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FTIR spectral signature of the effect of cardiotonic steroids with antitumoral properties on a prostate cancer cell line

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\textbf{Abstract}

\textsuperscript{1} \textbf{Abbreviations:} IR, infrared; FTIR, Fourier Transform infrared; CS, cardiotonic steroid; \\
PCA, Principal Component Analysis; PC, Principal Component; ESS, Error sum of square.
We show in the present work that the infrared (IR) spectrum of human PC-3 prostate cancer cells exposed to anticancer drugs could offer a unique opportunity to get a fingerprint of all the major biochemical components (DNA, RNA, proteins, lipids, etc.) present in the cells and to identify with high sensitivity the signature of the metabolic changes induced by anticancer drugs.

We investigated here the FTIR-related signatures of the effect of 4 structurally-related cardiotonic steroids (CS), i.e. ouabain, 19-hydroxy-2''-oxovoruscharin, hellebrin and 19-hydroxy-hellebrin on PC-3 cancer cells incubated between 0 and 36 h in the absence (control) or the presence of the CS. For each molecule a single spectral signature described the largest part of the time dependent modifications with a possible very minor second component. The spectral signatures characterizing the effects of each of the four CS were unique but very similar when compared to the signature of the effect of an intercalating anticancer drug, i.e. doxorubicin, selected as a positive reference compound in our study, suggesting a fully distinct set of cellular perturbations. The current study thus illustrates that Fourier Transform Infrared (FTIR) analyses can be used to identify, among the perturbations induced on a given cancer cell line, the features common to a group of anticancer compounds as well as features specific to every single drug.

**Key words:** IR spectroscopy, cancer, drug, PC-3, FTIR, cardiotonic steroid.
1. Introduction

A growing number of potential anticancer agents fails in the course of drug development process suggesting that the selection procedure for progression of molecules from early research stages through pre-clinical trials to the clinic requires significant improvement [1]. High throughput screening procedures remain essentially based either on the ability of new drugs to induce cell death over a panel of several cell lines or on their interaction with key parts of one or several biological pathways [1,2]. A technique able to provide a global fingerprint of drug mechanism of action on cancer cells could be of great interest in the development of therapeutic medicines with new modes of action. We suggest in the present study that the infrared (IR) spectrum of tumor cells exposed to anticancer drugs could offer a unique opportunity to get a signature of all the major biochemical components present in the cells and to characterize, with a high sensitivity, specific metabolic changes induced by new potential anticancer drugs.

Cardiotonic steroids (CS) are well known inotropic drugs for the treatment of congestive heart failure and atrial arrhythmia, and the mechanism of their positive inotropic effect is well characterized [3-6]. Chemically, CS are compounds presenting a steroid nucleus with a lactone moiety at position 17 [3,4]. Glycosylated CS contain a sugar moiety at position 3 [3,4]. The nature of the lactone ring at position 17 defines the class of CS: the cardenolides (with an unsaturated butyrolactone ring) and the bufadienolides (with a α-pyrene ring) [3,4]. Recent studies emphasized potent in vitro and in vivo antitumoral effects of these molecules (reviewed in [3-5]). The Na⁺/K⁺-ATPase, the so-called sodium pump, is an integral transmembrane protein found in all higher eukaryotic cells that uses energy from ATP hydrolysis to maintain a high K⁺ and low Na⁺ concentration in the cytoplasm [7]. Recently a non pumping pool of Na⁺/K⁺-ATPase had been evidenced and localised in caveolae [8], a raft like structure [9]. This pool of Na⁺/K⁺-ATPase was shown to be associated with different
signalling proteins and act as a signal transducer [10-12]. The sodium pump mediates CS-induced effects in a compound, concentration and cell type-specific manner [3,4,13,14]. Exciting recent findings have suggested additional signaling modes of action of the sodium pump, implicating CS in the regulation of several important cellular processes and highlighting potential new therapeutic roles for these compounds in various diseases, including cancer [3-6]. The increased susceptibility of cancer cells to these compounds supports their potential use as anticancer therapies, and the first generation of glycoside-based anticancer drugs are currently in clinical trials [3-6]. In addition, we have previously demonstrated that cardenolides are able to overcome MDR phenotype in cancer cells [15] as well as the intrinsic resistance of various types of cancers to apoptosis [16,17]. We have demonstrated that cardenolides exert in fact their anticancer activity through the targeting of the alpha subunits of the sodium pump [18] and that the alpha-1 subunit of the sodium pump is overexpressed in a large proportion of non-small-cell lung cancers [18], glioblastoma [16] and melanoma [19]. Because of their closely related structure and action, the CS family is particularly challenging when distinct modes of action are to be identified.

