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# Quantitative Structure – Activity Relationship Study on Saponins as Cytotoxicity Enhancers

Reneta Gevrenova[1], Alexander Weng[2], Laurence Voutguenne-Nazabadioko[3], Mayank Thakur[4] and Irini Doytchinova[5,*]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav st., 1000 Sofia, Bulgaria*

[2]*Molecular Immunology Unit, UCL Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, UK*

[3]*Institute of Molecular Chemistry of Reims, UMR CNRS 7312, Bâtiment 18, BP 1039, 51687 Reims cedex 2, France*

[4]*Institut für Laboratoriumsmedizin, Klinische Chemie und Pathobiochemie, Charité – Universitätsmedizin Berlin, Campus Virchow-Klinikum, Augustenburger Platz 1, D-13353 Berlin, Germany*

[5]*Department of Chemistry, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav st., 1000 Sofia, Bulgaria*

**Abstract:** Saponins enhance the cytotoxicity of the type I ribosome-inactivating protein (RIP-I) saporin. In the present study, the synergistic cytotoxicity of nine newly isolated gypsogenin-containing saponins from *Gypsophila trichotoma* in combination with RIP-I was evaluated *in vitro* and used to derive a quantitative structure – activity relationship (QSAR). The QSAR model distinguished two important structural features of the studied saponins necessary for their cytotoxicity enhancing activity: the branched trisaccharide moiety attached to C-3 should contain one xylose residue instead of an arabinose and the branched tetrasaccharide at C-28 should contain one acetyl group attached to the glucose residue.

**Keywords:** Cancer, cytotoxicity, saponins, saponin, targeted anticancer therapy, QSAR, PLS.



## INTRODUCTION

Saponins are a structurally diverse class of compounds occurring in many plant species which are chemically referred to as triterpenoid and steroidal glycosides [1]. They consist of non-sugar aglycone and one or more sugar chains linked by glycosidic and (or) ester binding to the aglycone. Saponins have been reported to possess anti-tumor properties, to inhibit angiogenesis and to induce tumor apoptosis [2-4]. Other studies have reported that saponins play a role in the reduction of tumor invasiveness and multidrug resistance [5], which suggests that they could represent alternative agents in cancer treatment. In addition, it has been shown that saponins combined with some conventional chemotherapeutic agents exerted synergistic inhibitory effect on tumor growth *in vitro* and *in vivo* [3]. Moreover, saponin-adjuvanted particulate vaccines were reported to have great potential as cancer immunotherapeutics [6]. Among other interesting properties of saponins are their roles in permeabilization and hemolysis [7].

Roots from *Gypsophila* species are an extremely rich source of triterpenoid saponins belonging to the group of GOTCAB saponins (glucuronide oleanane-type triterpenoid carboxylic acid 3,28-bidesmosides) [8-12]. Previous reports have shown that saponins from *Gypsophila oldhamiana* Miq. and *Gypsophila pilulifera Boiss. & Heldr.* display cytotoxic activity against different human cancer cell lines [13-15].

It has also been reported that *Saponinum album, G. paniculata* and *G. arrostii* Guss. var. *nebulosa* (Caryophyllaceae) saponins enhance the cytotoxicity of the type I ribosome-inactivating protein (RIP-I) saporin from *Saponaria officinalis* L. [7, 11, 16-18]. The principle of the synergistic cytotoxicity was adopted for saporin-based targeted toxins which are used in tumor therapy [12, 19]. Targeted toxins are proteins consisting of two components: a toxic protein and a cell-binding domain like the epidermal growth factor (EGF), which targets tumor associated antigens. Bachran et al. [20] have demonstrated that the targeted toxin in combination with *Saponinum album* enters cells via clathrin- and actin-dependent pathways [20]. Böttger *et al.* [21] have analyzed the structure – activity relationships of 56 saponins and their synergistic cytotoxicity with saporin and have defined the concept of ideal saponin. According to this concept, the ideal RIP-I synergistic saponin consists of an oleanane-type aglycone (gypsogenin or quillaic acid), a branched trisaccharide at C-3 including β-D-glucuronic acid, β-D-galactopyranose and β-D-xylopyranose and a branched tetrasaccharide at C-28 including desoxy-sugars such as β-D-fucose and/or α-L-rhamnose and acetyl residues.

