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Antiproliferative withanolides from several Solanaceous species

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Abstract

To date, our work on Solanaceous species (Datura wrightii, Jaborosa caulescens, Physalis hispida, P. longifolia, Vassobia breviflora, and *Withania somnifera*) has resulted in the isolation of 65 withanolides, 31 of which were new, as well as the semi-synthesis of a further 30 withanolides. Structure identification and MTS assay-based antiproliferative evaluation of these 95 compounds revealed that a Δ^2-1-oxo functionality in ring A; in conjunction with either a 5β,6β-epoxy or 5α-chloro-6β-hydroxy moiety in ring B; are the minimum structural requirements for withanolides to produce potent cytotoxic activity. Such structural-activity relationship analysis (SARA) also revealed that oxygenation (the –OH or –OR groups) at C-4, 7, 11, and 12; as well as C-14 to C-28; did not contribute toward the observed antiproliferative activity. Herein we present a complete overview of our work as it relates to the withanolides reported from 1965 to 2013.

Keywords

withanolide; Solanaceae; *Datura wrightii*; *Jaborosa caulescens*; *Physalis hispida*; *Physalis longifolia*; *Vassobia breviflora*; *Withania somnifera*; antiproliferative; cytotoxicity; structure activity relationship

1. Introduction

Withanolides are highly oxygenated steroids classified into 24 structural types (**I**–**XXIV**) derived from a C_{28} ergostane skeleton (Figure 1). The first compound in this class was reported in 1965 and called withaferin A (**1**) (Kupchan et al. 1965; Lavie et al. 1965) (Figure 2). Since this time approximately 900 withanolides have been reported, formed by ring fission, cyclization, or skeleton rearrangements of the steroid nucleus or the nine carbon side chain (Cao et al. 2014; Jin et al. 2012; Ma et al. 2007; Zhang et al. 2012a). The presence of these compounds are predominantly reported in the Solanaceous genera of Acnistus, Aureliana, Brachistus, Browallia, Datura, Deprea, Discopodium, Dunalia, Exodeconus, Hyoscyamus, Iochroma, Larnax, Lycium, Mandragora, Nicandra, Salpichroa, Saracha, Solanum, Trechonaetes, Tubocapsicum, Vassobia, and *Witheringia*. The primary sources of the natural withanolides come from extensively studied species in the genera *Jaborosa*, *Physalis*, and *Withania* (Chen et al. 2011; Misico et al. 2011; Zhang et al. 2012a).

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Interestingly, more than 13 (**I, III, V**–**VIII, XI, XVII, XIX**–**XXIV**) of the 24 known structural types have been reported in *Physalis* to date (Cao et al. 2014; Jin et al. 2012; Ma et al. 2007; Zhang et al. 2012a). Withanolides have also been isolated from several non-solanaceous plants, such as in members of the Dioscoreaceae (Kim et al. 2011), Fabaceae (Ray et al. 1994), Lamiaceae (Chen et al. 2011), Myrtaceae (Vankar et al. 2009), and Taccaceae (Chen et al. 2011), as well as the marine Alcyoniidae (Chao et al. 2011, Ksebati et al. 1988).

Unmodified withanolides (type **I**) are by far the most abundant withanolide category observed in nature, where approximately 580 have been reported in the Solanaceae alone. Due to their vast abundance in nature compared to the other varieties (**II**–**XXIV**), type **I** withanolides are further sub-categorized according to specific side chain variations (**Ia** δ-lactone; **Ib** δ-lactol; **Ic** and **Id** γ-lactone; **Ie** γ-lactol). Furthermore it is reasonable to suppose that type **I** withanolides are the biogenetic precursors to the more advanced structural types **II**–**XXIV** (Figure 1).

In recent years withanolides have gained significant scientific interest due to their structural and biological diversity, as well as their antitumor capacities, where greatest antiproliferative potency was observed in type **I** withanolides containing an α,β-unsaturated ketone in ring A; a 5β,6β-epoxy group in ring B; and a nine-carbon side chain with a δ-lactone. (Chen et al. 2011; Misico et al. 2011; Zhang et al. 2012a).

