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Structure–function relationship for saponin effects on cell cycle arrest and apoptosis in the human 1547 osteosarcoma cells: a molecular modelling approach of natural molecules structurally close to diosgenin

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Abstract—In this paper, eight natural molecules structurally close to diosgenin (five saponins: diosgenin, hecogenin, tigogenin, sarsasapogenin, smilagenin; two steroidal alkaloids: solasodine, solanidine; one sterol: stigmasterol) have been tested for their biological activities on human 1547 osteosarcoma cells. Differences in activity were studied in term of proliferation rate, cell cycle distribution and apoptosis induction. By using molecular modelling, two structural characteristics were calculated: spatial conformation and electron transfer capacity. The second property has been investigated by the HOMO repartition and the corresponding energy. Correlation between the experimental and the theoretical data permit us to highlight the importance of the hetero-sugar moiety and the 5,6-double bond in the biological activity (apoptosis and cell cycle arrest) on the human 1547 cell line. The importance of conformation at C-5 and C-25 carbon atoms was also discussed.

1. Introduction

Plant steroids have been thoroughly described for their pharmacological properties, including hypocholesterolemic,[1] antidiabetic[2] and antioxidant activities.[3,4] Particular attention has been given to their potential for cancer chemoprevention, especially as apoptosis inductors.[5–9] Apoptosis is considered to be the major process responsible for cell death in various physiological events.[10,11]

We had previously shown that diosgenin, a plant steroid, altered cell cycle and induced apoptosis in different human cancer cell lines[12–14] and in cultured human synoviocytes.[15] Diosgenin caused an inhibition of cell growth with cycle arrest and apoptosis induction by p53 activation;[13] the mechanism of action was caspase-3 dependent, but also this molecule caused a nuclear localization of apoptosis inducing factor with a fall of mitochondrial membrane potential.[13] Furthermore, diosgenin was the most effective as cell death inductor compared to the other two plant steroids (hecogenin and tigogenin) in the human osteosarcoma 1547 cell line.[16]

For the present study, we tested a series of eight natural molecules structurally close to diosgenin. The objective was first to investigate the different biological activities (proliferation rate, cell cycle distribution and apoptosis on human 1547 osteosarcoma cells) of five saponins (diosgenin, hecogenin, tigogenin, sarsasapogenin and smilagenin), two steroidal alkaloids (solasodine, the nitrogen-containing equivalent of diosgenin, and solanidine) and a sterol (stigmasterol) (Fig. 1). Secondly, using molecular modelling, we tried to establish structural criteria that could be implicated in the biological activity of these molecules.

Keywords: Saponins; Apoptosis; Cell cycle; 1547 cancer cells; Molecular modelling; Spatial conformation; HOMO.
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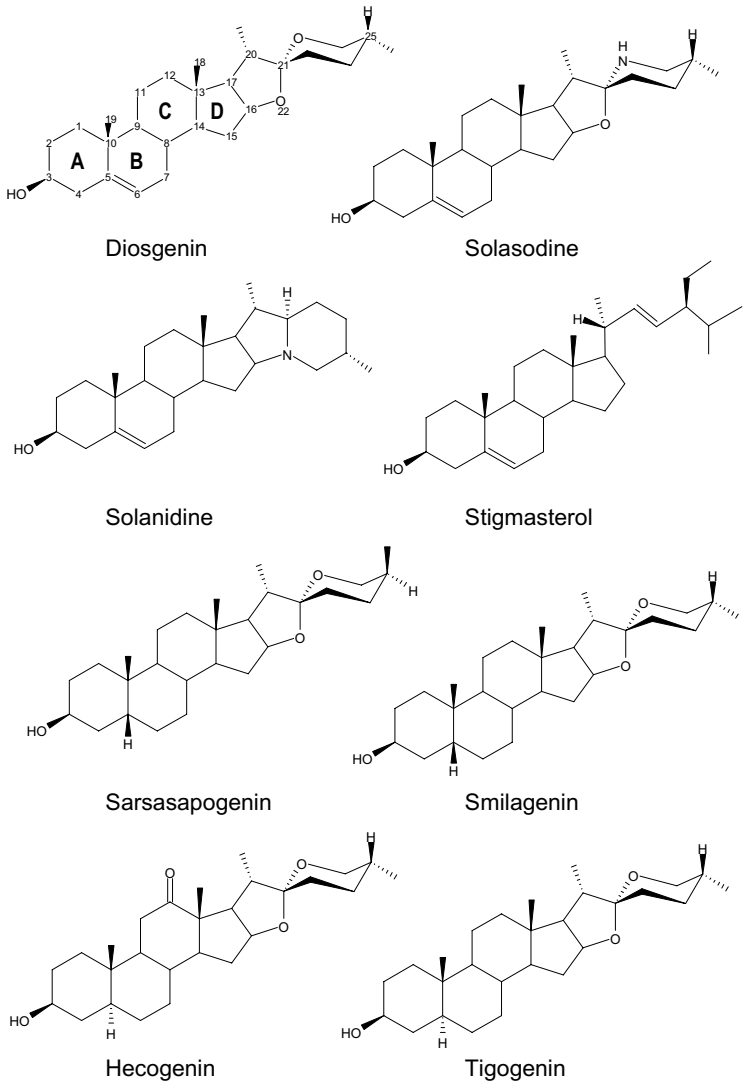

Figure 1. Structure of molecules.

