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Raman spectroscopy in multidisciplinary approaches applied to drug design

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This paper addresses the applications of Raman spectroscopy to the development of novel metal-based anticancer drugs, within the research activity carried out in the last few years at the Molecular Physical-Chemistry R&D Unit of the University of Coimbra (Portugal).

Raman spectroscopy, coupled to other complementary techniques such as infrared and inelastic neutron scattering spectroscopies, was used to characterize novel metallo drugs and to probe their interaction with key biomolecules and their impact on cellular metabolism and intracellular water (pharmacokinetic and pharmacodynamic profiles). Platinum and palladium complexes with poly amines were investigated, comprising more than one metal center and displaying a non-conventional inter play with DNA. New drug targets were explored, aiming at a multitarget approach with a view to improve chemotherapeutic outcome.

Keywords: Raman spectroscopy, Fourier transform infrared spectroscopy (FTIR), Inelastic neutron scattering spectroscopy (INS), Quasi-elastic neutron scattering spectroscopy (QENS), Anticancer drugs, Human cancer cells.

1 Introduction

The worldwide burden of cancer is increasing, being currently the second cause of death and expected to rise up to 22 million cases *per* year within the next two decades [1]. Since cancer is an age-related disease, this trend is likely to worsen due to the currently increasing life-expectancy. Chemotherapy is one of the main strategies in the fight against cancer, alongside with radiotherapy and surgery. In view of the high morbidity and mortality of this disease, new and more efficient therapeutic approaches are a pressing social and medical need, targeting malignant cells with minimal damage to healthy tissue and leading to an improved prognosis for oncology patients.

Over the past decade, intense research efforts have been carried out with a view to attain a molecular-level understanding of the processes underlying carcinogenesis, cancer progression, invasion and metastasis. This knowledge is crucial for designing new antitumor agents with an enhanced therapeutic activity coupled to lower deleterious side-effects and acquired resistance. Nonetheless, even though much progess has been achieved in this field there are still numerous issues to be addressed in order to improve cancer therapy, particularly regarding critical side effects and drug resistance. Several approaches have been followed in order to overcome these challenges, namely combination therapy (administration of two or more drugs simultaneously) [2], adjuvant therapy (e.g. with antioxidants) [3-10], drug encapsulation and targeting (using state-of-the-art tailored and biocompatible nanomaterials) [11] and multitarget strategies allowing a multimodal drug action [12-14].

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2 Metal-based anticancer drugs

While many drugs are organic in nature, inorganic molecules display a diverse chemistry and provide functionalities not accessible to purely organic compounds, thus being a valuable source of active agents in Medicinal Chemistry. Metal-containing compounds have been used as the therapeutic agents for millennia, even if a molecular basis knowledge of their activity has only been addressed with the advent of modern medicine in the early 20thcentury. These type of potencial antineoplastic agents have gained a progressively increasing interest in Medicinal Chemistry since the1970´s [14,15-27], following the pioneering work of Köpf on the antitumor activity of cyclopentadienyl metal complexes (in the late1970´s) [28] and the approval of cisplatin (*cis*-diamminodichloro-platinum(II), *cis*-Pt(NH_3)2Cl_2) as the first anticancer metallodrug (in 1978) [2].

Inorganic anticancer compounds encompass a wide variety of metal ions and ligands, as well as different coordination patterns and designs tailored to attain selectivity/specificity according to the type of receptor or biological target. The use of metals other than platinum and palladium may allow a distinct and more beneficial drug activation and reactivity profiles [29,30]. Due to their versatility, several transition metal complexes have been investigated as potential antineoplastic agents, namely of ruthenium (II)/ruthenium (III), gold (I)/gold (III), bismuth (III), rhenium (I), copper (II), gallium (III) and tin (IV), some of them having shown promising antitumor properties often *via* an interplay with non-genomic targets (e.g. topoisomerases, redox enzymes, thiol-proteins, mitochondria) [2,24,27,31-40].

