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Induction of Quinone Reductase by Withanolides

Rosana I. Misico,[†,⊥] Lynda L. Song,[†,⊥] Adriana S. Veleiro,[‡] Adriana M. Cirigliano,[‡] María C. Tettamanzi,[‡] Gerardo Burton,[‡] Gloria M. Bonetto,[§] Viviana E. Nicotra,[§] Gloria L. Silva,[§] Roberto R. Gil,[§] Juan C. Oberti,[§] A. Douglas Kinghorn,[*,†] and John M. Pezzuto[†]

Program for Collaborative Research in the Pharmaceutical Sciences and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina, and Departamento de Química Orgánica (IMBIV-CONICET), Universidad Nacional de Córdoba, 5000 Córdoba, Argentina

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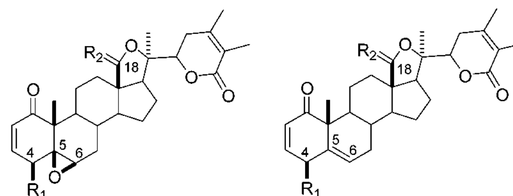
Thirty-seven naturally occurring withanolides (**1**–**37**), previously isolated in our laboratories, were evaluated for their potential to induce quinone reductase with cultured murine hepatoma cells (Hepa 1c1c7). Spiranoid (**29**, **32**) and 18-functionalized withanolides (**2**–**5**, **7**–**9**, **24**) were found to be potent inducers of the enzyme, while 5α-substituted derivatives exhibited weak activity. Preliminary studies were performed with compound **29** to evaluate enzyme-inducing capacity in multiple organ sites of BALB/c mice. Significant induction was observed in liver and colon, but not in lung, stomach, or mammary gland.

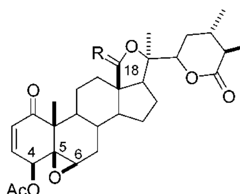
The in vivo fate of chemical carcinogens is determined at least in part by the balance between phase I enzymes (cytochromes P-450) that activate these species to highly reactive electrophilic metabolites capable of damaging DNA and phase II enzymes (e.g., glutathione transferases, NAD(P)H quinone oxireductase [QR], UDP-glucuronosyltransferases) that convert these reactive electrophiles to less toxic and more easily excretable products.[1–3] A wide variety of agents that protect against chemical carcinogenesis are also inducers of phase II enzymes in many animal cells and tissues, and there is convincing evidence that induction of phase II enzymes is an important mechanism responsible for cancer chemoprevention. It is therefore of interest that vegetables contain a variety of inducer molecules. A simple screening procedure involving measurement of quinone reductase activity in murine hepatoma cells has led to the isolation and identification of cancer chemopreventive agents such as sulforaphane from *Brassica oleracea* L. "broccoli" (Brassicaceae)[4] and several withanolides, constituents of the fruits of *Physalis philadelphica* Lam. "tomatillos" (Solanaceae).[5]

The withanolides are a group of natural C-28 steroids that occur mainly in plants of certain genera of the Solanaceae. In addition to enzyme induction potential noted above, withanolides have been studied previously for their antifeedant, antiinflammatory, antitumor, cytotoxic, and immunomodulating activities and for protection against CCl_4-induced hepatotoxicity.[6,7] They are built on an ergostane skeleton in which C-22 and C-26 are appropriately oxidized in order to form a δ-lactone or δ-lactol ring. Biogenetic transformations can produce highly modified compounds both in the steroid nucleus and the side chain, including the formation of additional rings. The withanolides may be classified according to their structural skeleton;[7] compounds within each group usually differ in the nature and number of oxygenated substituents and the degree of unsaturation of the rings. The diversity of pharmacological effects exhibited by these molecules is obviously a function of the differences in their chemical structure. However, convincing structure–activity relationships have not been developed previously for the withanolides.