FTIR spectroscopy was used successfully for decades as label free technique for the study of cells [20,21] as well as tissues [22-24]. Absence of reagent remains one of the main advantages of IR spectroscopy among other exploratory techniques such as fluorescence or microarrays. It senses every metabolite of the cells with a great sensitivity without disturbing its integrity and can therefore provide an accurate fingerprint of modifications occurring within the cells. In turn, IR spectroscopy can be used for bacteria or yeast typing at strain level [20,25,26]. With human samples, IR spectroscopy allowed rapid distinction between tumor and normal tissues [27-29] or powerful discrimination between cancer cell lines [30]. For example, our group established IR markers for determination of the in vivo aggressiveness level of glioma cell lines [30]. Recently, we evidenced that metabolic perturbations induced
by ouabain on the human PC-3 prostate cancer cell line at nM concentration can be monitored by infrared spectroscopy [31]. In the present study, we analyzed spectra of this human PC-3 prostate cancer cell line treated with four different CS during 5 different periods of time. Two of these CS are cardenolides and the remaining two bufadienolides. Each pair of cardenolides and bufadienolides included one natural and one hemisynthetic compound reduced in position 19 of the steroid nucleus (see Figure 1). As indicated above, CS share a common cellular receptor, the Na\(^+\)/K\(^-\)-ATPase, but their mechanisms of anticancer action might vary markedly from one compound to another [4,16,32]. In addition, these signatures were compared to the ones induced by doxorubicin, an intercalating agent that is widely used to combat various types of cancers [33] in order to evaluate spectral changes induced by a molecule belonging to a different family.
2. Materials and Methods

2.1. Compounds

Doxorubicin and hellebrin were purchased from Sigma-Aldrich S.A. (Bornem, Belgium). Ouabain was purchased from Acros Organics (Geel, Belgium). 19-hydroxy-2”-oxovoruscharin was derived from 2”-oxovoruscharin, a cardenolide identified in the African plant *Calotropis procera*, as detailed elsewhere [34]. In the same manner, 19-hydroxy-hellebrin has been derived from hellebrin as detailed elsewhere [35]. The structure of the CS’s is presented in Figure 1.

2.2. Cell culture

The human prostate cancer PC-3 (CRL-1435) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and was maintained according to the supplier’s instructions. Briefly, the cells were incubated at 37°C in sealed (airtight) Falcon plastic dishes (Nunc, Invitrogen SA, Merelbeke, Belgium) in a humidified atmosphere of 5% CO₂. The cells were kept in exponential growth in RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (an antibiotic/antimycotic solution), and 1% kanamycin to prevent mycoplasms. Medium and FBS were purchased from Gibco (Invitrogen, Merelbeke, Belgium). Penicillin/streptomycin and kanamycin solutions came from Sigma-Aldrich SA, (Bornem, Belgium). Drug concentrations used throughout this paper were the IC₅₀, defined as the drug concentration required for decreasing the cell population by half after 72 hours of growth [34]. The medium was replaced 24 hours before delivering the drug to prevent any stress induced by this handling.

For FTIR spectroscopy, cells were suspended after a five-minute treatment with trypsin (0.5g/L)/EDTA (0.2g/L) buffer (Gibco, Invitrogen SA, Merelbeke, Belgium). The reaction was stopped by adding 1 ml of culture medium. The cells were pelleted by a 2-minute
centrifugation (300g), and washed three times in isotonic solution (NaCl, 0.9%) to ensure complete removal of trypsin and culture medium. They were then suspended in the NaCl solution.