As part of our continuing investigations to discover drug leads from plant biodiversity, we have explored the antiproliferative potential of compounds present in several members of the Solanaceae family. The *in vitro* MTS cytotoxic bioassay guided fractionation of *Datura wrightii* Regel; *Jaborosa caulescens* Gillies & Hook; *Physalis hispida* (Waterf.) Cronquist; *P. longifolia* Nutt.; *Vassobia breviflora* (Sendtn.) Hunz; and *Withania somnifera* (L.) Dunal; led to the isolation and characterization of 65 withanolides (**1**–**65**) as well as a further 30 withanolide derivatives (**66**–**95)** (Figures 2–5). In this report, we provide a complete summary of our work as well as an overview of the structural types of withanolides reported from 1965 to 2013.

2. Withanolides isolated from natural sources

2.1. Withanolides of Vassobia breviflora and Withania somnifera

The first Solanaceae species we investigated was the Latin American spiny shrub *V. breviflora*, where cytotoxicity-guided purification led to the isolation and characterization of withaferin A (**1**) (Samadi et al. 2009; Samadi et al. 2010). Preliminary results revealed **1** as a promising chemotherapeutic candidate for antitumor therapy, and that further translational evaluation of **1** was warranted (Grogan et al. 2013; Samadi et al. 2012). However, difficulties arose in obtaining sufficient quantities of *V. breviflora* biomass needed to isolate the required amounts of **1** for such studies.

To overcome this obstacle we investigated the commercially available roots of *W. somnifera* (Solanaceae) from where **1** was first discovered. This species is popularly known as Ashwagandha or Indian ginseng and widely used in the traditional Ayurvedic system of

plant medicine for immune-modulation and anti-aging (Misra et al. 2008). To date more than 130 diverse withanolides, the vast majority of which possess type **Ia** skeleton (Figure 1), have been identified in *W. somnifera*, which represents the largest number of withanolides reported in a single species. In addition to **1**, our study of the species also resulted in the isolation and identification of the new withanolide 6α-chloro-5β,17α-dihydroxywithaferin A (**6**) in conjunction with further known [withanolide D (**2**), 27-hydroxywithanolide D (**3**); 5,6-deoxywithaferin A (**4**); 16β-acetoxy-6α,7α-epoxy-5α-hydroxy-1-oxowitha-2,17(20),24-trienolide (**5**); 6α-chloro-5β-hydroxywithaferin A (**7**); 2,3-didehydrosomnifericin or 5β,6α-dihydroxywithaferin A (**8**); withanolides A (**9**), 27-hydroxywithanolide A (**10**), and withanolide B (**11**); 27-hydroxywithanolide B (**12**), withanone (**13**); (22*R*)-5β-formyl-6β,27-dihydroxy-1-oxo-4-norwitha-24-enolide (**14**); 2,3-dihydrowithaferin A (**15**); 3-methoxy-2,3-dihydrowithaferin A (**16**); withanoside IV (**17**) and withanoside X (**18**)] withanolides (Figure 2) (Tong et al. 2011; Zhang et al. 2014a).

2.2. Withanolides of Physalis longifolia and P. hispida

Preliminary screening of our ethnobotanical library, which includes in excess of 200 species native to the U.S. Great Plain, suggested that withaferin A (**1**) was also present in the wild tomatillo *Physalis longifolia* Nutt. (Solanaceae), commonly known as “long leaf groundcherry” or “wild tomatillo” (Kindscher et al. 2012). In addition to **1**, our investigation on this species resulted in the isolation and identification of an array of new [withalongolides A-P (**19**–**34**)] and other known [2,3-dihydrowithaferin A (**15**), 3β-methoxy-2,3-dihydrowithaferin A (**16**), sitoindoside IX (**35**), viscosalactone B (**36**), 2,3-dihydro-3β-*O*-sulfate withaferin A (**37**), and 3α,6α-epoxy-4β,5β,27-trihydroxy-1-oxowitha-24-enolide (**38**)] withanolides (Figure 2) (Zhang et al. 2011; Zhang et al. 2012b).