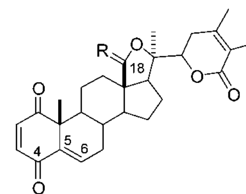


1 R_1 = OH; R_2 = H, OH; (18 *R/S*)
9 R_1 = OAc; R_2 = O

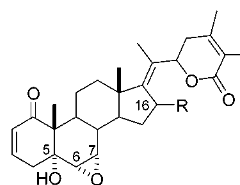
2 R_1 = OH; R_2 = H, OH; (18 *R/S*)
3 R_1 = OH; R_2 = H, OCH_3; (18 *R*)
4 R_1 = OH; R_2 = H, OCH_3; (18 *S*)
5 R_1 = OH; R_2 = O
6 R_1 = OAc; R_2 = H, OAc; (18 *S*)



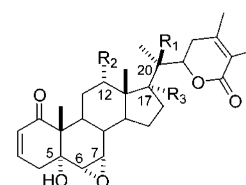
7 R = H, OH; (18 *R/S*)
8 R = O



10 R = H, OCH_3; (18 *R*)
11 R = H, OCH_3; (18 *S*)



12 R = βOH
13 R = αOH



14 R_1 = R_3 = H; R_2 = OH
15 R_1 = R_2 = H; R_3 = OH
16 R_1 = OH; R_2 = R_3 = H

Over the past several years, in the course of phytochemical studies, we have isolated a variety of known and novel withanolides from several plants in the Solanaceae, including *Datura ferox* L.,[8] *Dunalia brachyacantha* Miers.,[9] *Exodeconus maritimus* (Benth.) D'Arcy,[10] *Iochroma australe* Griseb.,[11] *Jaborosa araucana* Phil.,[12] *J. integrifolia*

* To whom correspondence should be addressed. Tel: (312)-996-6809. Fax: (312)-996-7391. E-mail: kinghorn@uic.edu.
† University of Illinois at Chicago.
‡ Universidad de Buenos Aires.
§ Universidad Nacional de Córdoba.
⊥ These authors contributed equally to this work.

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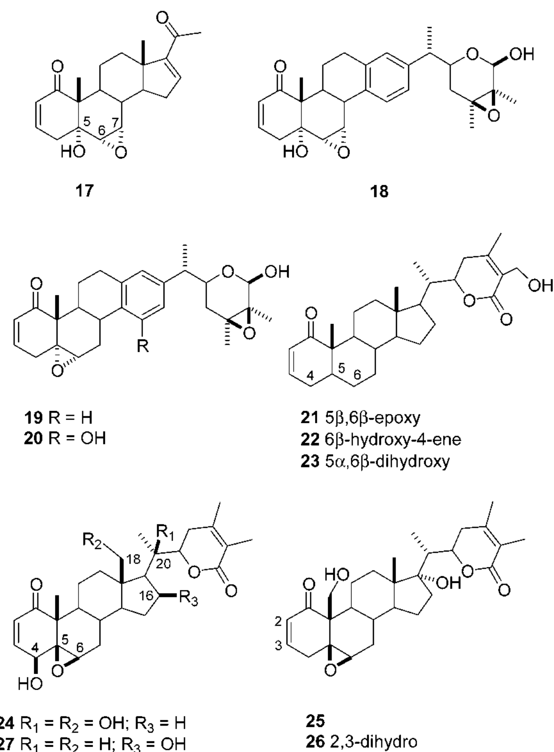
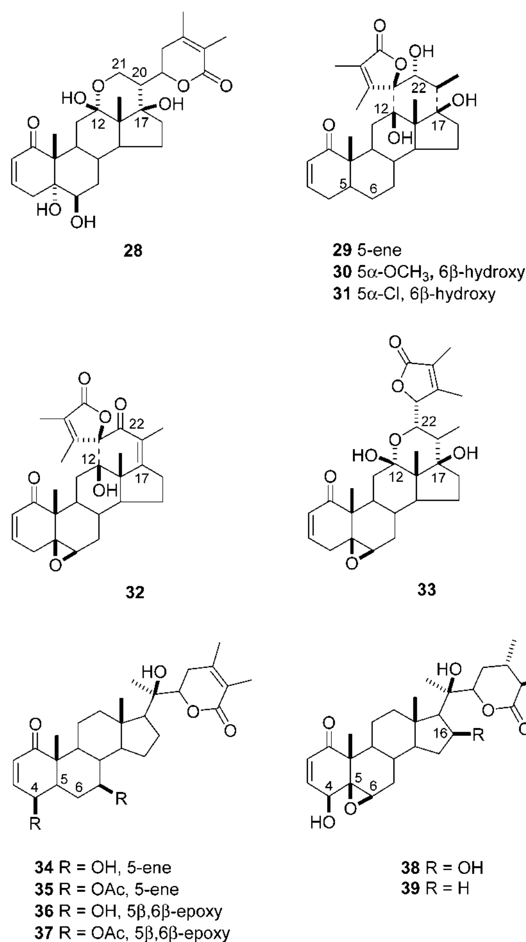
Lam.,[13] *J. leucotricha* (Speg.) A. T. Hunz.,[14] *J. odonelliana* A. T. Hunz.,[15,16] *J. runcinata* Lam.,[12] *J. sativa* (Miers) A. T. Hunz. & G. Barboza,[17] *Nicandra physaloides* (L.) Gaertn.,[18] *Salpichroa origanifolia* (Lam.) Thell,[19,20] and *Vassobia lorentzii* (Dammer) A. T. Hunz.[21] As an approach for the discovery of novel cancer chemopreventive agents, these pure withanolides (**1**−**37**) were tested for induction of quinone reductase with in vitro and in vivo models. Compilation of the results obtained with these withanolides has allowed a preliminary analysis of the structural features that might be responsible for inducing the activity of this phase II enzyme.