Recently, fifteen new GOTCAB saponins were isolated from the roots of *Gypsophila trichotoma* Wend. *var. trichotoma* [10, 22]. The saponins have a commonly found gypsogenin as an aglycone substituted at C-3 with a trisaccharide and at C-28 with an oligosaccharide bound to

*Address correspondence to this author at the Department of Chemistry, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav st., 1000 Sofia, Bulgaria; Tel: +35929236506; Fax: +35929879874;
E-mail: doytchinova@gmail.com

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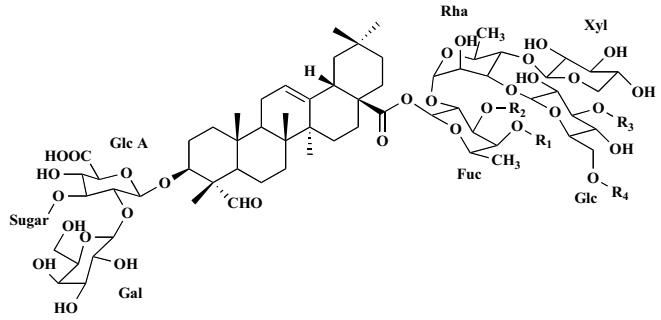



| saponin | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Sugar |
|---|---|---|---|---|---|
| 1 | $COCH_3$ | H | $SO_3H$ | H | Ara |
| 2 | H | $COCH_3$ | $SO_3H$ | H | Ara |
| 3 | $COCH_3$ | H | H | H | Ara |
| 4 | $COCH_3$ | H | H | $COCH_3$ | Ara |
| 5 | $COCH_3$ | H | H | $COCH_3$ | Xyl |
| 6 | H | H | H | H | Ara |
| 7 | CisMCin | H | H | H | Ara |
| 8 | TransMCin | H | H | H | Ara |
| 9 | $COCH_3$ | H | H | H | Xyl |

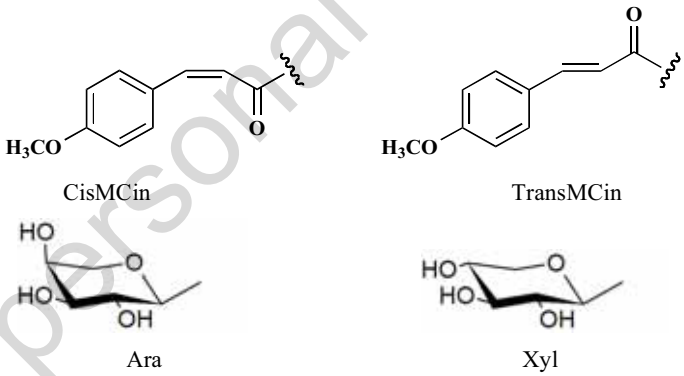



**Fig. (1).** Structures of the studied saponins.



arginin, methoxycinnamoyl or acetyl and (or) sulfate groups. In addition, the aminoacyl saponins showed a synergistic effect enhancing the cytotoxicity of the saporin-based targeted toxin in HER14 cells [22]. In the present study, we evaluated the synergistic cytotoxicity of the newly isolated saponins from *G. trichotoma* in combination with RIP-1 saporin and investigate quantitatively their structure – activity relationships.