Such unprecedented withanolide variety indicated that the *Physalis* genus was a good source of diverse withanolides and clearly suggested that other *Physalis* species were worthy of further exploration. This led to the phytochemical investigation of the North American herbaceous perennial *Physalis hispida* (Waterf.) Cronquist, commonly known as “prairie groundcherry”, which resulted in the isolation of new [withahisolides A–I (**39**–**47**)] as well as known [nicaphysalin E (**48**), nicandrenone (**49**), nicandrenone methyl ether (**50**), nicandrenone 12 (**51**); salpichrolides A (**52**), C (**53**), and N (**54**); physalindicanols A (**55**) and B (**56**)] withanolides (Figure 3) (Cao et al. 2014). In an effort to further diversify our withanolide library and better probe the withanolide antiproliferative structural activity relationship model, it was decided that different Solanaceous genera warranted further investigation, namely the species of *Datura* and *Jaborosa*.

2.3. Withanolides isolated from Datura wrightii and Jaborosa caulescens var. bipinnatifida

Since the *Datura* genus is renowned as a rich source of oxygen-substituted C-21 withanolides (Anjaneyulu et al. 1998), we initiated a study into North American herbaceous perennial *Datura wrightii* Regel, which resulted in the isolation of new [withawrightolide (**57**)] and known [withametelin L (**58**), daturilin (**59**), withametelin O (**60**), and withametelin F (**61**)] withanolides (Figure 4) (Zhang et al. 2013). Similarly, our investigation of the Chilean perennial shrub *Jaborosa caulescens* var. *bipinnatifida* also resulted in the isolation of both new [2,3-dihydrotrechonolide A (**62**); 2,3-dihydro-21-hydroxytrechonolide A (**63**)]

and known [trechonolide A (**64**), jaborosalactone 39 (**65**)] withanolides (Figure 4) (Zhang et al. 2014b).

3. Structural diversity among the naturally occurring withanolides

Withanolides (**1**–**65**) obtained from natural sources were characterized by 2D NMR as well as HRMS data, and compared against those reported in the literature. The structures of 13 (**3, 4, 6, 11, 19–21, 24, 33, 39, 40, 58**, and **62**) of these were subsequently confirmed by single crystal X-ray diffraction crystallography (Cao et al. 2014; Tong et al. 2011; Zhang et al. 2011; Zhang et al. 2012b; Zhang et al. 2013; Zhang et al. 2014a; Zhang et al. 2014b).

These naturally occurring withanolides (**1**–**65**) represent 8 of the 24 withanolide structural types, which include type **I** (**1**–**13**), **II** [presence of cyclopropane moiety in ring A in the steroidal nucleus (**57**)], **VI** [presence of six-membered ring D in the steroidal nucleus, (**39**–**45**)] **VII** [presence of re-arranged C-18 methyl group (**54**)], **VIII** [19-nor withanolides (**29**–**32**)], **XI** [4-nor withanolides (**24**)], **XII** [presence of re-arranged C-4 (**14**)], **XXIV** [absence of a C-13 to C-17 linkage (**46**)]. It should also be mentioned that at this juncture all reported naturally occurring type **VI** withanolides ubiquitously exhibited an aromatic ring D moiety (Anjaneyulu et al. 1998; Chen et al. 2011; Misico et al. 2011; Ray et al. 1994; Veleiro et al. 2005; Zhang et al. 2012a). However our recent identification of naturally occurring non-aromatic six-membered ring D withanolides (**39**–**41)** suggests that the drawing style of type **VI** withanolides should be altered to incorporate both the aromatic and non-aromatic ring D varieties as depicted (Figure 1) (Cao et al. 2014).

Even though oxygenated carbons at C-22 and C-26 were present in all isolates, there were still oxygenation pattern diversities amongst the withanolides (**1**–**65**). For instance, **32**, **55** and **56** lack oxygenation at C-1, whereas all other withanolides present common oxygenated carbons at C-1, C-22, and C-26 (Glotter 1991). Further oxygenated positions are observed at C-3 (**15**, **16**), C-4 (**1**–**3**), C-5 (**9**–**13**), C-6 (**1**–**3**, **5**, **8**), C-7 (**5**, **9**–**13**, **29**, **33**), C-11 (**21**), C-12 (**58**, **62**–**65**), C-13 (**41**, **46**), C-14 (**41**), C-16 (**5**), C-17 (**6**, **46**), C-19 (**19**, **20**, **25**), C-20 (**2**, **3**), C-21 (**57**–**61**, **63**, **65**), C-23 (**62**–**64**), C-24 (**39**–**42**, **57**–**61**), C-25 (**39**–**42**), C-27 (**1**, **2**, **19**), and C-28 (**28**–**32**). Furthermore, among the naturally occurring isolates (**1**–**65**) are a series uncommon 3-*O*-sulfated (**25** and **37**) and chlorinated (**6** and **7**) withanolides.