Results and Discussion

The potential of the withanolides (**1**−**37**) to induce QR activity in comparison with the activity of sulforaphane was tested. This system measures the specific activity of quinone reductase in murine hepatoma cells grown in 96-well microtiter plates and exposed to a range of concentrations of the tested compounds. Induction of QR activity was calculated from the ratio of specific enzyme activities of compound-treated cells in comparison with a solvent control. The concentrations required to double and quadruple QR activities in the cells, CD and CQ, respectively, were generated. To observe only the induction on QR and to avoid cytotoxic effects, the half-maximal inhibitory concentration of cell viability, IC_{50}, was also determined. Finally, from the ratio between the IC_{50} and CD or CQ values, chemopreventive indices (CI) were calculated. Such measurements not only predicted anticarcinogenic activity but also provided a reasonable index of potency and toxicity.

The induction of QR activity observed with withanolides **1**−**37** is summarized in Table 1. Data previously reported for withanolides **38** and **39** from *P. philadelphica* and sulforaphane are included for comparison. Of the withanolides examined in this study, compounds **2**, **3**, **5**, **22**, **24**, **26**, **29**, **32**, and **33** were promising inducers of QR activity with CI (IC_{50}/CD) values of 19.3, 10.0, 28.1, 11.1, >27.8, 7.2, >57.0, 28.9, and 28.7, respectively. Compounds **1**, **6**, **10**, **11**, **15**, **16**, **25**, and **28** failed to show induction of QR, and five additional compounds (**12**−**14**, **17**, and **23**) exhibited weak activity with CD values of 14.5−35 μM (Table 1).

From the subgroup of compounds with an oxidized methyl-18 isolated from *D. brachyacantha*[9] and *V. lorentzii*[21] (**1**−**11** and **24**), compounds **2**−**5**, **7**−**9**, and **24** were strong inducers of QR with CD values smaller than 1.05 μM, with most of them being more potent than sulforaphane (Table 1). The stereochemistry at C-18 did not affect inducer potency, since both withaphysalin H (18*R*, **3**) and withaphysalin I (18*S*, **4**) gave similar CD values (0.52 and 1.04 μM, respectively). When the 2,5-dien-1,4-diketone system was present, as in **10** and **11**, no induction of QR was found, although these compounds also presented a functionalized C-18. This finding as well as other drastic variations in activity associated with changes in the substitution pattern of rings A and B (e.g., compare **1** and **2**) indicates that these structural features have a decisive influence in promoting QR activity.