## MATERIALS AND METHODS

### Dataset of Saponins

The roots of *Gypsophila trichotoma* Wender var. *trichotoma* were procured from the Black Sea region (Balgarevo, Bulgaria). Triterpenoid saponins were isolated from *Gypsophila trichotoma* Wender var. *trichotoma* by solvent extraction (methanol, 25%) followed by low pressure liquid chromatography (RP-$C_8$) and semi-preparative HPLC (RP-$C_{18}$) as described elsewhere [10, 22]. Saponins were characterized on the basis of extensive NMR analysis ($^1$H, $^{13}$C NMR, COSY, TOCSY, ROESY, HSQC, HMBC, and HSQC- TOCSY), completed by analysis of HR-ESI-MS and ESI-MS$^n$, as well as polarimetry, thin-layer chromatography and high-performance liquid chromatography [10, 22]. The structures of the studied saponins are given in Fig. (**1**).

### Antitumor Assay

The antitumor assay was described in details elsewhere [7, 23]. Briefly, the tested saponins (5 μg/mL) were applied together with saporin-6 (30 nM) on mammary breast cancer cell line MDA-MB-231 (ATCC® HTB-26™) and the cell viability was continuously monitored by Real-Time Cell Analyzer (RTCA) (xCELLigence system, Roche Applied

**Table 1. Cytotoxic activities expressed by a cell index (*CI*) per 1 μM and indicator variables describing the chemical structure of studied saponins.**

| Saponin | *CI* | *1R* | *2COCH$_3$* | *3SO$_3$H* | *4COCH$_3$* | *Xyl* |
|---|---|---|---|---|---|---|
| 1 | 0.307 | 1 | 0 | 1 | 0 | 0 |
| 2 | 0.264 | 0 | 1 | 1 | 0 | 0 |
| 3 | 0.279 | 1 | 0 | 0 | 0 | 0 |
| 4 | 0.219 | 1 | 0 | 0 | 1 | 0 |
| 5 | 0.016 | 1 | 0 | 0 | 1 | 1 |
| 6 | 0.117 | 0 | 0 | 0 | 0 | 0 |
| 7 | 0.219 | 1 | 0 | 0 | 0 | 0 |
| 8 | 0.222 | 1 | 0 | 0 | 0 | 0 |
| 9 | 0.251 | 1 | 0 | 0 | 0 | 1 |

Science, Mannheim, Germany). The activities of the studied saponins were represented by a cell index (*CI*) per 1 μM after 128 hours of incubation.

### Quantitative Structure – Activity Relationship (QSAR) Protocol

The chemical structure of the studied saponins was described by five indicator variables: *1R*, *2COCH$_3$*, *3SO$_3$H*, *4COCH$_3$* and *Xyl*. If a substituent presents at a given position, the indicator variable takes value 1; otherwise it takes 0. For example, the descriptor *1R* takes 1 if there are $COCH_3$, cis-*p*-methoxycinnamoyl (CisMCin) or trans-*p*-methoxycinnamoyl groups (TransMCin) at position $R_1$. Similarly, descriptors *2COCH$_3$*, *3SO$_3$H*, *4COCH$_3$* take 1 if there are *COCH$_3$*, *SO$_3$H*, and *COCH$_3$* at positions $R_2$, $R_3$ or $R_4$, respectively (Fig. **1**). The descriptor *Xyl* takes 1 when a β-D-xylopyranose (β-D-Xylp) residue exists in the carbohydrate part attached to position C-3. The activities of the studied saponins are represented by a cell index (*CI*) per 1 μM after 128 hours of incubation. Low *CI* values correspond to highly active compounds. The QSAR model was derived by multiple linear regression (MLR) applying partial least squares (PLS) as implemented in SIMCA 13.0 (Umetrics Ltd.).

## RESULTS

The cytotoxicity enhancing activities of the studied saponins given as a cell index (*CI*) per 1 μM after 128 hours of incubation are shown in Table **1**. The highly active compounds have low *CI* values and *vice versa*. The studied saponins were also screened at concentrations of 5, 10 and 20 μg/mL for self-toxicity (data not shown). No toxic effects were observed up to 20 μg/mL for a period of 128 h after incubation for all saponins.