Two major characteristics could be implicated in the biological activity of molecules: spatial conformation and redox reactivity. Both characteristics could be investigated by molecular modelling. The first one has thoroughly been used in structure–activity relationship studies. The second one is more difficult to investigate but it is well known that redox transfers are the primary chemical reactions for explaining reactivity of molecules. The use of electronic properties improves the knowledge concerning this very important aspect: the repartition of electronic density, the repartition of molecular orbitals and the ionization potential (IP) permit us to predict the capacity of electron transfer.

So, we first showed experimental results on cell proliferation, cell cycle distribution and apoptosis in 1547 human osteosarcoma cells. Secondly, we detailed structural information obtained from calculations (conformations and electronic structures). We finally shed light on different criteria in relation with the role of saponins as inhibitors of cancer cell proliferation.

2. Results

2.1. Inhibition of 1547 proliferation

Cells were cultured in 10% FCS-medium with or without 40 μM compounds for 4 days and cell proliferation was evaluated by the MTT test. We had previously demonstrated that 40 μM diosgenin induced strong inhibition of 1547 cell proliferation at 24 h (86%, ($p < 0.05$))[12] whereas hecogenin and tigogenin moderately inhibited cellular growth (38% and 53%, respectively, $p < 0.05$).[16]

Under our experimental conditions, a decrease in proliferation was observed at 24 h for all saponins and steroidal alkaloids (range of inhibition was 77–96% ($p < 0.05$)) except for hecogenin and tigogenin for which the inhibition of proliferation was moderated (Fig. 2). Stigmasterol inhibited proliferation by 72% ($p < 0.05$).

In the presence of diosgenin, solasodine, sarsasapogenin, smilagenin or solanidine, proliferation of 1547 cells

decreased until 96 h contrary to hecogenin, tigogenin and stigmasterol.

2.2. Cell cycle analysis

To explain the inhibition of proliferation observed, we studied cell cycle distribution after 24 h treatment. Among the seven saponins tested, only three of them stopped the cell cycle: diosgenin, sarsasapogenin and smilagenin. Smilagenin and diosgenin blocked cells in G_0/G_1 phase (54.9% and 54.1% compared to 39.6% for control, $p < 0.05$, respectively), and the fraction of S phase cells consequently decreased at 24 h (21% and 16.3% respectively, compared to 40.2% for control, $p < 0.05$) (Fig. 3). Contrary to diosgenin and smilagenin, sarsasapogenin arrested cell cycle in G_2/M (52.6% compared to 20.3% for control, $p < 0.05$). With this compound, the number of cells in S phase was unmodified whereas the fraction of cells in G_0/G_1 was markedly diminished (6% compared to 39.6% for control, $p < 0.05$) (Fig. 3).

