

## Antiproliferative Activity of Withanolides against Human Breast Cancer Cell Lines

Rubén P. Machin,<sup>†</sup> Adriana S. Veleiro,<sup>‡</sup> Viviana E. Nicotra,<sup>§</sup> Juan C. Oberti,<sup>§</sup> and José M. Padrón<sup>\*-1</sup>

Unidad de Investigación, Hospital Universitario de Gran Canaria Dr. Negrín, Bco. Ballena s/n 35010, Las Palmas de Gran Canaria, Spain, Departamento de Química Orgánica and UMYFOR (CONICET-FCEN), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, C1428EGA Buenos Aires, Argentina, Departamento de Química Orgánica and IMBIV, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, 5000 Córdoba, Argentina, and BioLab, Instituto Universitario de Bio-Organica "Antonio González" (IUBO-AG), Universidad de La Laguna, C/Astrofísico Francisco Sánchez 2, 38206 La Laguna, Spain

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The in vitro antiproliferative activity of a series of 22 naturally occurring withanolides was examined against the T-47D, MCF7, MCF7/BUS, MDA-MB-231, and SK-Br-3 human solid tumor breast cancer cell lines. The most active compound showed GI<sub>50</sub> values in the range 0.16–0.71 μM. The aromatic withanolide **19** exhibited specific activity for the estrogen-receptor-positive cell lines (T-47D, MCF7, and MCF7/BUS). Overall, the results demonstrated the relevance of the substitution pattern on the A and B rings on the resultant antiproliferative activity.

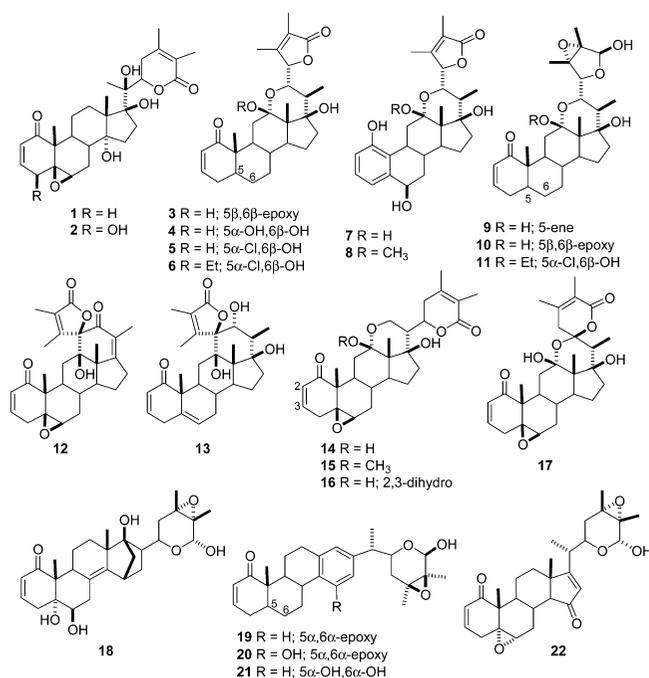
Withanolides are a group of natural C-28 steroids built on an ergostane skeleton, with a common feature of all having oxidation at C-1, C-22, and C-26.<sup>1–4</sup> These compounds have been isolated, largely but not exclusively, from genera belonging to the plant family Solanaceae.<sup>5,6</sup> The first withanolide to be characterized was withaferin A, isolated in 1965, almost simultaneously, by Lavie from *Withania somnifera* and by Kupchan from *Acnistus arbore-scens*. Both plants are known for their use in the treatment of cancer.<sup>7,8</sup> Withaferin A showed cytotoxicity in vitro and in vivo against several types of carcinoma.<sup>1,2</sup>

Withanolides may be classified into two major groups according to their structural skeleton: (a) compounds with a γ-lactone or γ-lactol side chain and (b) compounds with a δ-lactone or δ-lactol side chain. Biogenetic transformations can produce highly modified compounds, in both the steroid nucleus and the side chain. Several biological activities are known for these compounds, including antifeedant, anti-inflammatory, antitumor, cancer prevention, cytotoxic, and immunomodulating effects.<sup>1–4</sup>