Two principal components (*PC*) account for 66% of the variance in the set. The first *PC* accounts for 41.3%; the second *PC* adds 25.0%. The score and loadings plots are given in (Fig. **2**). The saponins **3**, **7** and **8** overlap in the score plot as they have the same values for all descriptors (Fig. **2a**). The most active compound (saponin **5**) is positioned in the third quadrant (lower left), the least active one (saponin **1**) is just opposite, in the first quadrant (upper right). The loadings plot (Fig. **2b**) points to descriptors contributing positively (right part) and negatively (left part) to *CI*. Descriptors *1R*, *2COCH$_3$* and *3SO$_3$H* increase *CI*, i.e. decrease activity; descriptors *4COCH$_3$* and *Xyl* decrease *CI*, i.e. increase activity. The derived QSAR model is:

$$CI = 0.472*1R + 0.033*2COCH_3 + 0.335*3SO_3H - 0.515*4COCH_3 - 0.379*Xyl + 2.328$$

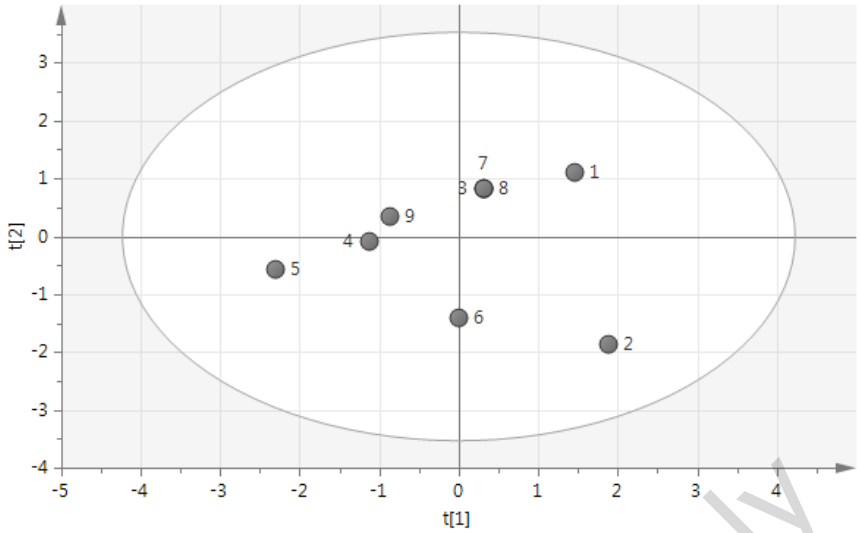
$$n = 9 \; r^2 = 0.708$$

This model clearly revealed several trends in the structure – activity relationships of studied saponins:

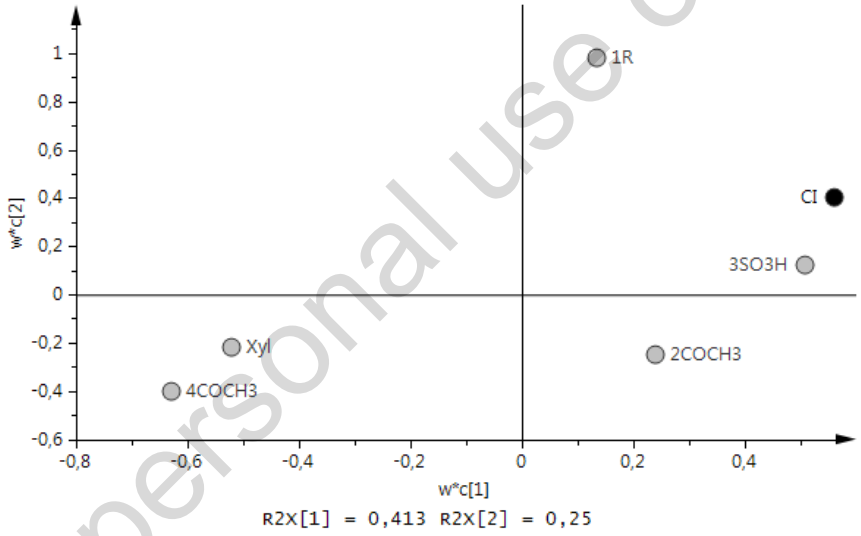
- the presence of acetyl, cis-*p*-methoxycinnamoyl or trans-*p*-methoxycinnamoyl groups at position $R_1$ increases *CI*, i.e. decreases activity;
- the presence of acetyl group at position $R_2$ increases *CI*, i.e. decreases activity;
- the presence of sulfonyl group at position $R_3$ increases *CI*, i.e. decreases activity;
- the presence of acetyl group at position $R_4$ decreases *CI*, i.e. increases activity;
- the presence of β-D-Xylp in the carbohydrate part attached to C-3 decreases *CI*, i.e. increases activity.