4. Withanolide artifacts and derivatives

4.1 Artificial withanolides resulted from intra/inter Michael addition

During investigation on the isolation and structural elucidation of withanolides, the instability of withanolides with Δ^2-1-oxo functionality was noticed when exposed to certain solvents. In depth analysis suggested, and subsequently proven via deuterated solvent experiments, that intramolecular (**66**, **67**) and intermolecular (**16**, **22**, **68**) Michael addition withanolide artifacts were formed from methanolic solutions of withaferin A (**1**), withalongolide A (**19**) and withalongolide B (**20**) (Figure 2) (Cao et al. 2013).

4.2. Derivatives prepared from the natural isolates

In order to better probe the withanolide antiproliferative structural activity relationship model, a total of 27 semi-synthetic withanolide derivatives (**69**–**95**) were prepared from natural isolates (**1**, **19**, **20**, and **33**) (Figure 5) (Zhang et al. 2011; Zhang et al. 2012b; Motiwala et al. 2013).

5. Antiproliferative evaluation of withanolide library

Extensive MTS-based assaying revealed that withaferin A (**1**) reduced cell viability with IC_{50} values in the 0.16–2.9 μM range against an array of human [brain (U-87, U-251), colorectal (DRO81-1), laryngeal (JHU-011), oral cavity (JMAR, MDA-1986, UM-SCC-2), skin (SK-MEL-28), and thyroid (B-CPAP, FTC-133, FTC-236, FTC-238, SW-1736, TPC-1)] and murine [brain (GL26) and skin(B16-F10)] carcinoma cell lines. Furthermore, mechanistic studies showed that **1** inhibits proliferation by inducing a dose-dependent G2/M cell cycle arrest while promoting cell death through both intrinsic and extrinsic apoptotic pathways (Grogan et al. 2013; Samadi et al. 2009; Samadi et al. 2010; Samadi et al. 2012).

Comparable IC_{50} values in the 0.26–2.9 μM range were also observed in several other natural withanolides [2,3-dihydro-3-*O*-sulfate withaferin A (**37**); withalongolide B (**20**), and withanolide D (**2**)]. Greater potency was observed in the acetylated derivatives withalongolide A 4,19,27-triacetate (**79**), withalongolide B 4,19-diacetate (**73**), and withalongolide O 4,7-diacetate (**71**), which collectively produced IC_{50} values in the 0.067 *n*M – 2.0 μM range (Zhang et al. 2011; Zhang et al. 2012b; Motiwala et al. 2013).

6. Structure-activity relationship analysis

A structure activity relationship analysis (SARA) was initiated on the basis of these results and compared against those reported in the literature. Through this process it became obvious that the most potent withanolides shared a common pharmacophore (ring-A Δ^2-1-oxo functionality; and ring-B 5β,6β-epoxy moiety) as evidenced in withaferin A (**1**), withanolide D (**2**), withalongolide B (**20**), withalongolide A 4,19,27-triacetate (**79**), withalongolide B 4,19-diacetate (**73**), and withalongolide O 4,7-diacetate (**71**) (Zhang et al. 2011; Zhang et al. 2012b).

Examination of structural variations in ring A revealed that potency was retained through replacement of the Δ^2-1-oxo with a 2,3-dihydro-3-*O*-sulfate-1-keto functionality, as this generated a water soluble pro-drug variety that spontaneously converted back to the bioactive Δ^2-1-oxo form (Xu et al. 2009). In contrast the presence of either a 2,3-dihydro-1-keto; 2,3-dihydro-3-oxymethy-1-keto; 1,3-dihydroxy; or Δ^3-1-oxo functionality significantly reduced or lost the antiproliferative activities compared to the bioactive Δ^2-1-oxo form (Figure 6).

