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The Cytotoxicity of Saponins Correlates with Their Cellular Internalization

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Saponins are conventionally (long before the full characterization of any single saponin structure) and literally (from the Latin word *sapo*) defined as any of various plant glycosides that form soapy lathers when mixed and agitated with water. These plant components are virtually all hemolytic, toxic to fish, have a bitter taste, and are used routinely as detergents, foaming agents, and emulsifiers.[1] The surfactant properties of saponins are exploited in biological research to permeabilize plasma and intracellular membranes to release cytosolic and membrane-bound proteins and to enhance the entry of proteins and other macromolecules across cell membranes.[2] Although most of the commercially available saponins in use are heterogeneous plant-derived preparations, and are even structurally disparate (for example, the saponins from *Gypsophila* and *Quillaja* plants are triterpene glycosides, whereas digitonins are steroid glycosides), it is believed that they all form complexes with cholesterol, which constitutes up to one molecule for every phospholipid present in the plasma membrane. Formation of such complexes is a key event toward membrane permeabilization.[2, 3] The unambiguous modern definition of saponins is based on their structural character; saponins are glycosides of steroids and triterpenes. Thousands of saponins have been characterized from not only terrestrial plants but marine species as well, displaying tremendous structural diversity and a wide spectrum of biological activities.[1b] However, all these structures and activities have been arbitrarily correlated to surfactant properties, regardless of the fact that many homogeneous saponins are neither foaming nor hemolytic; thus, the detailed structure–activity relationship (SAR) and mechanism of action are largely ignored.

The digitonin-like saponins constitute the largest class of the steroidal saponins, named spirostan saponins, which have conservative spirostan aglycones and sugar substitutions primarily at the 3-OH group of the spirostan structure.[1b] It was recently found that a quite common feature of spirostan saponins is the inhibitory activity against the growth of tumor cell lines which is sensitive to the structure of the sugar residues.[4] Dioscin, diosgenin-3-yl-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (chacotrioside), shown in Figure 1, represents one of the most common spirostan saponins, which has been isolated from some 20 genera, including many vegetables and medicinal plants. Dioscin is among the potent antiproliferative spirostan saponins, and preliminary studies show that dioscin and some of its congeners can induce the apoptosis of tumor cells.[4, 5] The apoptotic lesion induced by spirostan saponins might be instinctively correlated to their conventional membrane-perturbing properties. How this process takes place, then, emerges as an interesting question to address. Herein we report that, unexpectedly, the cytotoxicity of spirostan saponins correlates with their ability for cellular internalization.

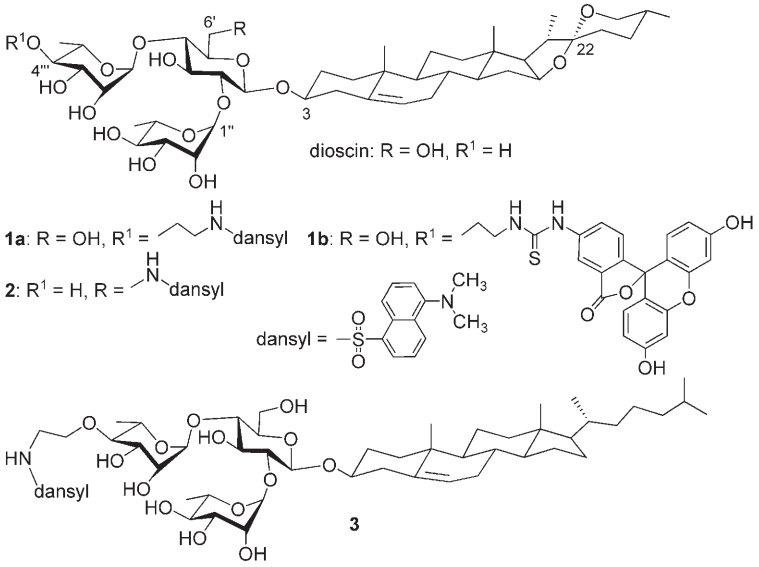

Figure 1. The spirostan saponin dioscin and its fluorescently labeled analogues **1**–**3**.