At present, the development of improved metal-based anticancer agents relies on a target-directed approach, with an emphasis on controlled delivery, as well as on drug reprofiling, with a view to enhance and extend cytotoxic activity with minimal toxicity [11,14,25,26,41-43]. Such an approach depends on reliable methodologies for determining structural and conformational behavior, as well as for probing interactions (at a molecular level) with biological receptors.

2.1 Platinum Drugs

The serendipitous discovery of the antitumor properties of cisplatin by Barnett Rosenberg, in the early 1960´s, is a milestone in the history of metal-based drugs [44,45]. Cisplatin is still one of the leading drugs in cancer management (used in *ca.* 50% of all chemotherapy strategies, usually in combination schemes) [46], specifically against head and neck, testicular, ovarian, cervical, prostate, bladder and lung carcinomas, as well as melanoma and lymphoma [2,47-49]. Since the introduction of cisplatin to oncology, in 1978 (trade name Platinol), only two other cisplatin-like compounds have been approved for worldwide routine clinical use – carboplatin (Paraplatin) and oxaliplatin (Eloxatin) (Fig 1). These have been designed upon structural modifications of the lead cisplatin, regarding both the metal coordination pattern and the labile ligands, in order to tailor the drug´s pharmacokinetics and attain a decreased toxicity and a minimal treatment-induced resistance.

The main mechanism of action of Pt-drugs is covalent binding of the metal centre (s) to the nitrogen atoms of DNA´s purine bases (mainly guanine at the most nucleophilic N7 atom, Fig 2), upon intracellular chloride hydrolysis, yielding short-range intra-and interstrand adducts [50-53]. This specific inter action is responsible for DNA conformational rearrangements and activation of several signal transduction pathways, that finally induce cell death (by apoptosis). Apart from their DNA-damaging effect, platinum compounds have also been found to bind to protein receptors, enzymes or membrane lipids [54,55], and to affect the cell´s biomechanical properties (e.g. cellular water dynamics) [50,56] which are closely related to malignant progression.

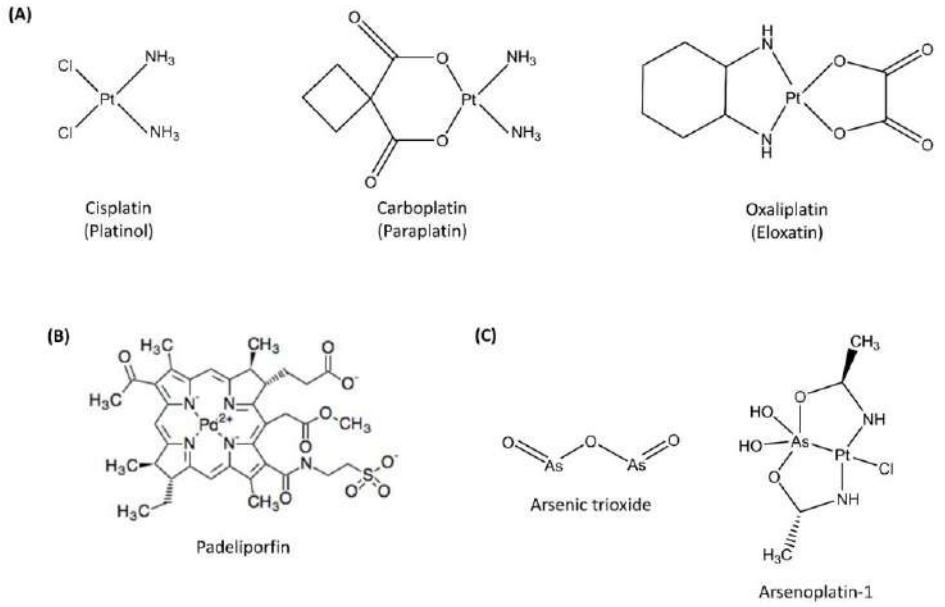

Fig 1. Schematic representation of: (A) platinum-based drugs currently approved for clinical use worldwide – cisplatin (Platinol), carboplatin (Paraplatin) and oxaliplatin (Eloxatin); (B) palladium anticancer agent Padeliporfin (Tookad); (C) arsenic trioxide (Trisenox) and Arsenoplatin-1.