2.3. In vitro overall growth determination

Overall cell growth was assessed by means of the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-diphenyltetrazolium bromide, Sigma, Bornem, Belgium) assay, as detailed elsewhere [34]. The cells were incubated for 72 h in the presence or the absence (controls) of the tested compounds. Drug concentrations ranged between 10^{-9} and 10^{-5} M (2 concentrations per log unit). Experiments were carried out in sextuplicate.

2.4. FTIR spectroscopy

All measurements were carried out on a Bruker Equinox 55 FTIR spectrometer (Bruker, Karlsruhe, Germany) equipped with a liquid N2-refrigerated Mercury Cadmium Telluride detector. All spectra were recorded by attenuated total reflection (for a review, see [36]). A diamond internal reflection element was used on a Golden Gate Micro-ATR from Specac (Orpington, UK). The angle of incidence was 45 degrees. A 0.5 µL of the cell re-suspended in about twice the volume of the pellet was deposited on the diamond crystal (about 3x10^4 cells per smear). The sample was quickly evaporated under N2 stream to obtain a homogenous film of whole cells, as ascertained by microscope examination. The FTIR measurements were recorded between 4000 and 800 cm^{-1}. Each spectrum was obtained by averaging 256 scans recorded at a resolution of 2 cm^{-1}. PC-3 cells were incubated in the presence of CS during 5 different exposure times: 0h, 6h, 12h, 24h and 36h. PC-3 cells were grown in culture flasks and always harvested in exponential phase to avoid possible metabolic changes occurring at the plateau [37]. Each condition was represented by at least 7 independent culture samples,
grown over a period of several months, to obtain a good statistical sampling. From each culture flask, 3 independent samples were harvested providing 3 independent spectra from c.a. \(3 \times 10^4\) cells. Overall around 20 to 25 spectra were recorded for each incubation time and drug.

### 2.5. Data analysis

The FTIR data were preprocessed as follows. Water vapour contribution was subtracted, and spectra were baseline-corrected and normalized for equal area between 1765 and 950 cm\(^{-1}\). The spectra were finally smoothed at a final resolution of 4 cm\(^{-1}\) by apodization of their Fourier transform by a Gaussian line.

Normality of the distribution of the absorbances was checked for each group at every wavenumber by a Kolmogorov-Smirnov test by comparison with a standard normal distribution, with a confidence level \(\alpha=0.5\%\) (not shown). The results demonstrated the normality of absorbance distributions. For each culture run in the presence of a CS, an additional culture of cells, free of any treatment, was grown in parallel and analysed. These untreated cells were first used as control for the quality of the cultures. If the IR spectra of a control displayed any statistically significant differences (\(\alpha=0.5\%\)) with previous controls, the experiment was repeated. Second, untreated cells spectra provided us with the reference FTIR fingerprint. Overall, more than 350 non treated cells spectra were recorded. Among them only 70, randomly picked up, are displayed in Figure 2 and used for further analyses.

In IR spectra, each wavelength is a variable, i.e., about 1000 wavelengths are associated with biological molecule absorptions. With at least nine spectra for each condition, the number of variables submitted to statistical analysis quickly becomes extremely large. Data are best handled after Principal Component Analysis (PCA), which is an unsupervised statistical method that enables a reduction of variables by building linear combinations of wavenumbers that vary together [38]. Diagonalization of the covariance matrix of the data provides new
variables, the so-called principal components (PC) holding all the correlated original variables on which original spectra are finally projected. The first principal component accounts for most of the variance present in the data set; the second is built with the residual variance and is uncorrelated to the first one. The subsequent components are constructed the same way and account for the residual variance. In practice, almost all the variance of the original data can be explained with three or four first PC, reducing the description of each spectrum to three or four numbers. Simultaneously, these weights allow an unsupervised classification of the spectra as such an observation does not suppose any a priori condition on these groups [38].

In the analyses reported here, the collection of spectra was mean-centred (the mean was removed from the individual spectra).

Ward’s linkage is another unsupervised method allowing hierarchical clustering of $n$ groups with minimum loss of information. It is based on the similarity of group members with respect to many variables [39]. The grouping is based on the error sum of square criterion (ESS). At each step of the grouping procedure, each possible union is considered and the two items with the lower ESS are grouped. The process is repeated until the number of groups is reduced from $n$ to 1 [40].