Hence, the synergistic cytotoxicity of the newly isolated saponins from *G. trichotoma* is facilitated by an acetylation at position $R_4$ and by the presence of β-D-Xylp residue instead of α-L-arabinopyranose (α-L-Arap) in the trisaccharide attached to C-3 of the aglycon. The summarized results from the QSAR study are given in (Fig. **3**).

In order to discriminate between the substituents at position $R_1$, descriptor *1R* was replaced by two descriptors *1COCH$_3$*




**a**




**b**

**Fig. (2).** The score plot (**a**). The first *PC* accounts for 41.3% of the variance in the dataset, the second PC – for 25.0%. The loading plot (**b**). X and Y descriptors are given as grey and black circles, respectively.

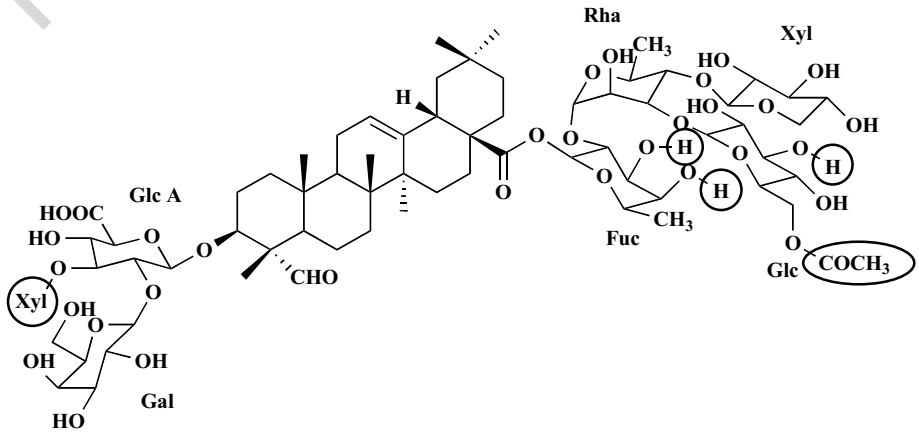



**Fig. (3).** The ideal saponin according to the QSAR model, derived in the present study. The synergistic cytotoxicity of saponins increases when $R_1$ = H, $R_2$ = H, $R_3$ = H, $R_4$ = $COCH_3$ and Sugar = Xyl.

and *1Cin* accounting for the presence of $COCH_3$ and *CisMCin/TransMCin*, respectively. The derived QSAR model (data not shown) showed that both descriptors have positive coefficients, i.e. decrease activity.

## DISCUSSION

Saporins are ribosome-inactivating proteins (RIPs) extracted from roots, leaves and seeds of *Saponaria officinalis*, commonly known as soapwort [24]. Among the several saporins isolated from *S. officinalis*, saporin-6 is the most abundant and stable. RIPs are a family of plant toxins, which specifically and irreversibly inhibit protein synthesis in eukaryotic cells by removing one or more adenine residues from ribosomal RNA [25]. RIPs are classified into two types, referred as type I and type II. Type I RIPs are single-chain proteins, while type II RIPs consist of an enzymatically active A-chain and a B-chain with cell-binding ability [25]. The lack of cell-binding domain in RIPs-I limits their membrane permeability and decreases their cytotoxicity. It was found that saponins enhance the toxicity of certain RIPs-I synergistically at submicellar concentration [16]. Saponins mediate the endosomal escape of RIPs-I after internalization [21].