As a part of a systematic investigation, a variety of withanolides from several plants in the Solanaceae growing in Argentina have been isolated.<sup>4,9–13</sup> In a previous study, a group of naturally occurring withanolides was evaluated for their potential to induce quinone reductase activity in vitro in hepatoma cells, and spiranoid and C-18-functionalized withanolides were found to be potent inducers of enzyme activity.<sup>14</sup> Herein, we report the antiproliferative activity of a series of 22 withanolides against a panel of human breast cancer cell lines (MCF7, MCF7/BUS, T-47D, MDA-MB-231, and SK-Br-3). This panel included widely used cell lines as in vitro models of breast cancer with distinctive molecular features,<sup>15</sup> in order to assess chemosensitivity to the natural products investigated.

A total of 22 withanolides were studied, with all compounds isolated previously being fully characterized.<sup>9–13,16–25</sup>

The in vitro cytotoxicity assay using tumor cell lines is a widely accepted preclinical model for the characterization of new plant-derived antitumor compounds, and a panel of human breast cancer cell lines was used to evaluate compounds **1–22**. These cell lines can be divided into two groups: those comprising cells possessing the estrogen receptor (ER) (T-47D, MCF7, and MCF7/BUS) and those lacking this receptor (MDA-MB-231 and SK-Br-3) [ER(+)



and ER(-), respectively]. The antiproliferative activity was studied using the SRB protocol.<sup>26</sup> The results, expressed as 50% growth inhibition (GI<sub>50</sub>),<sup>27</sup> are summarized in Table 1.

Overall, the data obtained showed that the majority of the compounds were able to induce growth inhibition in all cell lines, and certain preliminary structure–activity relationships can be inferred. The most active product was withanolide **1** (GI<sub>50</sub> = 0.16–0.71 μM), which exhibits an unmodified skeleton bearing an α,β-unsaturated ketone in the A ring and a 5β,6β-epoxy functionality in the B ring. The presence of a hydroxy group at position C-4 led to a slight decrease in activity (**1** vs **2**).

Compounds **3–11** belong to the trechonolide subgroup. They contain a hemiketal or ketal bridge formed between a C-22 OH and a C-12 ketone, resulting in a six-membered ring and a γ-lactone or γ-lactol system as a side chain. Similarly to withanolides **1** and **2**, the trechonolides with an α,β-unsaturated ketone in the A ring and a 5β,6β-epoxy functionality showed antiproliferative activity (**3** and **10**). No loss of activity was observed when the 5β,6β-epoxy functionality was replaced by a 5α-chloro,6β-hydroxy system (**5** and **6**). However, the presence of a 5α-hydroxy group (**4**) or a phenolic A ring (**7** and **8**) exerted a deleterious effect on the resultant

\* To whom correspondence should be addressed. Tel: +34-922316502, ext. 6126. Fax: +34-922318571. E-mail: jmpadron@ull.es.

<sup>†</sup> Hospital Dr. Negrín.

<sup>‡</sup> Universidad de Buenos Aires.

<sup>§</sup> Universidad Nacional de Córdoba.

<sup>1</sup> Universidad de La Laguna.

**Table 1.** In Vitro Antiproliferative Activity of Withanolides **1–22** against Human Breast Tumor Cell Lines<sup>a</sup>

