Fourier Transform Infrared (FTIR) spectroscopy to monitor the cellular impact of newly synthesized platinum derivatives

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Abstract. Platinum complexes remain widely used to combat various types of cancers. Three platinum complexes, cisplatin, carboplatin and oxaliplatin, are marketed for various oncological purposes. Additionally, nedaplatin, lobaplatin and heptaplatin have gained regionally limited approval for oncology purposes. Furthermore, various platinum derivatives are currently under clinical trials. More than 40 years after their discovery, however, the precise mechanism of action of platinum antitumor complexes remains elusive, partly because these compounds display numerous intracellular targets. Structure-activity-relationship analyses are therefore difficult to conduct to optimize the synthesis of novel platinum derivatives. The aim of the present study is to illustrate the potential of using Fourier Transform Infrared (FTIR) analyses to monitor the cellular modifications induced by the new platinum derivatives that we have synthesized. We show in the present study the advantages of combining an in vitro assay to determine the IC50 growth inhibition concentrations of a series of compounds belonging to a given chemical series and FTIR analyses carried out at the IC50 concentrations for each compound to identify potential hits within this series of compounds. The original pharmacological approach proposed here could, therefore, avoid large-scale pharmacological experiments to find hits within a given chemical series.

Introduction

Platinum complexes remain widely used to combat various types of cancers (1). Three platinum complexes, cisplatin, carboplatin and oxaliplatin, are marketed for oncological purposes (Fig. 1). Nedaplatin, lobaplatin and heptaplatin have gained regionally limited approval, and various other platinum derivatives are currently under clinical trials (1-3). However, platinum compounds present several drawbacks, such as numerous and serious side effects (4), a limited spectrum of activity, development of resistant cancer cells (5) and unfavorable pharmacokinetic profiles (6). Solutions to these problems have been partially found with oxaliplatin (belonging to the so-called third generation of antitumor platinum complexes), which is less toxic and especially active against several types of tumors known to be less responsive to cisplatin (7,8). These properties have been attributed to the 1,2-diaminocyclohexane moiety that is thought to strongly influence the biological profile of the drug (9). Starting from this hypothesis, numerous classes of new platinum complexes have been designed, among which platinum (IV) complexes (10), trans-complexes (11), polynuclear compounds (12) and hybrid complexes, i.e., platinum bound to biologically relevant molecules (13), are particularly interesting. However, more than 40 years after their discovery, the precise mechanism of action of platinum antitumor complexes remains particularly elusive, partly because these compounds display numerous intracellular targets (14,15). Structure-activity-relationship analyses are therefore difficult to conduct to optimize the synthesis of novel platinum derivatives. Our group has already synthesized various Pt(II) complexes structurally related to 1-phenyl-alkane-1,2-diamines (Fig. 1); those substituted by a fluorine atom at position 4 of the aromatic ring display significant anticancer activity in vitro (16-19).

The aim of the present study was to illustrate the potential of using Fourier Transform Infrared (FTIR) analyses to monitor the cellular modifications induced by the new platinum derivatives that we have synthesized and that are illustrated in Fig. 1. We recently made use of this novel...
approach to characterize the anticancer effects of sodium pump inhibitors (20).

Materials and methods

Chemistry. The complexes (labeled 4-10 in Fig. 1) have been synthesized using the methods we detailed previously (16-19). Briefly, the diamines have been obtained starting from diversely substituted benzaldehyde derivatives and through the hydrocyanation of their corresponding imines followed by the reduction of the nitrile (21). Then the complexes were isolated by reaction of the diamines with K₂PtCl₄ (precipitation, filtration, washing with 2 N HCl and drying at 60°C under vacuum) (22). All of these compounds have been characterized by ¹H- and ¹³C-NMR and IR (16-19,21,22).

Cancer cell lines, media and cell culture conditions. The cell lines used in this study included human U373 (ECACC, European Collection of Cell Culture, Salisbury, UK, code 89081403) and Hs683 (ATCC, American Type Culture Collection, Manassas, VA, USA, code HTB-138) glioblastoma lines, A549 (DSMZ, Deutsche Sammlung von Mikroorganismen and Zellkulturen; Braunschweig; Germany, code ATCC107) non-small cell lung cancer (NSCLC), MCF-7 (DSMZ code ACC115) and MDA-MB-231 (ATCC code HTB-96) breast cancer lines, OE21 esophageal cancer (ECACC code 96062201) and SKMEL-28 (ATCC code HTB-72) melanoma cell lines.