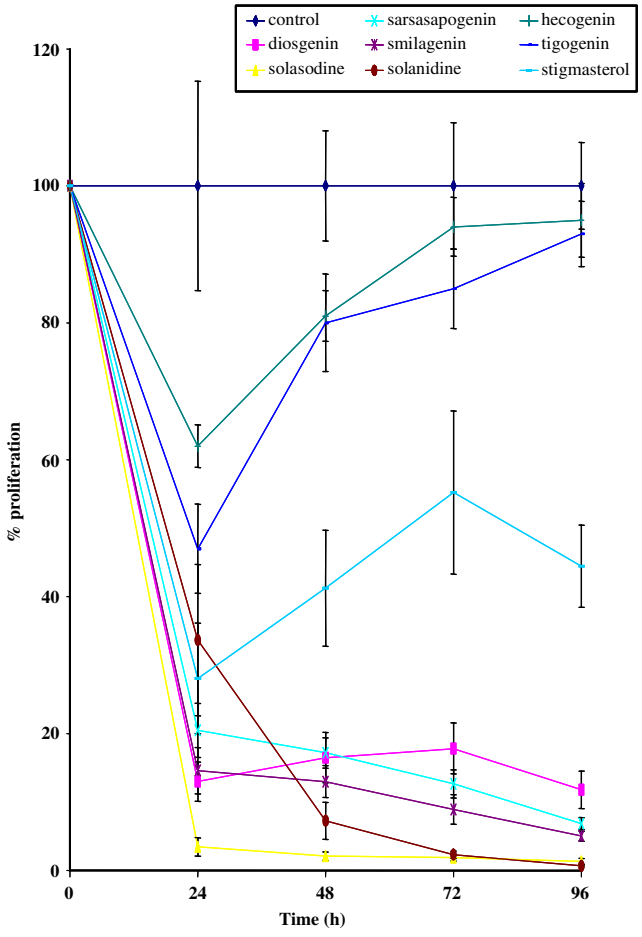

Figure 2. Cell proliferation under molecule treatment. After 72 h adherence, cells were cultured in 10% FCS medium and treated with 40 μM molecules for 24–96 h. Results are presented as percentage of control (untreated cells) and values are expressed as mean ± SD of six experiments (p-value relative to control group, $p < 0.05$).

2.3. Analysis of apoptosis marker: DNA fragmentation

Programmed cell death is another phenomenon that could explain inhibition of proliferation. This is studied by analysis of DNA fragmentation, the latest stage of apoptosis. After 24 h treatment, we confirmed that diosgenin induced apoptosis in 1547 cells (5.6-fold compared to control, $p < 0.05$).[12] Sarsasapogenin and solasodine also significantly enhanced DNA fragmentation (6.9-fold and 13.2-fold, respectively, compared to control, $p < 0.05$) (Fig. 4). The other saponins weakly generated mono- and oligo-nucleosomes and stigmasterol had no effect on DNA fragmentation compared to control (Fig. 4).

3. Discussion

Saponins have brawn in scientific attention due to their structural diversity and the significance of their biological activities. Some studies had revealed that differences in saponin structure including the type and number of sugar moieties attached by a glycosidic bond at C-3 (Fig. 1) influence biological responses.[17,18] Wang et al.[19] have compared dioscin, a spirostenol (diosgenin-3-*O*-[α-L-rhamnopyranosyl(1→2)][α-L-rhamnopyranosyl(1→4)]), and methyl protodioscin, a furostanol with 26-*O*-glycopyranoside. These authors have shown that only dioscin induces apoptosis although these two compounds are structurally close. They suggested that the structure of the spirostenol must play a critical role in the activity of dioscin.

In this study, we focused on four structural characteristics that had not been studied: the hetero-sugar moiety bonded at C-16 and C-17, the 5,6-double bond, the isomerization at C-25 and the osidic bond of diosgenin (Fig. 1). In the following, each paragraph first gives a short summary of the biological activities and the theoretical observations then highlights the exact criterion that could be related to the activity on cancer cells.

3.1. The role of the sugar moiety

In order to investigate the role of the sugar moiety and the adjacent hetero-cycle, we compared diosgenin, stigmasterol and solanidine, which all possess the same steroid ring (i.e., androst-5(6)-en-3β-ol) (Fig. 1).

Under our experimental conditions, a decrease in proliferation was observed with solanidine and diosgenin. Stigmasterol only slowed down the 1547 proliferation, indeed it decreased until 24 h and cells proliferated again. Such a surprising behaviour led us to perform apoptosis and cell cycle analysis. We observed no apoptosis and no cell cycle arrest with stigmasterol. Besides, solanidine moderately induced apoptosis but did not modify cell cycle distribution.

Superimposition of these three compounds showed that the conformations of the A-, B-, C- and D-rings are