Employing dioscin as a lead structure, we carried out a systematic SAR study on the cytotoxicity of spirostan saponins.[6] These results enabled us to prepare a group of the dansyl- and fluorescein isothiocyanate (FITC)-conjugated fluorescent probes **1a**, **1b**, **2**, and **3** with three levels of activity: strong, medium, and none, against the growth of tumor cells (see Supporting Information for the synthesis, characterization, and fluorescence properties of **1**–**3**). As shown in Table 1, the dioscin derivatives **1a** and **1b**, labeled with dansyl and FITC groups, respectively, at the 4‴-OH group showed strong inhibitory activities against the growth of tumor cell lines. In fact, both compounds were more potent than dioscin against H4 and HeLa cells, but slightly less potent toward A549 cells, with the lowest IC_{50} value (0.15 μM) observed for **1a** toward H4 cells. Replacement of the spirostan aglycone of **1a** with cholesterol abolished cytotoxicity; compound **3** showed no activity at concentrations up to 100 μM. The dioscin derivative **2**, labeled through the 6′-NH linkage, demonstrated much lower activities than dioscin toward the three tumor cell lines (IC_{50} values ~50 μM).

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Table 1. The inhibitory activity of dioscin and its fluorescently labeled probes **1**–**3** against the growth of tumor cell lines.[a]

Compd	IC_{50} [μM]		
	H4	HeLa	A549
1a	0.15 ± 0.10	1.08 ± 0.09	11.93 ± 0.31
1b[b]	6.31 ± 0.20	0.64 ± 0.14	9.87 ± 0.41
2	49.17 ± 2.60	36.57 ± 3.33	50.00 ± 5.54
3	≫ 100	≫ 100	≫ 100
dioscin	9.00 ± 0.45	2.90 ± 0.09	4.16 ± 0.73

[a] Measured at 550 nm (650 nm as reference wavelength) according to the standard MTT method, see Supporting Information for details. H4: human neuroglioma cell line; HeLa: Human cervical carcinoma cell line; A549: human alveolar epithelial cell line. [b] The absorbance of the fluorescent probes at 550 nm was subtracted from the total absorbance measured.

Determination of the activities of the fluorescent probes toward the tumor cells enabled us to choose appropriate concentrations to which cells could be exposed without causing significant disruption of their growth. For the potent compounds **1a** and **1b**, we first tried concentrations ≤ 0.5 μM (below the IC_{50} values) to treat HeLa cells, however, such low concentrations did not afford clear fluorescence. With an increase in the final concentration to 1 μM, the fluorescence of **1a** became apparent in HeLa cells after an incubation time of 30 min; fluorescence was enhanced markedly at 1 h and exhibited a juxtanuclear staining pattern (Figure 2a). However, after further incubation the compartmentalized fluorescence spread to the cytosol, and the cells then underwent a remarkable apoptosis-type morphological change that finally led to cell death. Similar phenomena were observed with compound **1b** (Figure 2b). Compound **2** was much less potent, and thus a working concentration of 10 μM could be used. The fluorescence of **2** in HeLa cells showed up at 4 h, grew brighter at 8 h, and then stayed almost unchanged for an additional 16 h (Figure 2c). The inactive compound **3**, however, did not display any cellular fluorescence at a concentration of 10 μM over an incubation time of 24 h (Figure 2d).