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 phase in MEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% kanamycin to prevent mycoplasmas. Cell culture medium and FBS were purchased from Gibco (Invitrogen, Merelbeke, Belgium). Penicillin/streptomycin and kanamycin solutions were provided by Sigma-Aldrich, SA (Bornem, Belgium).

Determining IC₅₀ in vitro growth inhibitory concentrations. The overall growth level of human and mouse cancer cell lines and human normal cell lines was determined using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Belgium) assay (23-25). Briefly, the cell lines were incubated for 24 h in 96-microwell plates (at a concentration of 10,000-40,000 cells/ml culture medium depending on the cell type) to ensure adequate plating prior to cell growth determination. The assessment of cell population growth by means of the MTT colorimetric assay is based on the capability of living cells to reduce the yellow MTT to a blue product, formazan, by a reduction reaction occurring in the mitochondria. The number of living cells after 72 h of culture in the presence (or absence: control) of the various compounds is directly proportional to the intensity of the blue color, which is quantitatively measured by spectrophotometry, in our case using a Biorad Model 680XR (Biorad, Nazareth, Belgium) at 570 nm (with a reference of 630 nm). Each experiment was carried out in sextuplicate.

Validation of MTT colorimetric assay-generated data by means of the crystal violet assay. The in vitro testing of the substances for antitumor activity in adherent growing cell lines was carried out on exponentially dividing human cancer cells according to a previously published microtiter assay (26,27). Exponential cell growth was ensured during the whole time of incubation. Briefly, 100 μl of a cell suspension was placed in each well of a 96-well microtiter plate at 7200 cells/ml (MCF-7) and at 3000 cells/ml (MDA-MB-231) of culture medium and incubated at 37°C in a humidified atmosphere (5% CO₂) for 6 days. By removing the medium and adding 200 μl of fresh medium containing an adequate volume of a stock solution of metal complex, the desired test concentration was obtained. All complexes tested were dissolved in DMF. Eight wells were used for each test concentration and for the control, which contained the corresponding amount of DMF. The medium was removed after reaching the appropriate incubation time. Subsequently, the cells were fixed with a solution of 1% (v/v) glacial dialdehyde in phosphate buffered saline (PBS) and stored in PBS at 4°C. Cell biomass was determined by means of a crystal violet staining technique as described earlier (26,27). The effectiveness of the complexes was expressed as corrected T/Ccorr [%] or t [%] values according to the following equation:

$$
\text{cytostatic effect: } T/C_{corr} [%] = \frac{(T - C_0)}{(C - C_0)} \times 100
$$

$$
\text{cytocidal effect: } t [%] = \frac{(T - C_0)}{C_0} \times 100
$$

where T (test) and C (control) are the optical densities at 590 nm of crystal violet extracts of the cells in the wells (i.e., the chromatin-bound crystal violet extracted with ethanol (70%), with C₀ being the density of the cell extract directly before treatment). For the automatic estimation of the optical density of the crystal violet extract in the wells, a microplate autoreader (Flashscan S 12; Analytik Jena, Germany) was used. The IC₅₀ values were calculated from the graphs using data taken after an incubation time of 96 h.

FTIR analyses. Human A549 NSCLC cells were detached by means of a 5-min treatment with trypsin/EDTA buffer (Gibco, Invitrogen SA). The reaction was then stopped by adding 1 ml of culture medium. The cells were pelleted by a 2-min centrifugation (300 g) and washed 3 times in isotonic solution (NaCl, 0.9%) to ensure complete removal of trypsin and culture medium. The cells were then suspended in 30 μl of a NaCl solution.