Correction of the spectra, Kolmogorov-Smirnov, Student t-tests, PCA and hierarchical classification were carried out by Kinetics, a custom made program, running under Matlab 7.1. (Matlab, Mathworks Inc). Confidence interval ellipses were computed with Statistica (Statsoft Inc.,Tulsa, U.S.A.).
3. Results

3.1. PC-3 cell spectra treated with different cardiotonic steroids.

IC_{50} values were measured on PC-3 cell line and were found to be 36 nM for ouabain, 9 nM for 19-hydroxy-2"-oxovoruscharin, 8 nM for hellebrin, 8 nM for 19-hydroxy-hellebrin and 372 nM for doxorubicin. For FTIR experiments, all compounds were used at their IC_{50} concentrations. This IC_{50} index represents the concentration that reduces by 50% the growth of cancer cells after 72 h in the presence of the drug. Figure 2 displays the FTIR spectra of PC-3 cells grown in the presence of the 4 CS at their IC_{50} as well as control spectra (untreated PC-3 cancer cells).

3.2. PCA analysis

PCA statistical analyses including the spectra displayed in Figure 2 are reported in Figure 3 for each molecule tested. Every point in the plot is the projection of one of the original spectrum presented in Figure 2. The different incubation times are identified by a unique colour and shape, and 95% confidence ellipses were computed for each group. While the cells exposed to the CS under study display a position on PC1 (the first principal component) / PC2 (the second principal component) projection distinct from the control untreated cells, significant overlap between spectra recorded at different incubation times is observed. Interestingly, the action of CS that are hydroxylated in position 19 still evolved continuously up to 36 hours of treatment while it stopped after 24 h for their natural parent compounds. As only the first principal component demonstrated a significant relation with treatment, the first principal component of each of the four PCA are presented on Figure 4A. These 4 principal components are relatively close in shape underlying the similarity of the spectral changes induced by the 4 CS with a positive contribution of 1765-1400 cm^{-1} IR spectral range, and negative contribution of region comprised between 1300 and 950 cm^{-1}. The features observed
on PC1 located from 1765 to 1400 cm\(^{-1}\) can be tentatively assigned to lipid and protein contributions [41-43] and the peak around 1400-1390 cm\(^{-1}\) to CH\(_2/\)CH\(_3\) vibrations from lipids and various protein side chains groups [42,44,45]. Small but significant differences between the CS under study can be observed between 1300 and 950 cm\(^{-1}\) where IR absorption bands mainly arise from polysaccharides or nucleic acids contributions [42,45]. A hierarchical ascendant classification of the PC1 clearly evidenced two subgroups, one containing natural compounds and the other their counterparts hydroxylated at position 19 (Figure 4B).

In a second step PCA decomposition was used to compare the effect of the different molecules after identical incubation times. Non treated PC-3 prostate cancer cells were always included as reference. As previously, 95% confidence intervals were computed and drawn as ellipses. Projections of spectra in the principal component space defined by PC1 (the first principal component) and PC2 (the second principal component) are reported on Figure 5.

Figure 5A illustrates metabolic modifications in PC-3 cells after 6 hours of treatment with CS: none of the CS induced significant metabolic modifications. Figure 5B shows that distinctions between untreated PC-3 cells and those exposed to a given CS become apparent, particularly along the first principal component axis, while no drug-to-drug difference could be evidenced. Figure 5C illustrates that complete separation between untreated and CS-treated PC-3 prostate cancer cells has been achieved after 24 h of exposure of the cancer cells to the CS. Again, no differences among the CS could be evidenced. Lastly, after 36 hours of incubation with the CS, Figure 5D clearly reveals that the two natural compounds, ouabain and hellebrin, caused similar alterations of the IR cell spectra, while the two compounds hydroxylated at position 19 were significantly distinct from their natural counterparts as well as between themselves. More precisely, 19-hydroxy-hellebrin (Figure 5D) still affected protein region i.e. amide I (1690-1620 cm\(^{-1}\)), amide II (1570- 1530) [42,43] and various CH\(_2/\)CH\(_3\) deformation mode arising from proteins (1468-1455 cm\(^{-1}\)) [42,45]. In addition to these areas, 19-hydroxy-2’’-
oxovorusharin (Figure 5B) also strongly modified the carbonyl associated with phospholipids (~1750-1700 cm⁻¹) [41,42] and regions associated with nucleic acids and glycosylations.