The presence of a 5α-hydroxy group, as in compounds **12**−**18**, **23**, and **28**, exerted a deleterious effect on the inducer activity. As a result, compounds bearing this group were much less potent (CD > 10 μM; Table 1) or inactive (e.g., **16** and **28**). This was evident when comparing compounds **21**, **22**, and **23** isolated from *J. integrifolia* that have the 27-methyl group oxidized to an alcohol.[13] Thus, jaborosalactone A (**21**) was as active as sulforaphane (CD = 0.29 μM), while cleavage of the epoxide to give the 5α-hydroxyl, as in compound **23**, decreased induction of QR (CD = 21.2 μM). Elimination of this group (as in **22**) restored the activity. In a similar way, the observed decrease in the induction activity for **19** and **20** may be

Table 1. Induction of Quinone Reductase Activity by Withanolides (**1**−**39**) and Sulforaphane[a]

compound	trivial name	CD (μM)	CQ (μM)	IC_{50} (μM)	IC_{50}/CD	IC_{50}/CQ
1	withaphysalin F	NI[b]	NI[b]	4.15	ND[c]	ND[c]
2	withaphysalin G	0.51	ND[c]	9.83	19.3	ND[c]
3	withaphysalin H	0.52	ND[c]	5.21	10.0	ND[c]
4	withaphysalin I	1.04	ND[c]	5.27	5.1	ND[c]
5	withaphysalin J	0.39	ND[c]	11.0	28.1	ND[c]
6	4,18-diacetylwithaphysalin G	NI[b]	NI[b]	5.10	ND[c]	ND[c]
7	NA[d]	<0.28	<0.28	0.39	>1.4	>1.4
8	NA[d]	<0.28	<0.28	0.42	>1.5	>1.5
9	NA[d]	<0.29	<0.29	0.57	>2.0	>2.0
10	withaphysalin K	NI[b]	NI[b]	7.29	ND[c]	ND[c]
11	withaphysalin L	NI[b]	NI[b]	0.65	ND[c]	ND[c]
12	exodeconolide A	15.8	>42.7	>42.7	>2.7	ND[c]
13	exodeconolide B	16.2	>42.7	>42.7	>2.6	ND[c]
14	withaferoxolide	14.5	40.8	>42.5	>2.9	ND[c]
15	withanone	NI[b]	NI[b]	NI[b]	ND[c]	ND[c]
16	withanolide A	NI[b]	NI[b]	>42.5	ND[c]	ND[c]
17	NA[d]	35.0	>58.4	>58.4	>1.7	ND[c]
18	nicandrenone	10.5	39.0	42.9	4.1	1.1
19	salpichrolide A	10.8	ND[c]	>42.9	>4.0	ND[c]
20	salpichrolide G	11.6	ND[c]	>42.9	>3.7	ND[c]
21	jaborosalactone A	0.29	0.68	1.83	6.3	2.7
22	jaborosalactone B	3.48	1.43	38.5	11.1	26.9
23	jaborosalactone D	21.2	>42.3	>42.3	>1.7	ND
24	18-hydroxywithanolide D	0.27	0.54	1.93	7.2	3.5
25	jaborosalactone L	NI[b]	NI[b]	NI[b]	ND[c]	ND[c]
26	jaborosalactone O	1.52	>42.3	>42.3	>27.8	ND[c]
27	16-deacetyliochromolide	<0.32	0.49	0.66	>2.1	1.3
28	jaborosalactone S	NI[b]	NI[b]	>39.8	ND[c]	ND[c]
29	jaborosalactone P	0.75	ND[c]	>42.7	>57.0	ND[c]
30	jaborosalactone 12	9.32	ND[c]	>39.0	>4.2	ND[c]
31	jaborosalactone 10	7.32	ND[c]	>38.0	>5.2	ND[c]
32	jaborosalactone 1	0.28	1.27	8.08	28.9	6.3
33	trechonolide A	0.27	1.26	7.74	28.7	6.1
34	7-hydroxy-14-deoxywithanolide U	0.47	2.66	1.66	3.5	0.62
35	7-acetyl-14-deoxywithanolide U	0.27	ND[c]	0.81	>3.0	ND[c]
36	NA[d]	0.51	ND[c]	1.27	2.5	ND[c]
37	NA[d]	0.26	ND[c]	0.33	1.3	ND[c]
38	withaphysacarpin	0.43[e]	1.9[e]	4.8[e]	11.1[e]	2.5
39	24,25-dihydrowithanolide D	0.70[e]	3.8[e]	5.5[e]	7.8[e]	1.4
	sulforaphane	0.49[e]	10.6[e]	11.7[e]	23.9[e]	1.1