Similar analysis of ring-B revealed that replacement of the 5β,6β-epoxy with an 5α-chloro-6β-hydroxy moiety retained potency. Conversely the presence of a Δ^5; 5α,6α-epoxy; 5β-chloro-6α-hydroxy; or 5α-hydroxy-6α,7α-epoxy functionality significantly reduced or lost the antiproliferative activity (Figure 6).

Among the withanolide structural types examined only type **I** produced significant antiproliferative potency, whereas types (**II**, **VI**, **VIII**, **XI**, **XII**, and **XXIV**) devoid of the aforementioned pharmacophore were inactive against the antiproliferative assays tested.

Due to the diverse oxygenated patterns of the withanolides obtained, the –OH and –OR groups at carbons C-4, 7, 11, 19, 20, and 27 could be directly evaluated by comparing the data of withaferin A (**1**), 7-hydroxywithaferin A (**33**), 11-hydroxywithaferin A (**21**), 19-hydroxywithaferin A (**19**), jaborosalatone V 19,27-diacetate (**88**), 27-deoxywithalongolide A (**20**), and 27-*O*-glycopyranoside-withaferin A (**35**). In addition, SARA indicated that the –OH and –OR groups on carbons C-4, 7, and 11–28 were noncontributory toward antiproliferative activity, although acetylation of the hydroxyl group will increase the cytotoxicity (Zhang et al. 2011; Zhang et al. 2012a).

7. Conclusions

Identification and antiproliferative evaluation of a total of 95 diverse withanolides (**1**–**95**) revealed a series of bioactive compounds with IC_{50} values in the 0.07–2.9 μM range, which could be potentially explored as antitumor agents. Structural-activity relationship analysis (SARA) confirmed the importance of the presence of a Δ^2-1-oxo functionality in ring A, a 5β,6β-epoxy or 5α-chloro-6β-hydroxy grouping in ring B for cytotoxic activity. These studies also revealed that the –OH or –OR moieties at C-4, 7, and 11–28 were noncontributory toward antiproliferative activity. We believe that in the future our ongoing withanolide-based research will contribute to revealing the full mechanism of action of these promising antitumor therapeutics.

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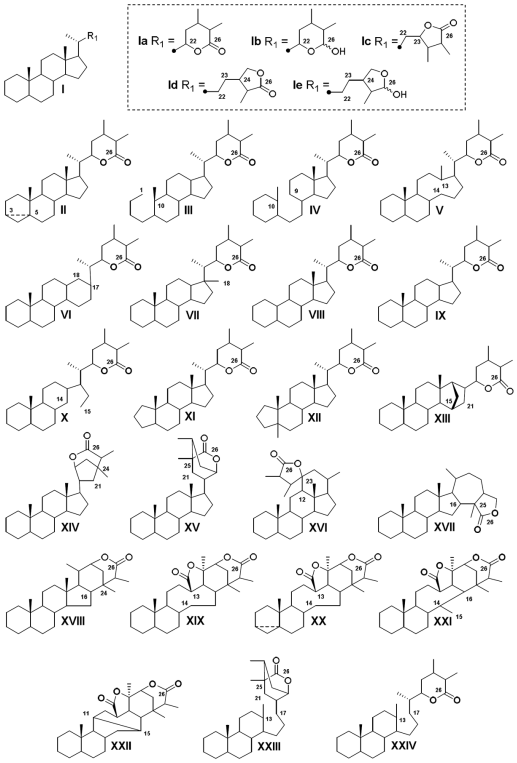

Figure 1.
The withanolide types (**I–XXIV**) isolated between 1965 and 2013.