As spectrum projection onto PC1 always showed the best separation between untreated and CS incubated cells (Figure 5), the first principal components of each PCA were displayed on Figure 6. Overall, the PC1 obtained for each incubation time have similar shapes. Yet, some significant variations occurred in the IR region comprised between 1300 and 950 cm⁻¹, assigned to nucleic acids and sugars.

### 3.3. Incremental Student t-tests

Student t-tests were computed to compare successive incubation times (6h - untreated cells, 12h - 6h, 24 h - 12h, 36h - 24h). For each molecule tested, Figure 7 reports differences between mean spectra obtained at two successive incubation times. Positive and negative peaks are representative of molecules respectively more or less abundant in the cells after exposure to the drug for the longer time. Thicker curves indicate wavenumbers where the absorbance are statistically different between the two conditions (Student t-test, α=0.5%).

Interestingly, during the first 6 hours, limited but statistically different changes can be observed in the ester C=O stretching of phospholipids at 1740 cm⁻¹ [41] as well as the 1690-1650, 1450 cm⁻¹ and 1230 cm⁻¹ regions. Between 12 h and 6 h and even more dramatically between 12 and 24 h large amplification of the effects observed earlier occurred. The intensity of these latter modifications was also relatively large and almost all the IR regions were affected by CS treatment after 24 h. Yet, only small differences among CS were observed in the difference profiles, indicating similar modification of the chemical composition of the cells during the first 24h. In the next 12 hours, the picture changed drastically. During this latest period, only small further spectral changes could be observed on PC-3 cell spectra exposed to natural compounds. On the contrary 19-hydroxy-2”-
oxovoruscharin and 19-hydroxy-hellebrin induced further marked spectral changes. 19-hydroxy-hellebrin still affected the protein spectral region as confirmed by further modification of amide I and II range. In addition, 19-hydroxy-2”-oxovoruscharin also heavily affected the lipid and nucleic acids/sugar spectral range.

3.4. Comparison of spectral modifications induced on PC-3 cells by cardiotonic steroids and doxorubicin.

In order to evaluate the relative specificity of the differences among the CS observed above, PC-3 cells were treated with a molecule belonging to a completely different family of anticancer drug: doxorubicin. This drug is known to inhibit topoisomerase II and induce lethal damage in DNA double strands that lead to apoptosis [33]. As for CS treatment, PC-3 cells were incubated at doxorubicin IC$_{50}$ concentration. Differences in spectra between a 36 h-period of incubation in the absence (control) or the presence of doxorubicin versus CS are illustrated in Figure 8A. It can be observed that all spectral signatures derived from a CS treatment were relatively similar in comparison with the doxorubicin treatment signature. These various spectra were compared by means of an ascendant hierarchical classification. Figure 8B displays the result of this unsupervised classification. As expected, the effects of ouabain and hellebrin spectral signature are highly similar, and 19-hydroxy-2”-oxovoruscharin and 19-hydroxy-hellebrin linked up further, while they are still closer than doxorubicin-induced signature, underlying the discrimination potential of the FTIR approach.
4. Discussion

We demonstrate in the current study the potential usefulness of IR spectroscopy to monitor metabolic modifications induced in cancer cells by compounds displaying high chemical similarities. The human PC-3 prostate cancer cell line was selected in the current study in order to monitor, by means of FTIR analyses, CS-induced metabolic effects because this cell line displays actual sensitivity to cardiotonic steroids both in vitro [29] and in vivo [46].

FTIR monitoring of CS-induced metabolic effects in PC-3 prostate cancer cells was carried out at the IC$_{50}$, in vitro growth inhibitory concentration calculated after 72 h of cell culturing in the absence (control) or the presence of each CS. This experimental approach enabled to ensure a maximum normalization process in terms of compound concentration when monitoring metabolic changes in PC-3 cancer cells. The choice of the drug concentration to be analyzed remains indeed a critical issue as impact on cell metabolism may depend on its concentration [47,48].