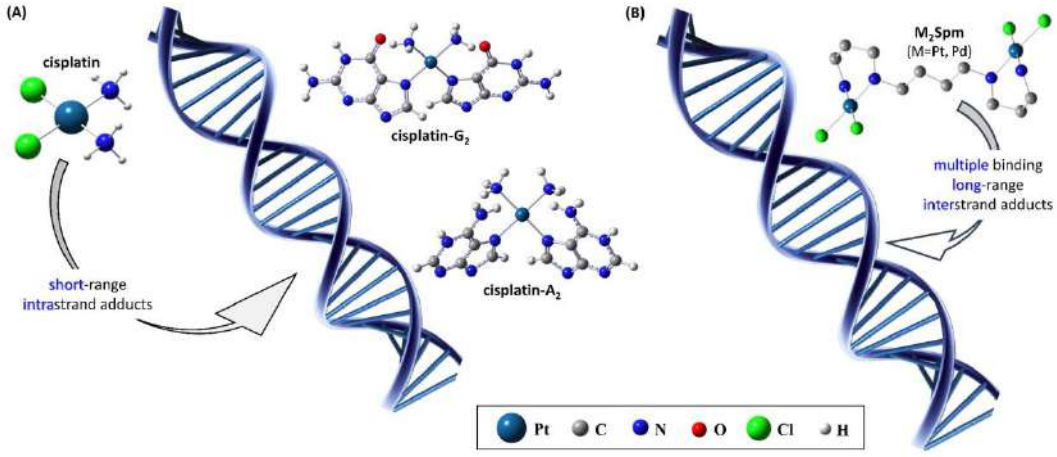

Fig 2. Schematic representation of the interaction between a Pt (II)-drug and DNA, for mononuclear (A) and polynuclear (B) agents.

However, metallodrugs still present major handicaps that severely limit its clinical use and chemotherapy success, thus affecting millions of oncological patients: low bioavailability, dose-limiting and cumulative toxicity, and acquired resistance [54,57-60]. The latter is a multifactorial process, associated to reduced drug accumulation at the target, increased drug efflux, drug inactivation (e.g. by parallel reactions with sulphur-containing biomolecules such as glutathione or metallothioneins), enhanced DNA repairing mechanisms, alteration of the drug target and/or changes in proteins that signal apoptosis. Major side-effects, in turn, are myelosuppression, nephrotoxicity, ototoxicity, hepatotoxicity and neurotoxicity. These drawbacks

triggered an intense search for new generation agents displaying an improved pharmacological profile, with an emphasis on novel drug delivery and targeting schemes [27,43,61-64]. Among these, liposomal and polymeric formulations have been developed, namely Lipoplatin (liposomal-encapsulated cisplatin) [65] and ProLindac (nanopolymer conjugated oxaliplatin) [66], which are currently in advanced human clinical trials (Fig 3). Nevertheless, up to this date only *ca.* 20 platinum agents have entered clinical trials, from which several (*ca.* 14) have been discontinued upon discovery of severe toxicity [43,47].