compound <sup>b</sup>	ER(+)			ER(-)	
	T-47D	MCF7	MCF7/BUS	MDA-MB-231	SK-Br-3
<b>1</b> <sup>16</sup>	0.16 (±0.02)	0.31 (±0.10)	0.42 (±0.04)	0.24 (±0.12)	0.71 (±0.13)
<b>2</b> <sup>17</sup>	2.2 (±0.5)	1.0 (±0.1)	1.1 (±0.2)	2.1 (±0.9)	1.7 (±0.8)
<b>3</b> <sup>18</sup>	4.3 (±1.1)	3.9 (±0.8)	3.1 (±0.6)	6.3 (±0.9)	1.9 (±0.2)
<b>4</b> <sup>19</sup>	>100	>100	>100	>100	>100
<b>5</b> <sup>10</sup>	3.5 (±0.4)	2.4 (±0.3)	2.6 (±0.4)	3.2 (±0.4)	2.5 (±0.4)
<b>6</b> <sup>10</sup>	3.9 (±0.6)	3.0 (±0.3)	3.0 (±0.3)	3.7 (±0.1)	2.5 (±0.6)
<b>7</b> <sup>11</sup>	>100	>100	>100	>100	>100
<b>8</b> <sup>11</sup>	>100	>100	>100	>100	>100
<b>9</b> <sup>12</sup>	30 (±9.0)	14 (±1.1)	18 (±3.7)	19 (±1.8)	18 (±1.2)
<b>10</b> <sup>13</sup>	6.5 (±1.4)	3.6 (±0.4)	2.6 (±0.1)	8.7 (±0.4)	2.3 (±0.3)
<b>11</b> <sup>13</sup>	13 (±2.1)	3.9 (±0.7)	5.1 (±1.1)	10 (±0.4)	3.4 (±0.2)
<b>12</b> <sup>20</sup>	3.4 (±0.1)	3.2 (±0.3)	3.3 (±0.3)	2.5 (±0.8)	2.0 (±0.5)
<b>13</b> <sup>21</sup>	61 (±27)	35 (±2.4)	33 (±2.8)	49 (±14)	29 (±3.0)
<b>14</b> <sup>10</sup>	4.1 (±0.7)	2.9 (±0.4)	2.8 (±0.3)	3.4 (±0.2)	2.6 (±0.4)
<b>15</b> <sup>10</sup>	3.9 (±0.6)	2.9 (±0.2)	2.7 (±0.3)	2.8 (±0.2)	2.0 (±0.7)
<b>16</b> <sup>10</sup>	38 (±7.1)	25 (±7.4)	29 (±4.2)	35 (±4.3)	34 (±7.0)
<b>17</b> <sup>9</sup>	3.7 (±0.1)	2.8 (±0.3)	2.9 (±0.3)	3.5 (±0.9)	2.5 (±0.7)
<b>18</b> <sup>22</sup>	>100	>100	>100	>100	>100
<b>19</b> <sup>23</sup>	18 (±3.1)	9.4 (±2.7)	13 (±1.5)	>100	>100
<b>20</b> <sup>24</sup>	14 (±2.6)	5.1 (±0.5)	5.7 (±2.6)	14 (±0.7)	3.4 (±0.2)
<b>21</b> <sup>25</sup>	>100	43 (±5.2)	29 (±3.4)	>100	>100
<b>22</b> <sup>25</sup>	17 (±6.0)	22 (±1.4)	13 (±2.0)	21 (±3.8)	21 (±5.9)
5-fluorouracil	47 (±18)	8.3 (±1.2)	6.2 (±0.5)	54 (±5.4)	3.0 (±0.8)
camptothecin	2.0 (±0.5)	0.28 (±0.14)	0.21 (±0.04)	1.3 (±0.15)	0.15 (±0.04)

<sup>a</sup> Expressed as GI<sub>50</sub> values given in  $\mu$ M and determined as means of two to five experiments; standard deviations are given in parentheses.

<sup>b</sup> Reference for compound isolation and characterization.

cytotoxicity. Furthermore, a 5,6-unsaturated functionality (**9**) led to a decrease in activity. Similar growth inhibition profiles could be observed for the  $\gamma$ -lactones (**3**, **6**) and the  $\gamma$ -lactols (**10**, **11**).