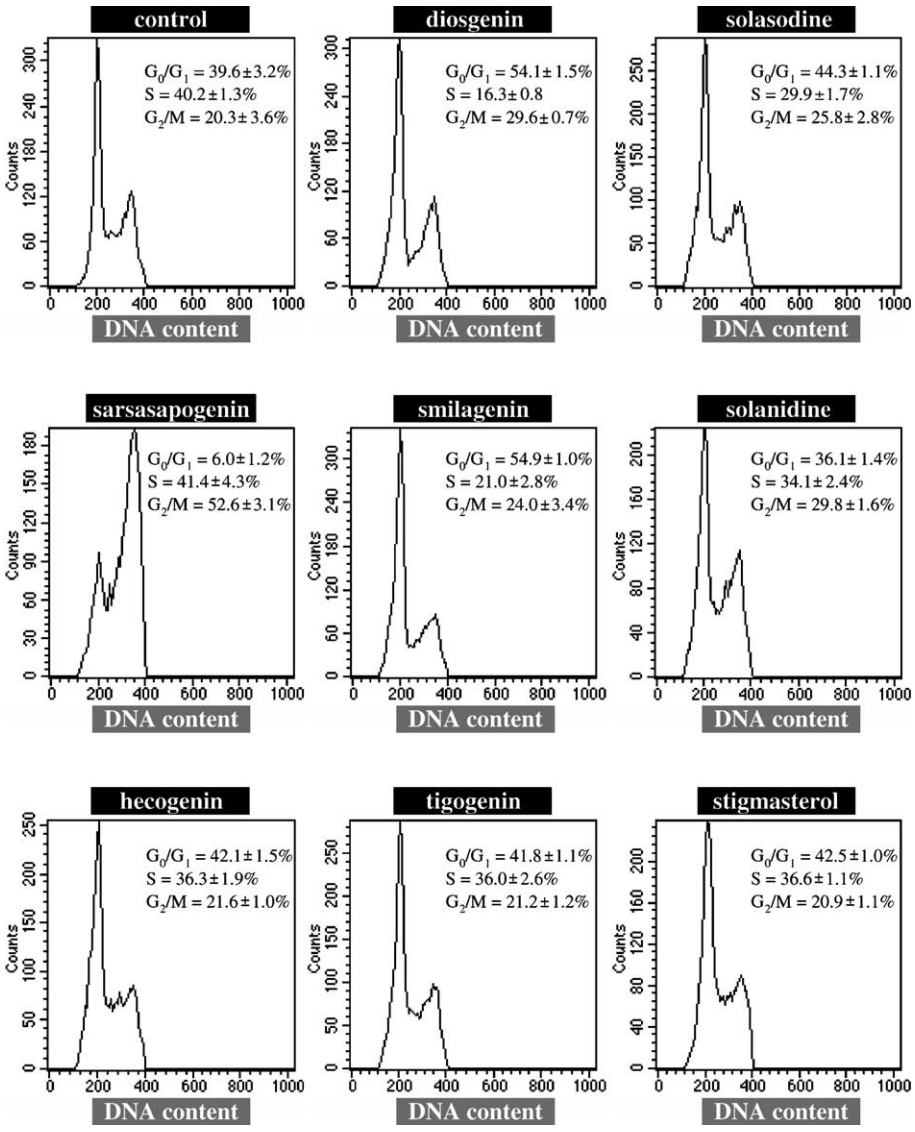

Figure 3. Cell cycle distribution of 1547 cells after molecule treatment. Cells were treated or not with 40 μM molecules for 24 h. Cell phase distribution was determined by PI staining and facs analysis. The experiments were performed three times, representative results are shown.

identical (Fig. 5a), whereas the moieties that were bonded with the D-ring are geometrically different.

As expected, HOMO was localized on the 5,6-double bond for diosgenin and stigmasterol (Fig. 6a). For solanidine, HOMO was localized on the N-atom. However, reactivity of the 5,6-double bond was preserved in this molecule by the presence of the HOMO-1 (Fig. 6b) with an energy close to that of HOMO of diosgenin and stigmasterol. So electron transfer capacity (energy and site) is the same for the three molecules.

In conclusion, diosgenin, stigmasterol and solanidine are three molecules with the same steroid moiety (the same configuration: androst-5(6)-en-3-ol and the same spatial conformation of cycles A, B, C and D) and with almost the same electronic configuration, but with different biological activities. This demonstrated the importance of the hetero-sugar moiety for the compounds based on an androst-5(6)-en-3-ol skeleton, especially in cell cycle arrest.

3.2. The role of the 5,6-double bond

From our previous study,[16] we already speculated that the 5,6-double bond probably played a significant role in biological effects. In the present study, we decided to focus on this structural characteristic to shed light on the reactivity of this site. To do so, diosgenin, smilagenin and tigogenin were compared in the light of the biological results, because they only differ in the presence (diosgenin) or not (smilagenin and tigogenin) of the 5,6-double bond (Fig. 1). Smilagenin and tigogenin are the two corresponding stereoisomers (5β and 5α, respectively).

Diosgenin and smilagenin strongly inhibited cell proliferation whereas tigogenin only induced moderate inhibi-