Saponins are amphiphilic molecules with surfactant activity. It was assumed that their cell permeability is due to the interaction with the membrane cholesterol leading to a rearrangement of the phospholipid bilayer and pore formation [26]. When saporin is applied in combination with saponins, its cytotoxic activity *in vitro* increases up to 100,000 times [16]. Recently, Böttger et al. [21] have suggested the most important structural features for the development of synergistic cytotoxicity between saponins and RIP-I.

Our results confirm and further develop Böttger's concept. The investigated saponins contain the aglycone gypsogenin, a branched trisaccharide chain at C-3 containing β-D-glucuronic acid, β-D-galactopyranose, and β-D-Xylp or α-L-Arap and a branched tetrasaccharide chain at C-28 consisting of β-D-fucopyranose (β-D-Fucp), α-L-rhamnopyranose, α-L-Arap and β-D-glucopyranose (β-D-Glcp), some of them partially acetylated or sulfonated (Fig. **1**). The QSAR study showed that the presence of a monoacetylated β-D-Glcp residue (substituent $R_4$) at C-28 tetrasaccharide and of β-D-Xylp at C-3 trisaccharide instead of α-L-Arap increases the cytotoxic activity of saporin. The presence of a substitutent in the β-D-Fucp (substituents $R_1$ and $R_2$) and a sulfonated β-D-Glcp (substituent $R_3$) attached to C-28 sugar chain decreases the synergistic cytotoxicity.

The least active compound in the set is saponin **1** (*CI* = 0.307). It is monoacetylated in β-D-Fucp and sulfonated in β-D-Glcp moiety at the sugar chain attached to C-28, and contains α-L-Arap at C-3 sugar chain. The most active compound saponin **5** (*CI* = 0.016) has monoacetylated β-D-Fucp and β-D-Glcp at C-28 tetrasaccharide and a β-D-Xylp residue at C-3 trisaccharide. Very similar to saponin **5** is saponin **4** having an α-L-Arap residue at C-3 trisaccharide instead of β-D-Xylp. However, the activity of saponin **4** is almost 14 times lower than the activity of saponin **5** (*CI* = 0.219 and *CI* = 0.016, respectively). This emphasizes the presence of β-D-Xylp in the branched trisaccharide attached to C-3 as an important new structural feature of saponins for cytotoxicity enhancing activity. The only difference between β-D-Xylp and α-L-Arap is the orientation of 4-OH group: in β-D-Xylp it is in equatorial position, in α-L-Arap – in axial. As such tiny structural difference results in a significant difference in activity, one might suppose a precise steric interaction with the biological target. It was found that equatorial hydroxyls formed more hydrogen bonds than axial groups [27]. Because of the higher accessible surface of equatorial groups, there is a definite tendency for them to be involved in two hydrogen bonds, whereas axial are more likely to form only one. The other β-D-Xylp-containing saponin in the set is compound **9**. However, it contains no acetyl group in the β-D-Glcp attached to C-28 and its activity is almost 16 times lower than the activity of saponin **5** (*CI* = 0.251 and *CI* = 0.016, respectively). Thus, the acetylated β-D-Glcp residue at C-28 sugar chain is another important structural feature for the synergistic cytotoxicity of saponins.

In summary, a QSAR model was derived in the present study using multiple linear regression by partial least squares. The model distinguished the most important structural features of gypsogenin-containing saponins acting as cytotoxicity enhancers of the RIP-I saporin. The branched trisaccharide moiety attached to C-3 should contain one β-D-Xylp residue instead of an α-L-Arap. The branched tetrasaccharide at C-28 should contain one acetyl group attached to the β-D-Glcp residue. The fact that the saponins themselves are non-toxic but in a combined application with a RIP-I show synergistic cytotoxicity makes them perspective drug candidates for targeted anticancer therapy.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

## ACKNOWLEDGEMENTS

Declared none.

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