[a] QR activity was determined with Hepa lclc7 cells as described in the Experimental Section. CD, concentration required to double QR activity; CQ, concentration required to increase QR activity 4-fold; IC_{50}, concentration to inhibit cell growth by 50%; CI, chemoprevention index (IC_{50}/CD or IC_{50}/CQ). [b] NI = no induction. [c] ND = not determined. [d] NA = trivial name not available. [e] Data taken from ref 5.

correlated with the presence of a 5α-oxygenated substituent (the 5α,6α-epoxide). Jaborosalactone S (**28**) belongs to a subgroup of withanolides that possess, in addition to the typical δ-lactone side chain, a six-membered hemiketal ring. Although this compound was the only one available representing its subgroup, the 5α-hydroxy group might be responsible for the lack of activity.

Compounds **29**−**32** contain a side chain with a carbon−carbon bond between C-12 of the intact steroid nucleus and C-23, and a spiranoid γ-lactone at the latter position. The additional ring thus formed has a 17,22-dihydroxy system, as in compounds **29**−**31**, or a 17(20)-ene-22-keto system, as in jaborosalactone 1 (**32**). In this group of withanolides, jaborosalactone P (**29**) and jaborosalactone 1 (**32**) are the most interesting, with CI (IC_{50}/CD) values of >57.0 and 28.9, respectively (Table 1). It is noteworthy that compound **29** displayed activity comparable to sulforaphane but no cytotoxicity at the highest concentration tested, resulting in a superior induction ratio for quinone reductase. The deleterious effect of a 5α-hydroxyl previously discussed may be extended to other 5α-substituents. Within the spiranoid withanolides, the presence of either a 5α-methoxy or a 5α-chloro substituent (compounds **30** and **31**) resulted in poorer induction in terms of CD values relative to **29** and **32**.

Trechonolide A (**33**) was the only compound available from this subgroup with a hemiacetal bridge formed between a 22-OH and a 12-ketone, resulting in a stable six-membered ring with a β-oriented 12-OH group and a γ-lactone system as a side chain. With CD and IC_{50} values of 0.27 and 7.74 μM, respectively, a favorable CI of 28.7 (Table 1) was obtained. Finally, the 7-hydroxy and 7-acetoxy derivatives **34**−**37** exhibited high induction activity, but also high cytotoxicity, resulting in low CI values.

In each subgroup of withanolides analyzed, we have shown that some substituents may lead to changes in quinone reductase activity. Within the limits of our data, several observations may be made. The current results indicate that a functionalized methyl-18 plays an important role in improving QR activity, as in compounds **2**−**5**, **7**−**9** and **24**. On the other hand, the presence of 5α-substituents, as in compounds **12**−**20**, **23**, **28**, **30**, and **31**, results in lower activities. In general, spiranoid- and trechonolide-type withanolides exhibit good QR induction.

In terms of CI values, some of the compounds described above compare favorably with sulforaphane, a known chemopreventive agent (Table 1). In fact, when the CI value is calculated on the basis of CQ, some of the isolates may be viewed as superior to sulforaphane (Table 1). Among these agents, jaborosalactone P (**29**) was one of the most promising in terms of inducing potency and low toxicity. To further evaluate the potential of jaborosalactone P (**29**), a preliminary study was performed to test the capacity of this agent to induce steady-state levels of quinone reduc-

