H-1-(3-Bromophenyl)-2,7,7-trimethyl-4,5,7,8-tetrahydropyrrolo[3,2-c]azepin-4-thione (4d'). mp 234-235 °C; IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1174; ¹H-NMR (CDCl₃, 200 MHz) δ 0.99 (H-10, 10'), 1.97 (H-9), 2.33 (H-8), 3.16 (H-6), 6.62 (H-3), 7.11-7.45 (Ar-H), 9.18 (exchangeable with D₂O, N-H); MS (EI) m/z (relative intensity) 362 (M⁺, 100), 364 (M⁺+2, 100); HRMS (EI) Calcd for C₁₇H₁₉N₂BrS 362.0452, Found 362.0458.

6H-1-(3-Chlorophenyl)-2,7,7-trimethyl-4,5,7,8-tetrahydropyrrolo[3,2-c]azepin-4-thione (4e'). mp 201-203 °C: IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1174; ¹H-NMR (CDCl₃, 200 MHz) δ 0.99 (H-10, 10'), 1.97 (H-9), 2.31 (H-8), 3.16 (H-6), 6.65 (H-3), 7.11-7.48 (Ar-H), 8.71 (exchangeable with D₂O, N-H); MS (EI) m/z (relative intensity) 318 (M⁺, 100), 320 (M⁺+2, 38); HRMS (EI) Calcd for C₁₇H₁₉N₂ClS 318.0957, Found 318.0965.

6H-1-(R-Phenyl)-2,7,7-trimethyl-4-methylsulfanyl-4,5,7,8-tetrahydropyrrolo[3,2-c]azepines (4a'-g'). Compounds **4a'-g'** have been prepared following a reported procedure. The spectral data were in agreement with the reported data.⁵ Compounds **4a'**, **4b'**, **4c'**, **4f'** and **4g'** are yellow amorphous solid⁵ and they were used without further purification. Spectral data for the unknown methylsulfanylazepines **4d'** and **4e'** are described below

1-(3-Bromophenyl)-2,7,7-trimethyl-4-methylsulfanyl-4,5,7,8-tetrahydropyrrolo[3,2-c]azepine (4d'). mp 133-135 °C: ¹H-NMR (CDCl₃, 200 MHz) δ 0.95 (H-11, 11'), 1.97 (H-9), 2.26 (H-8), 2.43 (H-10), 3.51 (H-6), 6.29 (H-3), 7.11-7.63 (Ar-H); MS (EI) m/z (relative intensity) 376 (M⁺, 100), 378 (M⁺+2, 100); HRMS (EI) Calcd for C₁₈H₂₁N₂BrS 376.0609, Found 376.0618.

1-(3-Chlorophenyl)-2,7,7-trimethyl-4-methylsulfanyl-4,5,7,8-tetrahydropyrrolo[3,2-c]azepine (4e'). mp 118-120 °C: ¹H-NMR (CDCl₃, 200 MHz) δ 1.04 (H-11, 11'), 1.98 (H-9), 2.31 (H-8), 2.36 (H-10), 3.57 (H-6), 6.34 (H-3), 7.08-7.49 (Ar-H); MS (EI) m/z (relative intensity) 332 (M⁺, 100), 334 (M⁺+2, 38); HRMS (EI) Calcd for C₁₈H₂₁N₂ClS 332.1114, Found 332.1120.

3-(R-Phenyl)-2,5,5-trimethyl-9a-methylsulfanyl-9-phenoxy-4,5,6,8,9,9a-hexahydro-3H-azeto[1,2-a]pyrrolo[3,2-c]azepin-8-one (1a-g). Compounds **1a-g** have been prepared following a reported procedure. The spectral data were in agreement with the reported data.^{5,7} **1a**, mp 195-196 °C (lit.³ 194-195 °C); **1b**, mp 183-186 °C (lit.⁵ yellow oil); **1c**, mp 178-180 °C (lit.⁵ 177-179 °C); **1f**, mp 171-173 °C (lit.⁵ 170-171 °C); **1g**, mp 184-185 °C (lit.⁵ 181-183 °C). Spectral data for the unknown azeto-pyrrolo azepinone **1d** and **1e** are described below

3-(3-Bromophenyl)-2,5,5-trimethyl-9a-methylsulfanyl-9-phenoxy-4,5,6,8,9,9a-hexahydro-3H-azeto[1,2-a]pyrrolo[3,2-c]azepin-8-one (1d). mp 159-162 °C: IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1755; ¹H-NMR (CDCl₃, 200 MHz) δ 0.91 (H-12), 0.95 (H-11), 1.9 (H-10), 2.1 (H-4b), 2.19 (H-12), 2.69 (H-4a), 3.05 (H-6b), 3.66 (H-6a), 5.5 (H-1), 5.8 (H-9), 7.01- 7.60 (Ar-H); MS (EI) m/z (relative intensity) 510 (M⁺, 9), 512 (M⁺+2, 9); HRMS (EI) Calcd for C₂₆H₂₇N₂O₂BrS 510.0977, Found 510.0988

3-(3-Chlorophenyl)-2,5,5-trimethyl-9a-methylsulfanyl-9-phenoxy-4,5,6,8,9,9a-hexahydro-3H-azeto[1,2-a]pyrrolo[3,2-c]azepin-8-one (1e). mp 162-164 °C: IR (CHCl₃) $\nu_{\max}$ (cm⁻¹) 1756; ¹H-NMR (CDCl₃, 200 MHz) δ 0.91 (H-12), 0.94 (H-11), 1.89 (H-10), 2.17 (H-4b), 2.19 (H-12), 2.61 (H-4a), 3.04 (H-6b), 3.74 (H-6a), 5.51 (H-1), 5.8 (H-9), 7.08- 7.43 (Ar-H); MS (EI) m/z (relative intensity) 466 (M⁺, 12), 468 (M⁺+2, 5); HRMS (EI) Calcd for C₂₆H₂₇N₂O₂ClS 466.1482, Found 466.1489.

Cytotoxic activity

Tumoral cell lines were supplied by the National Cancer Institute. The cytotoxicity assays were carried out at 5000 to 7500 cells/ml. as reported by Skehan et al and Monks et al using the

sulforhodamine **B** (SRB) protein assay to estimate cell growth.^{8,9} Compounds were dissolved in DMSO which has not effect on the inhibition has shown by the control. The % inhibition of the growth described for all compounds were obtained from three different experiments. The percentage growth was evaluated spectrophotometrically in a Bio kinetics reader spectrophotometer. Daunomycine was used as reference. This compound under the described conditions gave 100% of inhibition. Each experiment was made two times by triplicate.

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