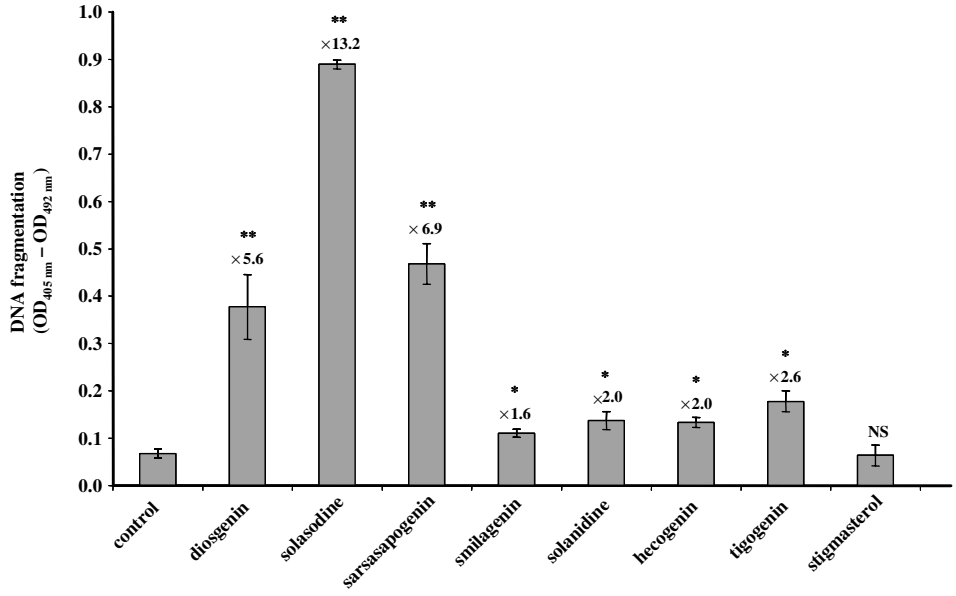

Figure 4. DNA fragmentation after saponins, steroidal alkaloids or stigmasterol treatment. Apoptosis was quantified on floating and adherent cells using ELISA. Apoptotic ratios (top of bars) were determined as sample absorbance/control absorbance. Values are expressed as mean ± SD (p-value relative to control group, $^{*}p < 0.05$ and $^{**}p < 0.01$). NS, not significant.

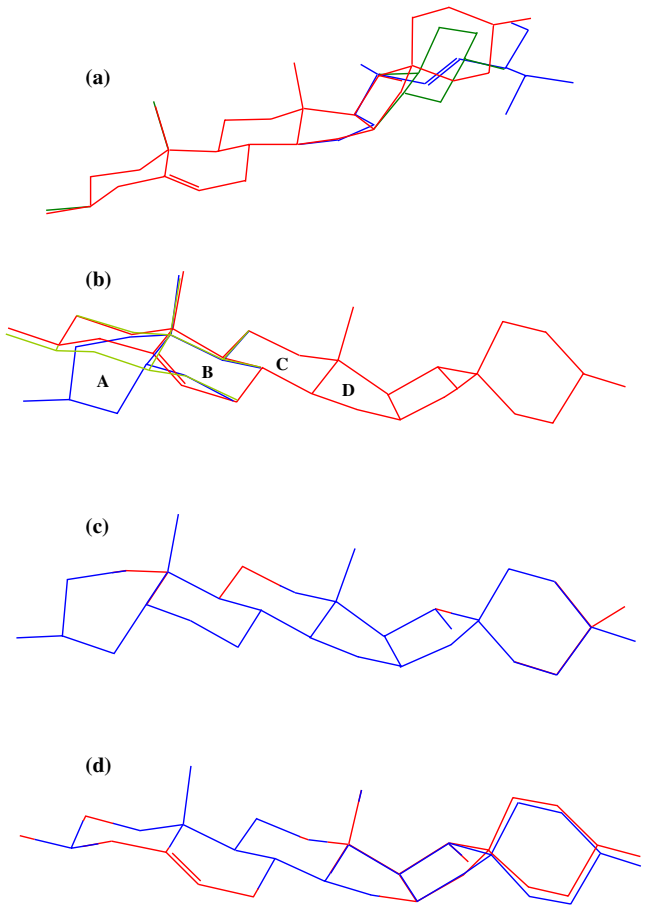

Figure 5. Superimposition of (a) diosgenin (red), stigmasterol (blue) and solanidine (green); (b) diosgenin (red), smilagenin (blue) and tigogenin (green); (c) smilagenin (blue) and sarsasapogenin (red); (d) solasodine (blue) and diosgenin (red).

tion. With regard to apoptosis and cell cycle data, the three molecules have different biological behaviours. Smilagenin and tigogenin only weakly induced apoptosis, compared to diosgenin. Both diosgenin and smilagenin arrested cell cycle in G_0/G_1, whereas tigogenin did not block it.

Our conformational studies showed that there are only weak differences in the spatial geometry between diosgenin and tigogenin (Fig. 5b). On the contrary, the presence of a 5β H-atom (smilagenin) triggered a strong distortion of the A-ring (Fig. 5b). With regard to this result, we conclude that such difference in the spatial conformation of the A- and B-rings did not influence the activity on cell cycle, because diosgenin and smilagenin caused cell cycle arrest in the same phase. No such conclusion could be established concerning the pro-apoptotic effect of saponins because smilagenin and tigogenin only weakly induced apoptosis, compared to diosgenin.