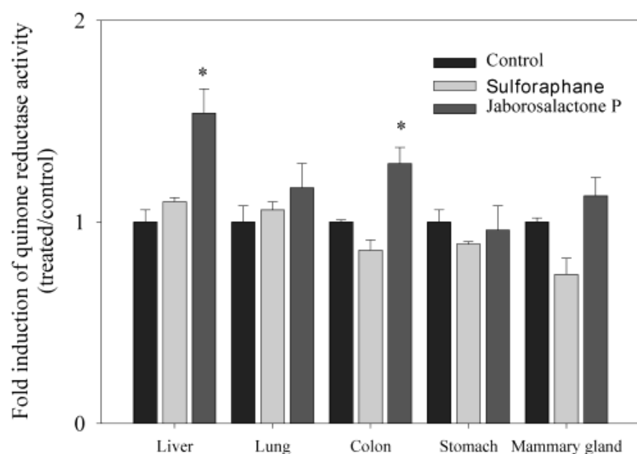


Figure 1. Effect of jaborosalactone P (**29**) and sulforaphane on quinone reductase activity in various organ sites of the mice. *: treatment groups were significantly different from the basal control group ($p < 0.05$) using Student's *t*-test with $n = 3$.

tase in multiple organ sites of BALB/c mice. Sulforaphane was used for comparison in this in vivo study. The scope of these tests was limited due to limited supplies of test compounds. Nonetheless, as shown in Figure 1, with jaborosalactone (**29**)-treated mice, significant induction was observed in liver and colon, but not in lung, stomach, or mammary gland. However, no significant induction was observed in all organ sites of mice treated with sulforaphane. The lack of inducing activity of sulforaphane in this experiment was possibly due to the dose level employed or the duration of the treatment period. Notably, however, the results of this in vivo study further confirm our in vitro findings that withanolides can function as potent phase II enzyme inducers. We are currently isolating larger quantities of selected withanolides in order to conduct more extensive in vivo evaluations.

Experimental Section

Chemicals. Test compounds were isolated and identified by the authors as referenced above from the species *Datura ferox* (**14**), *Dunalia brachyacantha* (**7**−**9**, **27**), *Exodeconus maritimus* (**12**, **13**, **15**−**17**), *Iochroma australe* (**34**−**37**), *Jaborosa araucana* (**33**), *J. integrifolia* (**21**−**23**), *J. leucotricha* (**25** and **26**), *J. odonelliana* (**29**−**31**), *J. runcinata* (**32**), *J. sativa* (**28**), *Nicandra physaloides* (**18**), *Salpichroa origanifolia* (**19**, **20**), and *Vassobia lorentzii* (**1**−**6**, **10**, **11**, and **24**). Prior to biological testing, all compounds were analyzed by TLC on Si gel 60 F254 (Merck) plates using hexanes−EtOAc (4:1, 1:1, or 2:3 v/v) as mobile phase. Spots were visualized by spraying with 10% H_2SO_4 in EtOH and heating. A purity greater than 95%, as verified by ^{1}H NMR spectroscopy, was considered acceptable.

Biological Assays for the Induction of Quinone Reductase (QR) with Cultured Mouse Hepatoma Cells. For the evaluation of pure isolates as inducers of QR, cultured mouse Hepa 1c1c7 cells were used as described previously.[22,23] In brief, 96-well plates were seeded at a density of 4000 cells/mL (200 μL/well) and incubated for 24 h at 37 °C in a CO_2 incubator. The medium was then changed, and test compounds, dissolved in 10 μL of 10% DMSO, were introduced and serially diluted in a concentration range of 0.15−20 μg/mL. The cells were incubated for an additional 48 h. Quinone reductase activity was measured by the NADPH-dependent menadiol-mediated reduction of MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] to a blue formazan. Protein levels were determined in a duplicate set of plates using crystal violet staining and subsequent measurement at 595 nm.[23] Induction of QR activity was calculated from the ratio of specific enzyme activities of compound-treated cells in comparison with a solvent control. Enzyme activity was expressed as CD, concentration required to double the specific activity of QR; CQ, concentration required to quadruple the specific activity of QR; IC_{50}, half-maximal inhibitory concentration of cell viability; and CI (chemoprevention index), IC_{50}/CD, or IC_{50}/CQ.

Induction of Phase II Enzyme Activities in Animal Tissues. BALB/c mice ($n = 3$) were treated with test agents for 3 consecutive days (2.5 mg in 0.2 mL of sesame oil/mouse /day, i.g.), and the changes in quinone reductase activities were assessed in liver, lung, colon, stomach, and mammary gland, as described previously.[24]

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