The staining patterns of **1a** and **1b** (before the morphological change of the cells) and **2** were quite similar (Figure 2). The juxtanuclear morphology of the fluorescence implies that the

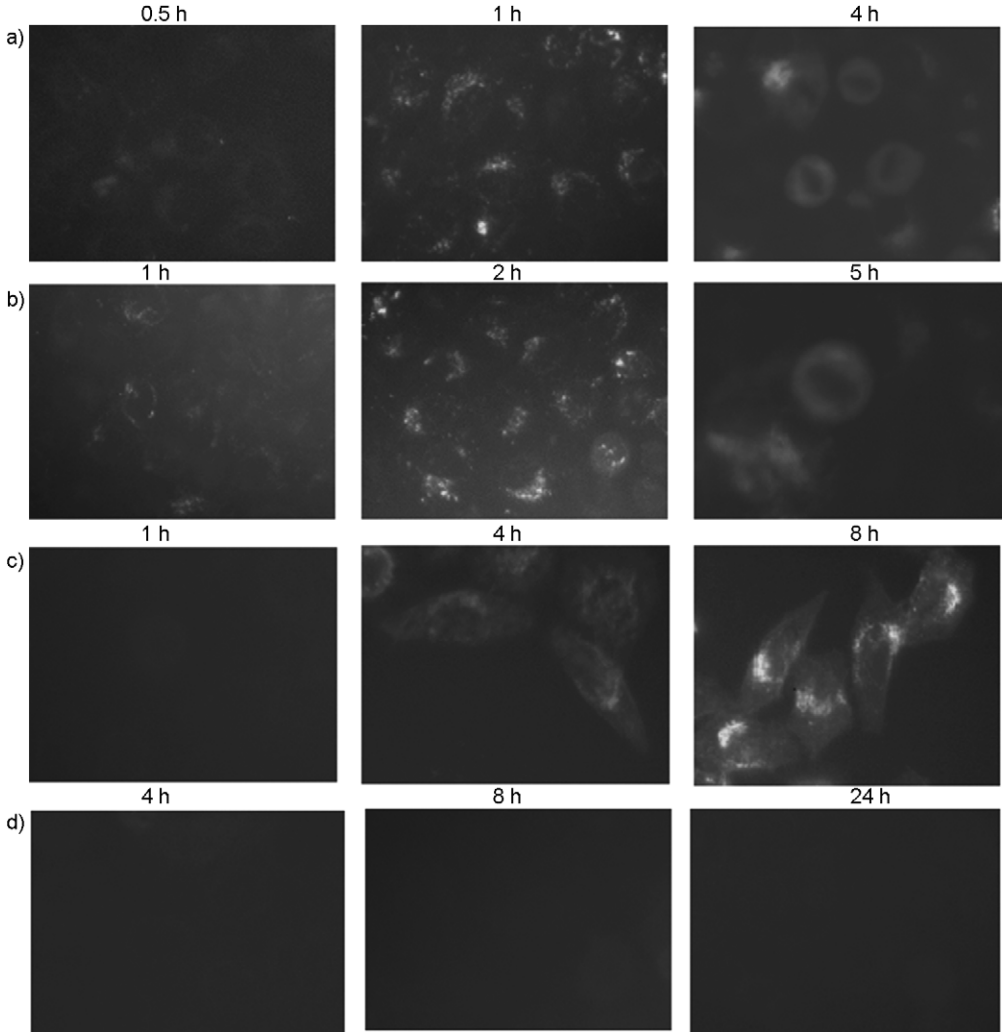

Figure 2. Fluorescence microscopy images of HeLa cells incubated with the fluorescent probes a) **1a** (1 μM), b) **1b** (1 μM), c) **2** (10 μM), and d) **3** (10 μM) for the times indicated; original magnification: 40 ×.