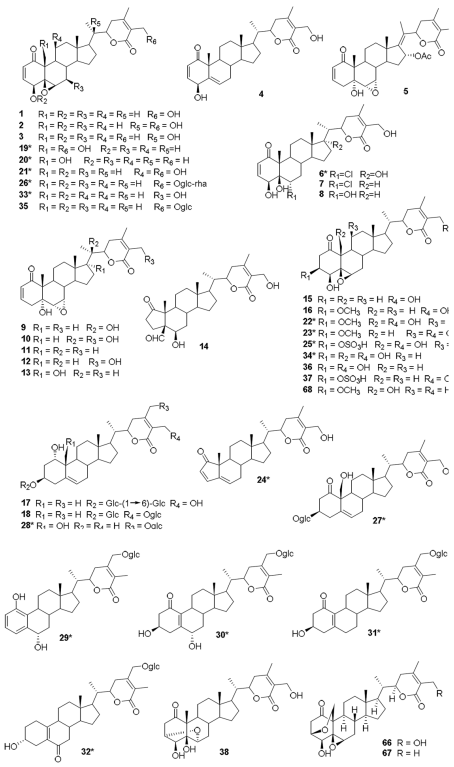

Figure 2.

New* (**6**, **19–34**, **66–68**) and known (**1–5**, **7–18**, **35–38**) withanolides from *Physalis longifolia* (**1**, **15**, **16**, **19–38**), *Vassobia breviflora* (**1**), *Withania somnifera* (**1–18**) and withanolide artifacts (**66–68**).

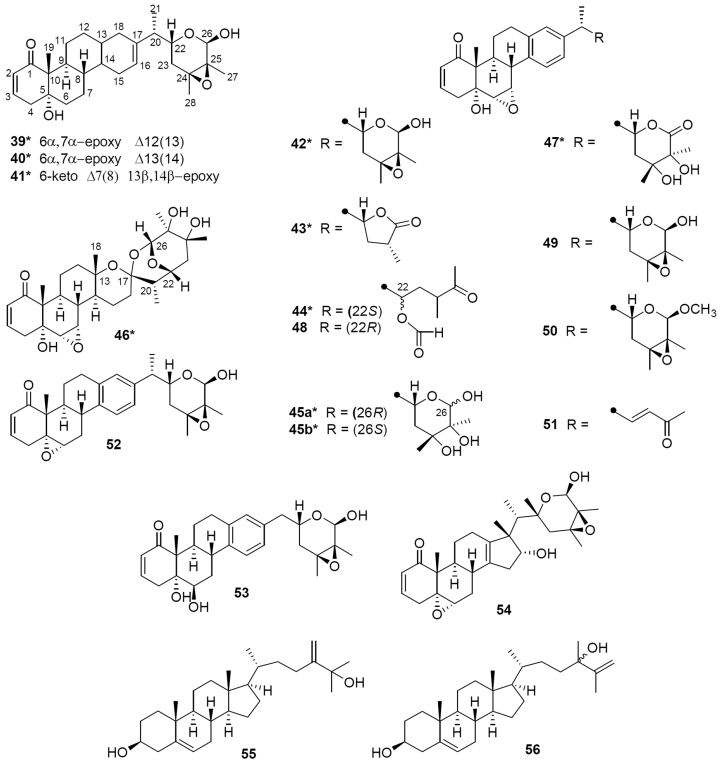

Figure 3.

New* (**39**–**47**) and known (**48**–**56**) withanolides from *Physalis hispida*.

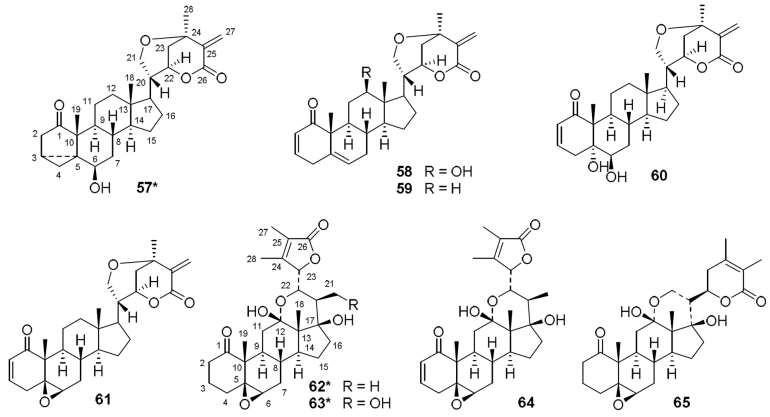

Figure 4.