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, as detailed elsewhere (20,28). Briefly, a diamond internal reflection element was used on a Golden Gate Micro-ATR from Specac (Orpington, UK), and the angle of incidence was found to be 45 degrees. Then 0.5 μl of cell pellet was resuspended in about twice the volume of the initial pellet and was deposited on the diamond crystal (about 3x10⁴ cells per smear). The sample was quickly evaporated under a stream of N₂ to obtain a homogenous film of whole cells, as ascertained by microscope examination. The FTIR measurements were recorded between 4,000 and 800 cm⁻¹. Each spectrum was obtained by averaging 256 scans recorded at a resolution of 2 cm⁻¹. For statistical validation, the cells were grown in...
three independent boxes for each experiment, and three samplings were taken from each box, generating a total of nine spectra per condition. For each case, 3 boxes of non-treated cells were grown, collected and analyzed in the same conditions as the treated ones for a control, generating a total of 9 non-treated cell spectra.

The FTIR data were pre-processed as follows. First, the water vapor contribution was subtracted, and then the spectra were baseline-corrected and normalized for equal area between 1765 and 950 cm\(^{-1}\). Finally, the spectra were smoothed at a final resolution of 4 cm\(^{-1}\) by apodization of their Fourier transforms by a Gaussian line. The processing of the spectra and Wilcoxon tests were carried out by means of specific software generated in our lab running in Matlab (Mathworks, Inc.).

Results

Determining IC\(_{50}\) in vitro growth inhibitory concentrations. The in vitro antitumor activity of compounds 1 and 4-7 (Fig. 1) have been determined in four human apoptosis-resistant and
two human apoptosis-sensitive cancer cell lines. The four human apoptosis-resistant cancer cell lines included the U373 astroglioma (29,30), the A549 NSCLC (31), the SKMEL-28 melanoma (32) and the OE21 esophageal cancer (33) cell lines. The two apoptosis-sensitive cancer cell lines included the MCF-7 breast cancer (34) and the Hs683 oligodendroglioma (30,35) cell lines.

The data in Table I indicate that apoptosis-resistant and apoptosis-sensitive cancer cell lines displayed similar (p<0.05; Student's t-test) levels of sensitivity to the various compounds under study. This result meant that the compounds under study overcame, at least partly, the natural resistance to apoptosis displayed by many cancers associated with poor prognoses.

We used cisplatin (1) as a positive reference compound in the current assay, and the data in Table I reveal that compounds 4-7 displayed \textit{in vitro} anticancer activity about 10-fold more pronounced against the various cancer cell lines under study than did cisplatin, except the U373 glioblastoma and the OE21 esophageal cancer cell lines, which displayed similar sensitivity to both cisplatin and compounds 4-7. We then decided to proceed with a second evaluation of the \textit{in vitro} anticancer activity of the compounds under study by means of the crystal violet assay, as detailed below. This second series of experiments were carried out to avoid any false-negative or false-positive data that could be generated by the MTT colorimetric assay (36).

Validation of MTT colorimetric assay-generated data by means of the crystal violet assay. The time activity curves obtained with the human MCF-7 breast cancer cell line are characterized by a fast onset of antiproliferative effects. At the highest concentrations, the maximum of activity was achieved after an incubation time of 48 h. In contrast, this effect was only observed at the end of the test (144 h) with

\begin{align*}
\text{cytostatic effect: } \frac{T}{C} \times 100 = \frac{[T(C_0) - C_0]}{C_0} \\
\text{cytocidal effect: } \% = \frac{[T(C_0)]}{C_0} \times 100
\end{align*}

where T (test) and C (control) are the optical densities at 590 nm of crystal violet extract of the cells in the wells (i.e., the chromatin-bound crystal violet extracted with ethanol (70%) with C_0 being the density of the cell extract directly before treatment).

Figure 2. Time-dependent cytotoxicity of representative platinum compounds (5 and 7) determined in the crystal violet assay. Cell biomass was determined by means of a crystal violet staining technique. The effectiveness of the complexes is expressed as corrected T/C corr [%] or t [%] values according to the following equations:

\begin{align*}
\text{cytostatic effect: } &\frac{T}{C} \times 100 = \frac{[T(C_0) - C_0]}{C_0} \\
\text{cytocidal effect: } &\% = \frac{[T(C_0)]}{C_0} \times 100
\end{align*}
another human breast cancer cell line, MDA-MB-231. As depicted in Fig. 2, five concentrations were used to calculate the IC50 value of each compound. Independent of the substituent in the aromatic ring, IC50 values of 2-4 μM were calculated after an incubation time of 96 h. A marginal preference in activity could be determined with the 4-F substituted complex 7, which caused cytotoxic effects at 20 μM and was cytostatic at 5 and 10 μM (144 h). Furthermore, it must be mentioned that the exchange of the fluorine (compounds 5, 6 and 7) by a trifluoromethyl substituent (compounds 8, 9 and 10) did not change the absolute cytotoxicity of these compounds in the MCF-7 breast cancer cell line. As seen in Fig. 2, only the kinetics of activity of these compounds slightly changed. In contrast, the influence of the substitution pattern was more pronounced in the MDA-MB-231 breast cancer cell line. As seen in Fig. 2, the complexes were generally less active compared to MCF-7 cells with the 2-F (5) and the 3-CF3 (9) substituted complexes showing the highest IC50 values of 13.9 and 9.1 μM, respectively (Table II).

The IC50 of complexes 4-7 were also calculated by means of the MTT colorimetric assay. Similar data have been obtained between the MTT colorimetric assay (Table I) and the crystal violet assay results (Table II). When present, differences between the data generated by the two assays resulted either from the different types of measurements, i.e., viability (MTT assay) versus cell mass (crystal violet assay) measurements, or from the different incubation times used for the two assays, i.e., 72 h for the MTT colorimetric assay versus 96 h for the crystal violet assay.

**FTIR analyses.** Human A549 NSCLC cells were cultured for 3 days in the presence of compounds 4-10 (Fig. 1) at their IC50 in vitro growth inhibitory concentrations as determined by the MTT colorimetric assay (Table I). Three independent cell cultures (i.e., three independent culture flasks) and three samples per cell culture (for a total of 9 samples) were analyzed for each compound. The 81 spectra obtained for the 9 compounds are presented in Fig. 3C. The high quality of the signal (signal/noise ratio >3000) allowed the highlight of very subtle changes induced by different compounds. Statistical analyses were performed between 1765 and 950 cm−1. This spectral area contained vibrations arising from all biological molecules (37) and provided an accurate fingerprint of cell metabolism at the time of the measurement. Thus, each of the 9 measurements available for a given compound was described by 816 wavenumbers. In other words, each sample was located at a unique position in an 816-dimensional space.

To better visualize differences in locations in this 816-dimensional space between the various experimental conditions, principal component analysis (PCA) was applied to locate each sample into a bi-dimensional space defined by the first two PCA factors that described more than 80% of the variance.
its effectiveness has been hampered by toxic side effects and significant clinical benefit for several types of solid tumors, (38,39). However, despite the fact that cisplatin has achieved achievement and ushered in a new era in cancer treatment the discovery of cisplatin in the 1960s represented a landmark basis for treatment of several common tumors (38). Indeed, chemotherapy (38,39), and platinum-based regimens are the anticancer activity has had an enormous impact on cancer cancers (38). The development of metal complexes with that have the potential to be broadly active across multiple although targeted therapeutics have had effects in some rearrangements, and many silent and active mutations (38).

Discussion

The plasticity and instability of the cancer genome is impressive and is characterized by gene amplifications and deletions, rearrangements, and many silent and active mutations (38). Although targeted therapeutics have had effects in some diseases, there remains a large role for new cytotoxic agents that have the potential to be broadly active across multiple cancers (38). The development of metal complexes with anticancer activity has had an enormous impact on cancer chemotherapy (38,39), and platinum-based regimens are the basis for treatment of several common tumors (38). Indeed, the discovery of cisplatin in the 1960s represented a landmark achievement and ushered in a new era in cancer treatment (38,39). However, despite the fact that cisplatin has achieved significant clinical benefit for several types of solid tumors, its effectiveness has been hampered by toxic side effects and tumor resistance that often leads to the occurrence of secondary malignancies (39). Cisplatin was indeed one of the first chemotherapeutic agents to exhibit broad efficacy in solid tumors, and it remains among the most widely used agents in the treatment of cancer (3,38,39). Its achievements have inspired great efforts to design similarly effective platinum agents that overcome the three main limitations of cisplatin: toxicity, tumor resistance and poor oral bioavailability (3). However, 40 years after the initial discovery of cisplatin, only two other platinum agents have garnered US FDA approval: carboplatin and oxaliplatin (3,40,41). Although hundreds of promising agents were tested in clinical trials during the 1990s, only oxaliplatin made it past clinical development (3). Additionally, nedaplatin, lobaplatin and heptaplatin have gained regionally limited approval (40). For a brief period, the economic cost of these unsuccessful efforts retarded further efforts to develop new agents (3). Despite these challenges, however, two new exciting platinum agents have been brought to Phase III trials: satraplatin for hormone-refractory prostate cancer (3,42) and picoplatin for small-cell lung cancer (3). If successful, they may inspire a new effort to bring better-designed platinum agents to market (3).