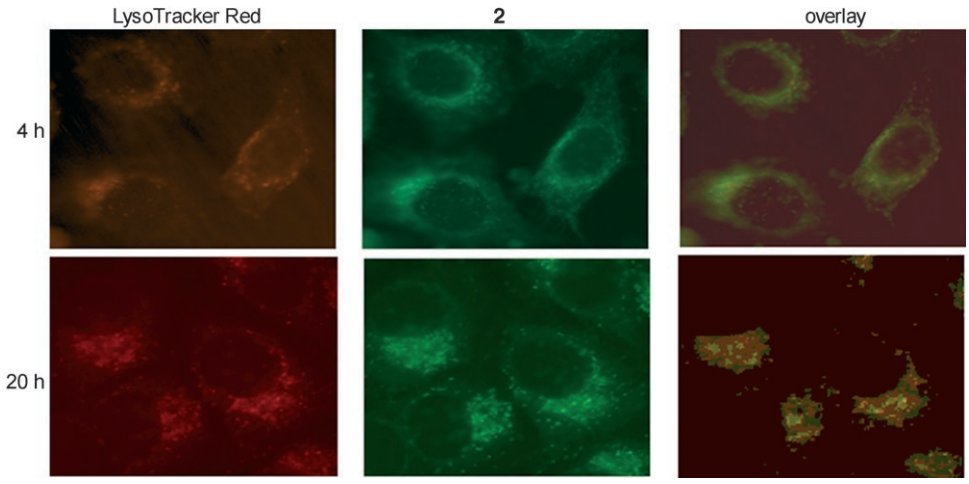

Figure 3. Fluorescence microscopy images (100 ×) of LysoTracker Red (50 nM, right-hand column, red) and **2** (1 μM, middle column, green) in HeLa cells over 24 h. Before the appropriate imaging time, LysoTracker Red was added to the cells and incubated for 40 min; top row: 4 h, bottom row: 20 h.

probes might be localized in the lysosomes. Thus, LysoTracker Red (Molecular Probes, Eugene, OR, USA), a specific probe for acidic compartments such as lysosomes and endosomes,[7] was used to confirm this point. As shown in Figure 3, the fluorescence of **2** (in HeLa cells) overlapped predominantly with that of the LysoTracker dye from 4 to 24 h, indicating their co-localization in the acidic organelles. We also examined the staining of probes **1**–**3** toward H4 and A549 cell lines and the co-localization of **2** with LysoTracker in these cells; similar results as with HeLa cells were observed (see Supporting Information for details).

The above observations clearly indicate that the propensity for cellular internalization of the steroidal saponin derivatives **1**–**3** correlates with their cytotoxic potency. The potent compounds **1a** and **1b** entered into cells faster than the less potent compound **2**, and the inactive compound **3** could not enter at all. Based on the conventional perception of saponins, we anticipated that these surfactant molecules might anchor onto the plasma membrane, with insertion of their steroidal aglycone moieties into the lipid bilayer (in complexation with the cholesterol therein) and their sugar groups exposed at the cell surface. However, we only discerned a blurry peripheral staining before clear observation of fluorescence in the cytosol for **1** and **2** (Figure 2a–c), indicating a quick transmembrane crossing for the saponin derivatives **1** and **2**. For the cholesterol glycoside **3**, surprisingly, fluorescence was found only in the media, indicating no membrane binding at all. Because cholesterol has been proven to be a good membrane anchor,[8] an explanation for this particular result is that the bulky 3-*O*-chacotriosyl residue in **3** prohibits the cholesterol moiety from insertion into the membrane. After entering the cells, the steroidal saponin derivatives **1** and **2** were shown to be compartmentalized specifically in the acidic organelles. As this cellular distribution was found to be independent of the fluorescent moieties (dansyl and FITC groups) present, it should be the parent structure of the saponins that determines cellular internalization and subcellular localization.

In summary, based on the extensive SAR studies on the cytotoxicity of spirostan saponins, we managed to prepare a set of fluorescently labeled saponin derivatives **1**–**3**, showing three levels of potency against the growth of tumor cells. The cellular behavior of these probes demonstrates that the cytotoxic potency of spirostan saponins correlates with their propensity for cellular internalization. The internalized saponins were observed to be specifically localized to the acidic organelles, implying that the accumulation of saponins in lysosomes leads to cell death.[9] Thus, the work presented herein has disclosed a new facet of these ancient natural surfactants, in that they could constitute a mechanistically new class of anticancer agent. The present saponin probes might be valuable tools for further studies on the cellular effects of saponins.

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