Concerning the electronic structures, differences at C-5 originate a smaller IP for smilagenin (IP = 10.20 eV) and tigogenin (IP = 10.20 eV) than for diosgenin (IP = 9.40 eV). This difference is clearly due to the presence of the C-5–C-6 double bond in diosgenin. Additionally, variations in HOMO repartition were observed (Fig. 6a). Regarding diosgenin and smilagenin, neither IP nor HOMO repartition influenced the activity on cell cycle. Indeed, the electronic structure was different for diosgenin and smilagenin whereas these two molecules caused arrest in the G_0/G_1 phase.

Since difference in conformation and electronic structure did not influence the activity on cell cycle, it is not relevant to draw any conclusion. This is just a first clue that permits us to suggest that for explaining the activity of

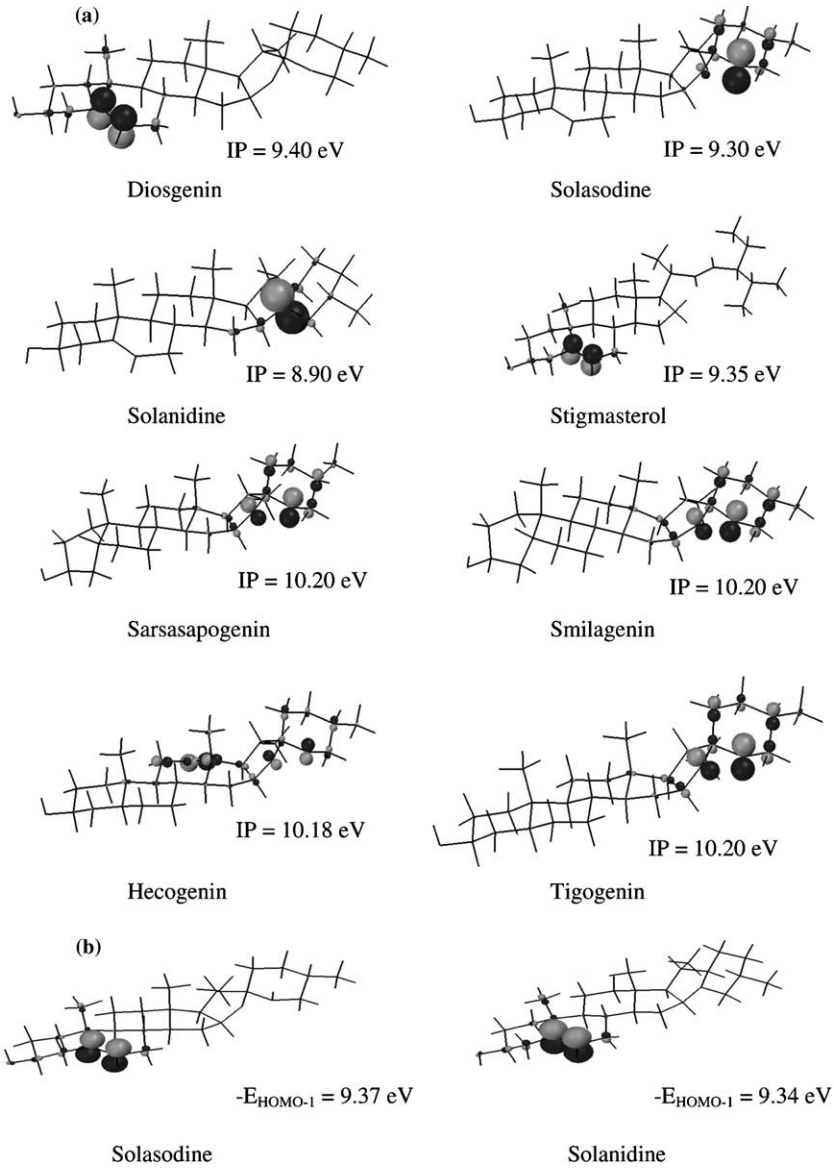

Figure 6. (a) HOMO repartition and IP (energy of the HOMO) of the different molecules. (b) Repartition and energy of the HOMO-1 of solasodine and solanidine.

diosgenin and smilagenin, there are two possibilities: either these two molecules did not bind to a receptor or the receptor binding is essentially governed by the hetero-sugar moiety.

On the other hand, the apoptosis rate could be influenced by the electronic structure, because there are significant differences in IP and HOMO repartition between diosgenin, an apoptosis inductor, and the other two, which only weakly induced apoptosis.

It must be stressed that apoptosis is a very complicated phenomenon, which implicates a large number of possible mechanisms. So, at this stage of our study, we only consider the presence of the 5,6-double bond as a good criterion for inducing a certain form of apoptosis.

Biological as well as molecular modelling results for hecogenin confirmed this hypothesis. Indeed, the configuration at C-5, IP value (10.18 eV), effect on apoptosis and cell cycle were the same for hecogenin and tigogenin (Fig. 6a).