Compared to the parent withanolide **1**, compounds **12** and **13** bear an additional six-membered ring and a spiranoid  $\gamma$ -lactone side chain. In agreement with previous observations, the replacement of the 5 $\beta$ ,6 $\beta$ -epoxy functionality with a 5,6-unsaturated group produced a decrease of the activity (**12** vs **13**). Compounds **14–16** belong to the sativilide subgroup, and they also possess an additional six-membered hemiketal or ketal ring. In this case, the bridge is formed between a C-21 OH and a C-12 ketone, leaving a  $\delta$ -lactone system as a side chain. Product **14**, which has an  $\alpha,\beta$ -unsaturated ketone in the A ring and a 5 $\beta$ ,6 $\beta$ -epoxy functionality, was active, in agreement with the results mentioned above, while the loss of the  $\alpha,\beta$ -unsaturated system in the A ring (**16**) resulted in a decrease of the antiproliferative activity.

Direct comparison of the GI<sub>50</sub> values of **3**, **10**, **12**, **14**, and **17**, all of which possess an additional six-membered ring, allows the conclusion to be made that the type of side chain does not affect the resultant activity. Although **18** was the only compound in this series with a bridge formed between carbons 15 and 17, the 5 $\alpha$ -hydroxy group seems to be responsible for the lack of activity, as was observed also for compound **4**.

On considering withanolides with an aromatic D ring (**19–21**), it was observed that the activity of compound **19** depended on the cell line tested. Thus, **19** showed specific activity against ER(+) cell lines, while it was inactive for ER(-) cells. Indeed, the differential activity exhibited by the nonaromatic withanolide **22**, structurally related to **19**, suggested that the antiproliferative activity showed by the latter compound depends in part on the presence of an aromatic ring. The presence of a hydroxy group on the aromatic D ring (compound **20**) partially restored the cytotoxic activity against the ER(-) cell lines. In the case of compound **21**, the cleavage of the epoxide to give a 5 $\alpha$ -hydroxy derivative decreased activity for the ER(+) cell lines, but did not affect the ER(-) cell line inhibitory activity.

Taken as a whole, the data led to the conclusion that in withanolides with an additional six-membered ring between positions 12 and 17 of the ergostane skeleton, the activity is independent of the side chain. The current results indicate that cytotoxic activity

depends mainly on the substitution pattern of the A and B rings, with the presence of an  $\alpha,\beta$ -unsaturated ketone and a 5 $\beta$ ,6 $\beta$ -epoxy system being important for activity. This is in agreement with previous results that showed 4  $\beta$ -hydroxywithanolide E (**2**) as a promising anticancer agent on the basis of its activity for B-16 melanoma and L-1210 leukemia cells.<sup>1,2</sup> A decrease in the activity was observed in the presence of a 5 $\alpha$ -hydroxy group, a 5,6-unsaturated functionality, or a phenolic A ring. On the other hand, the cleavage of the 5 $\beta$ ,6 $\beta$ -epoxide to give a 5 $\alpha$ -chloro-6 $\beta$ -hydroxy functionality does not affect activity.

It is noteworthy that antiproliferative activity does not depend on a particular tumor cell line, except in the case of the withanolides with an aromatic D ring. The selectivity showed by compound **19** toward hormone-dependent ER(+) cell lines when compared to ER(-) negative cell lines is notable. This specificity could suggest an estrogen-signaling-mediated mechanism in which compound **19** would act as inhibitor of the ER-dependent pathway, essential for the proliferation in these hormone-dependent breast cell lines. This potential antiestrogenic effect suggests the need to perform specific bioassays oriented to elucidate the intrinsic antiestrogenic activity of D ring-aromatic withanolides.