The results reported here present two remarkable features. First, the evolution of the spectra after 6, 12 and 24h is essentially characterized, for each CS molecule, by the same set of spectral features, i.e. there is a single set of chemical modifications in the cell (though affecting several types of molecules) which is continuously amplified in time. This is unexpected as biologists are used to observe cascades of different events, one triggering the next one when a threshold is reached. This is not observed here, considering the time period, the time resolution and the potential of FTIR to distinguish chemical modifications in entire cells. While this lack of details observed by FTIR is most certainly due to a lack of time and chemical resolution, it is good news for diagnostic purpose as it indicates that FTIR signatures are not strongly dependent on the incubation time. In turn, these signatures are expected to be particularly robust.
The second remarkable feature observed in the data is the high similarity of the spectral signatures determined for the four CS tested. This feature emphasizes the robustness of the FTIR with respect to the mechanism of action. In the present case, all cardiotonic steroids bind to a single receptor, i.e. the Na⁺/K⁺-ATPase [3-5]. This robustness is further highlighted when comparing the signatures of CS and doxorubicin treatment. Doxorubicin was chosen because it is a well characterized molecule known to induce a completely different mechanism in cancer cells. It does not interact with the Na⁺/K⁺-ATPase but is rather an inhibitor of topoisomerase II.

The FTIR approach revealed itself capable of evidencing subtle but significantly distinct metabolic modifications brought by the four CS. The first clear distinction appeared when comparing the two natural CS (ouabain and hellebrin) with two hemisynthetic derivatives chemically reduced in position 19 (19-hydroxy-2”-oxovoruscharin and 19-hydroxy-hellebrin). Indeed, the metabolic modifications brought by ouabain and hellebrin in PC-3 cancer cells evolved during the first 24 h of observation, and then stopped (Figure 3 A,C). In contrast, the metabolic modifications brought by the two hemisynthetic derivatives continuously evolved during the 36 hours of FTIR observation carried out during the present study (Figure 3 B,D). We did not proceed with longer time of observation because during these first 36 h-period of FTIR analyses at IC₅₀ we observed no PC-3 cell death as revealed by a Trypan Blue assay (> 90% of PC-3 cells were alive in any experimental condition; data not shown). In contrast, significant PC-3 cell death appeared after 48 h. At these IC₅₀ concentrations, cancer cells die from sustained autophagy and/or lysosomal membrane permeabilization at the 72 h of cell culturing in the presence of the CS [16,17].

In contrast to the two natural CS, i.e. ouabain and hellebrin, metabolic modifications still occurred between the 24th and the 36th h of PC-3 cell culturing in the presence of either 19-hydroxy-2”-oxovoruscharin or 19-hydroxy-hellebrin (Figure 7 B,D). The reason for the lack
of further activity after 36 h for natural compounds is unclear. One hypothesis is that the natural molecules could be degraded either in the culture medium or by the cells. Yet, the effect induced up to 24 h was not reversed as could be expected upon degradation and return to normal metabolism. Indeed, reversal of the process would have resulted in a difference spectrum between 36 and 24 h incubation which would have been the mirror image of the 24-12 h difference spectrum. The absence of such a spectral signature rather suggests the alternative hypothesis that the cells reached a new metabolic state allowing them to live in the presence of the natural CS, even though growing at a slower pace.

In conclusion, a single set of spectral modifications characterizes most of the effects of the four CS tested on PC-3 prostate cancer cells. The same set of modifications is continuously amplified in the course of the time to reach a plateau after 24 h for ouabain and hellebrin where the cells appear to find a new equilibrium. The effect of the 19-hydroxylated compounds is further amplified up to 36 h. Even though unique when compared to signature induced by an anticancer drug from another family, subtle differences were recognized by FTIR spectroscopy among the different CS and allowed to point out a definite particular effect of hydroxylation in position 19. These results suggest that infrared spectroscopy could provide a unique signature of cellular pathways triggered by the molecules tested and could offer an objective selection criterion for drugs with original modes of action.
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Figure captions

Figure 1: Structure of Cardiotonic Steroids used in this study. A: Ouabain, B: 19-hydroxy-2”-oxovoruscharin, C: Hellebrin, D: 19-hydroxy-hellebrin. A and C are natural products. B and D are chemically modified molecules by hydroxylation of respectively A and B at position 19.