3.3. Influence of the stereoisomery at C-25

As well as smilagenin, sarsasapogenin (the C-25 isomer of smilagenin, Fig. 1) inhibited cell proliferation. However, with regard to apoptosis and cell cycle data, we showed that these two molecules have different biological behaviours. Sarsasapogenin induced strong apoptosis whereas smilagenin weakly induced cell death. Both sarsasapogenin and smilagenin arrested cell cycle, however the mode of action seemed to be very different: sarsasapogenin arrested cells in G_2/M and smilagenin in G_0/G_1, as diosgenin. Except the difference at C-25 and as it is fully expected for the rest of the molecule, no conformational and electronic structure differences have been observed (Figs. 5c and 6a, respectively), according

to our calculations. Thus this small structural characteristic was the only one able to play a role in the G_2/M arrest, instead of G_0/G_1. Such behaviour is very specific of a receptor binding like activity, indicating the importance of the conformation at C-25.

3.4. The role of the osidic bond

We tested a molecule, solasodine that was structurally identical to diosgenin, except that the O-atom of the sugar moiety was replaced by a N-atom (Fig. 1). Our calculation demonstrated that there was no difference in conformation between these two molecules (Fig. 5d). Solasodine did not induce cell cycle arrest but, surprisingly, generated large DNA fragmentation. From quantum calculations, one could notice that the HOMO was localized on the N-atom whereas it was localized on the 5,6-double bond for diosgenin (Fig. 6a). However, it was interesting to note that the HOMO-1 was very close to the HOMO of diosgenin, in respect to the AM1 energy (9.37 eV and 9.40 eV, respectively), and the spatial repartition (Fig. 6b and a, respectively). Thus this electronic configuration (presence of the redox site on 5,6-double bond) could favour apoptosis for diosgenin as well as for solasodine. Concerning the cell cycle arrest, it seemed that the presence of an O-atom of the sugar moiety was necessary for the action of these compounds. Nevertheless, the last conclusion is not sufficient to explain cell cycle arrest because, for example, tigogenin and hecogenin (with no 5,6-double bond) did not possess this activity as previously described.

4. Conclusion

In conclusion we tested a series of molecules that possess very similar structures. Our molecular modelling study demonstrated that their spatial conformations were close to each other, except for saponins with 5β H-atom (smilagenin and sarsasapogenin). So, we focused on small structural variations, including HOMO distribution, interchanging of an O-atom by N-atom. We also looked for different stereoisomers (at C-5 and C-25).

The main results could be summed up as follows: biological activity was attributed in part to the presence of the hetero-sugar moiety and the 5,6-double bond. In that, diosgenin possessed these two criteria and exhibited strong apoptosis and cell cycle arrest. Nevertheless, absence of the 5,6-double bond could be compensated by other structural characteristics, as in the structure of sarsasapogenin that possesses a saturated 5,6-double bond with a 5β conformation. Indeed, we showed that this molecule had an important biological activity: same effect on apoptosis induction but cell cycle arrest in different phase, compared to diosgenin.

We analyzed geometrical and quantum characteristics and we tried to relate them with biological activities. The HOMO (and its energy) is an important parameter and has already been used for explaining the antioxidant activity of polyphenolic compounds.[20] Other parameters that must be taken into account are more geometrical: importance of the substituent moiety at C-17 and specific isomerization at C-5 and C-25. In order to identify the role of these structural criteria, future studies need to bring out information concerning the implication of the receptors in the mechanism of action of saponins and steroidal alkaloids, in human osteosarcoma cells.

5. Experimental

5.1. Cell line, cell culture and treatment

The 1547 human osteosarcoma cell lines were kindly provided by Professor M. Rigaud (Laboratoire de Biochimie, Faculté de Médecine de Limoges, France). The 1547 were seeded at 4×10^3 cells/cm^2 and grown in Eagle's minimum essential medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (FCS) (Gibco BRL), 100 U/mL penicillin and 100 μg/mL streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Cell viability was determined by the trypan blue dye exclusion method. For all experiments, cells were allowed to adhere and grow for 3 days in culture medium prior to exposure to molecules. A stock solution of 10^{-2} M for each molecule was prepared in ethanol and diluted in culture medium to give a final concentration of 40 μM. The same amount of ethanol (<0.4%) was added to control cells. Recently, we showed that 40 μM diosgenin caused a dramatic decrease in proliferation of osteosarcoma 1547 cells.[12,16] For this reason and to compare the effects of all molecules, we chose to work at the same concentration.