## Experimental Section

**Chemicals. Test Compounds.** 12-*O*-Ethyljaborosolactol **25** (**11**), 12-*O*-ethyljaborosolactone **42** (**6**), 12-*O*-methyljaborosolactone **38** (**15**), 12-*O*-methyljaborosolactone **45** (**8**), 4 $\beta$ -hydroxywithanolide E (**2**), jaborosolactol (**9**), jaborosolactol **20** (**18**), jaborosolactol **24** (**10**), jaborosolactone **1** (**12**), jaborosolactone **38** (**14**), jaborosolactone **39** (**16**), jaborosolactone **42** (**5**), jaborosolactone **45** (**7**), jaborosolactone **46** (**17**), jaborosolactone **P** (**13**), jaborotetrol (**4**), salpichrolide A (**19**), salpichrolide C (**21**), salpichrolide D (**22**), salpichrolide G (**20**), trechonolide A (**3**), and withanolide E (**1**) were isolated and identified by the authors as reported earlier. Prior to biological testing, all compounds were analyzed by TLC on silica gel 60 F254 (Merck) plates using hexane–EtOAc as mobile phase. Spots were visualized by spraying with 10% H<sub>2</sub>SO<sub>4</sub> in EtOH and heating. Purity greater than 95%, as verified by <sup>1</sup>H NMR spectroscopy, was considered acceptable.

**Biological Assays.** All starting materials were commercially available research-grade chemicals and used without further purification. RPMI 1640 medium was purchased from Flow Laboratories (Irvine, UK), fetal calf serum (FCS) was from Gibco (Grand Island, NY), trichlo-

roacetic acid (TCA) and glutamine were from Merck (Darmstadt, Germany), and penicillin G, streptomycin, dimethyl sulfoxide (DMSO), and sulforhodamine B (SRB) were from Sigma (St Louis, MO).

**Cells, Culture, and Plating.** The human solid tumor cell lines MCF-7, T-47D, MDA-MB-231, and SK-Br-3 were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and MCF-7BUS cells were kindly provided by Dr. Nicolás Olea Serrano from the University of Granada (Spain). Cells were maintained in 25 cm<sup>2</sup> culture flasks in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum and 2 mM L-glutamine in a 37 °C, 5% CO<sub>2</sub>, 95% humidified air incubator. Exponentially growing cells were trypsinized and resuspended in antibiotic-containing medium (100 units penicillin G and 0.1 mg of streptomycin per mL). Single cell suspensions displaying >97% viability by trypan blue dye exclusion were subsequently counted. After counting, dilutions were made to give the appropriate cell densities for inoculation onto 96-well microtiter plates. Cells were inoculated in a volume of 100 μL per well at densities of 7500 (MDA-MB-231), 12 000 (MCF-7,T-47D, MCF-7/BUS), and 15 000 (SK-Br-3) cells per well, based on their doubling times.

**Chemosensitivity Testing.** Chemosensitivity tests were performed using the SRB assay of the U.S. NCI with slight modifications. Briefly, pure compounds were initially dissolved in DMSO at 400 times the desired final maximum test concentration. Control cells were exposed to an equivalent concentration of DMSO (0.25% v/v, negative control). Each agent was tested in triplicate at different dilutions in the range 1–100 μM. The test compound treatment was started on day 1 after plating. Drug incubation times were 48 h, after which time cells were precipitated with 25 μL of ice-cold 50% (w/v) trichloroacetic acid and fixed for 60 min at 4 °C. Then, the SRB assay was performed. The optical density (OD) of each well was measured at 492 nm, using a PowerWave XS Absorbance microplate reader (BioTek). Values were corrected for background OD from wells containing only medium. The percentage growth (PG) was calculated with respect to untreated control cells (*C*) at each of the drug concentration levels based on the difference in OD at the start (*T*<sub>0</sub>) and end of drug exposure (*T*), according to the NCI protocol. Therefore, if *T* was greater than or equal to *T*<sub>0</sub> the calculation was  $100 \times [(T - T_0)/(C - T_0)]$ . If *T* was less than *T*<sub>0</sub>, denoting cell killing, the calculation was  $100 \times [(T - T_0)/(T_0)]$ . The effect was defined as percentage of growth, where 50% growth inhibition (GI<sub>50</sub>), total growth inhibition (TGI), and 50% cell killing (LC<sub>50</sub>) represent the concentration at which PG is +50, 0, and -50, respectively. With these calculations, a PG value of 0 corresponded to the amount of cells present at the start of drug exposure, while negative PG values denoted net cell kill.

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