To successfully achieve the development of new clinically efficient platinum complexes, several attempts have been made to obtain quantitative structure-activity relationships (43,44). The aim of the authors was to find general rules for guiding the design of new Pt complexes, but this approach has proven to be especially difficult. In particular, investigation of the mechanism of action of platinum complexes remained a particularly tough task. Owing to the nature of the platinum atom, which is a highly reactive Lewis acid, numerous cellular components are prone to bind the complexes, including nucleic acids (DNA and RNA), proteins and phospholipids (45). Despite this large variety of targets, however, it is commonly admitted that DNA is the most relevant biological structure responsible for their anticancer effects (3,38-42,45), which has been proven by the analysis of the cellular responses triggered by platinum complex treatment, mainly in terms of protein expression (14,46-48). Upon binding of the complexes, several peptides are recruited to recognize the DNA lesions and trigger apoptosis, as well as to repair the nucleic acid structure and allow cancer cell survival (46-48). Parallel to this, cancer cells undergoing apoptosis lose their membrane integrity and release molecules that are constitutive of the cellular frame-work (46-48). In addition, it has been demonstrated that the tumoral resistance toward anticancer platinum complexes can be increased by high concentrations of thiol-containing molecules, such as methionine (49), cysteine, glutathione (50) and metallothioneins (51). Taken together, these cellular events induce strong qualitative and quantitative modifications of the cellular content of the above-mentioned compounds. It is therefore expected that the "chemical signature" of cancer cells after treatment with the complexes could be different when the mechanisms of action or resistance are divergent, which is why we used a FTIR approach in the current study to investigate whether the chemical signatures in whole cancer cells (we chose the A549 NSCLC cell line as a model, 31) would significantly differ from the novel compounds (compounds 4, 5, 6, 8, 9 and 10 in Fig. 1) generated from compound 7 as a scaffold (Fig. 1).
The determination of the IC\textsubscript{50} \textit{in vitro} growth inhibitory concentration by means of two distinct tests (Tables I and II, Fig. 2) suggested that the anticancer activity displayed by all compounds under study were roughly similar, at least \textit{in vitro}. FTIR analyses then revealed that the mechanisms of action of the novel compounds we generated did not actually diverge from that displayed by compound 7, as illustrated in Fig. 3. Fig. 3B shows that compound 7 presented the largest spectral dissimilarities when compared with the spectra of untreated cells. Fig. 3D shows different spectra characterizing metabolic changes induced by each molecule tested in this study. Compound 7 clearly led to the deepest metabolic changes in intensity, as well as in the number of wave-numbers that significantly differed (\(a=0.5\%\)). The main changes appeared between 1765 and 1500 cm\(^{-1}\) in the spectral range. This area is mainly dominated by contributions arising from lipids (around 1740 cm\(^{-1}\) (37)) and proteins (the Amide I and II, roughly comprised between 1700 and 1500 cm\(^{-1}\) (37)). Difference peaks were positive, suggesting that A549 NSCLC cells were enriched in these two components against the rest of the cellular content after incubation with compound 7.

In conclusion, we proposed to combine an \textit{in vitro} assay to determine the IC\textsubscript{50} growth inhibitory concentrations of a series of compounds belonging to a given chemical series and FTIR analyses carried out at the IC\textsubscript{50} concentrations for each compound to identify potential hits within this series of compounds. The current study showed that we did not obtain a lead with a significant divergent activity when modifying a novel platinum derivative. Interestingly, the original pharmacology approach proposed here could avoid large-scale pharmacological experiments to find hits within a given chemical series.

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