Compounds studied in this work were diosgenin ([25*R*]-5-spirosten-3β-ol), hecogenin ([25*R*]-5α-spirostan-3β-ol-12-one), tigogenin ([25*R*]-5α-spirostan-3β-ol), sarsasapogenin ([25*S*]-5β-spirostan-3β-ol), smilagenin ([25*R*]-5β-spirostan-3β-ol), solasodine (solasod-5-en-3β-ol), solanidine (solanid-5-en-3β-ol) and stigmasterol (5,22-stigmastadien-3β-ol) (Sigma) (Fig. 1).

5.2. Cell proliferation assay

Measurement of cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated in 96-well culture plates and grown 72 h before treatment with 40 μM molecules for 24–96 h. MTT was carried out daily as previously described[21] and experiments were performed in sextuple assay.

5.3. Apoptosis quantification: DNA fragmentation

Cells were cultured in six-well culture plates. After 24 h treatment, apoptosis was quantified on pooled cells (floating and adherent) using the 'cell death' enzyme-linked immunosorbent assay (ELISA) (Cell Death Detection ELISA^{plus}, Roche Diagnostics). Cytosol extracts were obtained according to the manufacturer's protocol and apoptosis was measured as previously described.[22]

5.4. Cell cycle analysis

Cells were seeded in 75 cm^2 tissue culture flasks and cultured in 10% FCS medium without or with molecules (40 μM) for 24 h. Floating and adherent cells were combined and cell viability was determined by the trypan blue dye exclusion method. For DNA content analysis, 10^6 cells were fixed and permeabilized in 70% ethanol, washed in phosphate-buffered saline (PBS, pH 7.4) treated with RNase (40 U/μL, Boehringer) and stained with propidium iodide (PI) (50 μg/mL). Flow cytometric analyses were performed as previously described.[22]

5.5. Statistical analysis

Statistical analysis of differences was carried out by analysis of variance (ANOVA) using StatView 5.0. A p-value of less than 0.05 (Fisher's PLSD test) was considered to indicate significance.

5.6. Method of calculations

Only few studies have reported structural data on saponins and sterols. Because crystal structure was available for diosgenone,[23] we first performed our calculations on this molecule to test the method we used.

In order to explore the potential energy surface of saponins, we chose a simulated annealling procedure. This is a general method of function optimization, which was proposed by Kirkpatrick et al.[24] and has been used for conformation research.[25,26] Indeed, starting the annealling process at a sufficient temperature allowed to cross over all the energy barriers and to reach the global minimum. The exact procedure we used is described below.

The system was first blocked during 10 ps at 800 K. Therefore several conformations were generated and the most stable was selected. It was used for the following step that consisted in blocking the temperature of the structure at 750 K during 10 ps. The temperature was then decreased in such a way to 300 K, in 50 K steps. The same procedure was repeated twice and so we selected the most stable conformation, which was optimized again at 0 K.

To our knowledge, this study is one of the first that proposed a systematic theoretical investigation on saponins. In that, we needed to establish the most reliable method for such structures. Potential energy surface was thus investigated on diosgenone at the molecular mechanic (MM) level using different force fields: esff, cvff and cff95. All three methods reached the same conformation, close to the experimental geometry. The average of differences in distance, Δr, between the theoretical MM structure and the crystal structure was 0.010 Å. The average of differences in angle, $\Delta\theta$, between the theoretical MM structure and the crystal structure was 1.40°.

From this geometry, we afterwards tested the semi-empirical method AM1.[27] AM1 gave conformations close to those obtained with MM ($\Delta r = 0.011$ Å and $\Delta\theta = 1.24°$).

Subsequently, all the electronic calculations were performed from AM1 conformations. We also investigated the potential energy curve versus the torsion angle defined by C-2–C-3–OH (Fig. 1). This highlighted a very flat energy curve with low energy barriers and led us to conclude that at room temperature the 3-OH group can freely rotate, if it is not engaged in any chemical bond (receptor binding, H-bond and Van der Waals interactions with solvents…).

Quantum chemistry calculations gave electronic structure (especially IP) that is used to investigate a certain form of reactivity; precisely, it is characteristic of redox reactivity. In this paper we looked at the highest occupied molecular orbital (HOMO). According to the Koopmans theorem, IP equal $-E_{HOMO}$ (energy of the HOMO). HOMO is a molecular orbital estimated on the basis of the Hartree–Fock approximation. It is sometimes a good approximation but, concerning the electron behaviour, the electron density is more realistic. However in our case, calculations provided almost the same spatial repartitions for electron density and HOMO. So we decided to look at the orbital scheme, that is, HOMO but also HOMO-1 when it is close to HOMO. In such a case, the two levels must be taken into account and could give more information concerning the reactivity of the molecule.

Molecular mechanic was performed using Discover implemented in the Insight II software, semi-empirical calculations were performed with Mopac.

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