New* (**57**, **62**–**63**) and known (**58**–**61**, **64**–**65**) withanolides from *Datura wrightii* (**57**–**61**) and *Jaborosa caulescens* (**62**–**65**).

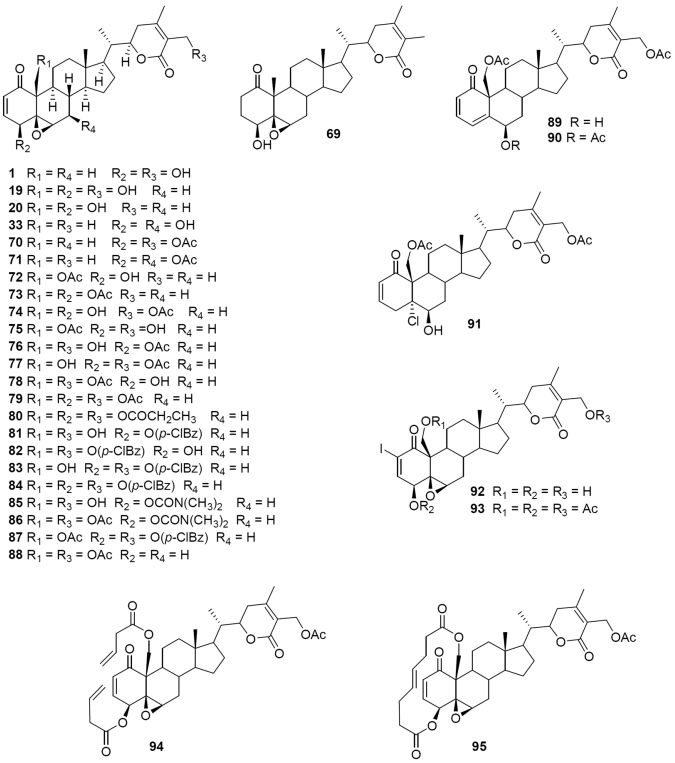

Figure 5.

Derivatives **69**–**95** prepared from the natural products withaferin A (**1**), withalongolides A (**19**), B (**20**) and O (**33**)

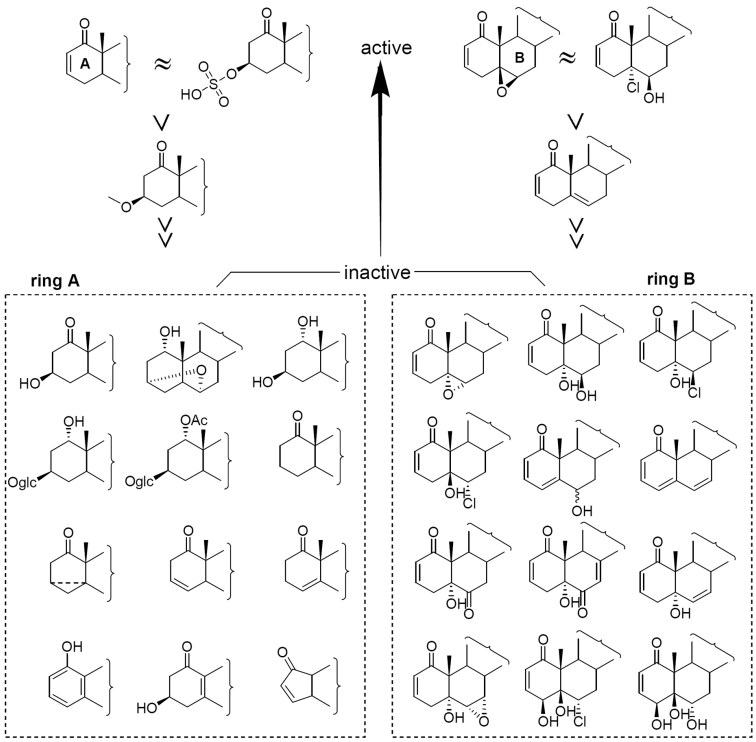

Figure 6.

Structural variations in rings A (left) and B (right) of the withanolides are closely related to the observed antiproliferative activity.