Figure 2: Series of infrared spectra of PC-3 cells untreated or exposed to different CS for a period of time indicated in the right margin. Each spectrum was an independent measure of about 3 x 10⁴ cells. For each condition (CS, incubation time) 20 to 25 spectra were recorded from at least 7 independent cell cultures. Untreated cell spectra were recorded for each condition (time + drug). Among them, only 70 untreated cell spectra were randomly selected and displayed here. For better readability, spectra were offset along the absorbance axis.

Figure 3: PCA scores on PC1 and PC2 computed for each drug including all exposure times. A: Ouabain, B: 19-hydroxy-2”-oxovoruscharin, C: Hellebrin, D: 19-hydroxy-hellebrin. Projection of untreated cells recorded for each incubation time were drawn in black filled circles. Squares: 6h exposure to CS, diamond, 12h; triangle, 24h; empty circles, 36h. The percentage of variance in the data explained by each PC is reported in brackets. 95% confidence ellipses are reported.

Figure 4: PCA loadings (PC1) obtained for each drug. A: Curves # 1, 2, 3, 4 are the PC1 respectively obtained from the PCA presented in Figure 3 A, B, C, D respectively. B. Hierarchical classification of PC1 displayed in Figure 4A. Classification was based on Euclidean distances.
Figure 5: PCA scores on PC1 and PC2 computed for each incubation time including all drugs. Projection of spectra arising from untreated cells and those exposed to the drug during: A: 6h; B: 12h; C: 24h; D: 36h. I: Projection of untreated cell spectra are drawn as black filled circles. II: 19-hydroxy-2”-oxovoruscharin treatment related cell spectra are drawn in red (□); III: 19-hydroxy-hellebrin, in blue (∆). IV: Hellebrin, in purple (▲); V: Ouabain in green (■). The percentage of variance in the data explained by each PC is reported in brackets. 95% confidence ellipses are reported.

Figure 6: PCA loadings (PC1) obtained at each incubation time. A. Curves 1, 2, 3, 4 are the PC1 obtained in Figure 5 A, B, C, D respectively. B. Hierarchical classification of PC1 displayed in Figure 6A. Classification was based on Euclidean distances between spectra. Abscissa units are arbitrary as all spectra were rescaled.

Figure 7: Incremental Student t-test. Each spectrum is the difference between two average spectra obtained from successive pairs of experimental conditions. Spectra are offset to the top for better readability. Thicker lines indicates wavenumbers statistically different (α=0.5%) according to a Student t-test. Curves from the bottom to the top are difference spectra respectively calculated for 6h-non treated cells, 12h-6h, 24h-12h, 36h-24h. A: Ouabain, B: 19-hydroxy-2”-oxovoruscharin, C: Hellebrin, D: 19-hydroxy-hellebrin. Note that all the original spectra were rescaled, as detailed in Material and Methods. In turn, intensity of the peaks can be directly compared.

Figure 8: Comparison of the spectral changes induced by cardiotonic steroids and doxorubicin. A: Student t-test between the mean spectrum obtained for each drug after 36 h
incubation and non treated cell spectra. Spectra were offset to the top for better readability. Thicker lines indicates wavenumbers statistically different with a Student t-test ($\alpha=0.5\%$). Difference spectra (drug minus control) are: 1. doxorubicin - control; 2. 19-hydroxy-2”-oxovoruscharin - control; 3. 19-hydroxy-hellebrin - control; 4. hellebrin - control; 5. ouabain - control. B. Hierarchical classification of difference spectra computed on the difference spectra shown in A. Classification is based on Euclidean distances between spectra. Abscissa units are arbitrary as all spectra were rescaled.
References


Fig. 1

Natural compounds

Cardenolides

Synthetic compounds

Bufadienolides
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.

A

Absorbance (A.U.)

Wavenumber (cm\(^{-1}\))

36h
24h
12h
6h

B

6 hrs
12 hrs
24 hrs
36 hrs
Fig. 7.
Fig. 8.