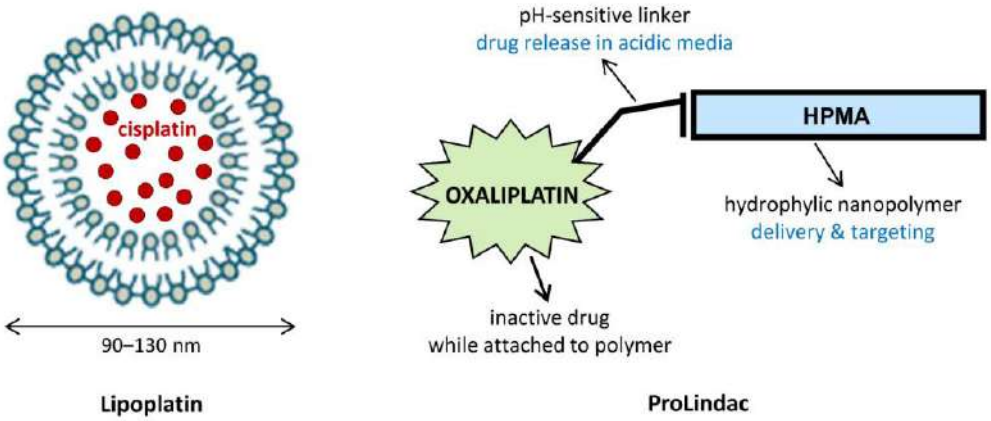

Fig 3. Schematic representation of Lipoplatin (liposome-encapsulated cisplatin) and ProLindac (polymeric formulation of oxaliplatin). (HPMA – N-(2-hydroxypropyl) methacrylamide).

2.2 Palladium Drugs

Since the pioneer work of Khan *et al* [67] and based on the structural and chemical similarity between the Pt (II) and Pd (II) ions, several Pd (II) compounds have been studied as potential antitumor drugs, these being currently one of most widely investigated metal-based group of compounds (*ca.*850)–the fifth after platinum, copper, gold and ruthenium complexes [68-71].

A careful choice of leaving group(s) and strongly coordinating ligands is essential – mostly polydentate compounds such as polyamines, phenantrolines, diphenylphosphines or thiosemicarbazones – in order to counterbalance the high lability of Pd (II) (as compared to Pt (II)) and hinder rapid intracellular hydrolysis and drug inactivation *via* metal ion displacement by biological sulphydryl groups. Hence, in order to modulate activity and toxicity polydentate amines with some degree of lipophilicity have been used as coordinating moieties (*e.g.* diaminocyclohexane, phenanthroline, spermidine and spermine), as well as N-containing aromatic rings (able to intercalate into DNA, apart from covalently binding), coupled to chloride or nitrate leaving groups.

New Pd-based complexes, as well as palladium for platinum substitution in previously developed Pt- compounds, are DNA-damaging agents that have often shown a high antineoplastic activity coupled to less deleterious side-effects and lack of cross-resistance relative to platinum clinical drugs [72-87]. Some dinuclear Pd-chelates, in particular, displayed a higher cytotoxicity than their mononuclear counterparts [76,78,82,84,85]. The activity of Pd- agents follows mechanisms of action generally distinct from those associated to cisplatin-like compounds, and is ruled by structure-activity relationships (SARs) that are not necessarily the same as those previously established for their Pt (II) homologues [70]. In fact, the conventional SARs for metal-based drugs have gradually been broken as research progressed: good antineoplastic activity has been verified for complexes without NH groups or having a *trans* geometry, while multinuclear and polycationic agents (interacting with DNA both covalently and non-covalently) have emerged as promising alternatives [62,88,89]. This leads to a different spectrum of clinical activity *via* biological targets beyond

DNA (e.g. enzymes, lysosomes, proteosomes), allowing for an improved efficiency against platinum-resistant tumors.

Among the Pd-systems currently under study, Padeliporfin (water soluble Tookad ®) has been approved for clinical use in the European Union in 2017 (Fig 1). This is a photodynamic therapy (PDT) agent highly effective against localised prostate cancer, and the first Pd (II)-drug in routine oncological treatment [62,90-94].

2.3 Polynuclear Polyamine Platinum and Palladium Complexes

Linear aliphatic polyamines are low molecular-weight saturated compounds with a very high conformational freedom and a dual hydrophilic-lipophilic character [95-98], that can act as chelating ligands with a considerable affinity for transition metal ions such as Pt (II) and Pd (II) [41,62,77,78,98-102]. Among these, the biogenic polyamines putrescine (Put, $H_2N(CH_2)_4NH_2$), spermidine (Spd, $H_2N(CH_2)_3NH(CH_2)_4NH_2$) and spermine (Spm, $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) are essential endogenous molecules that modulate key biochemical processes such as cellular proliferation and differentiation, and were found to be closely associated with carcinogenesis and neoplastic growth [103-108].

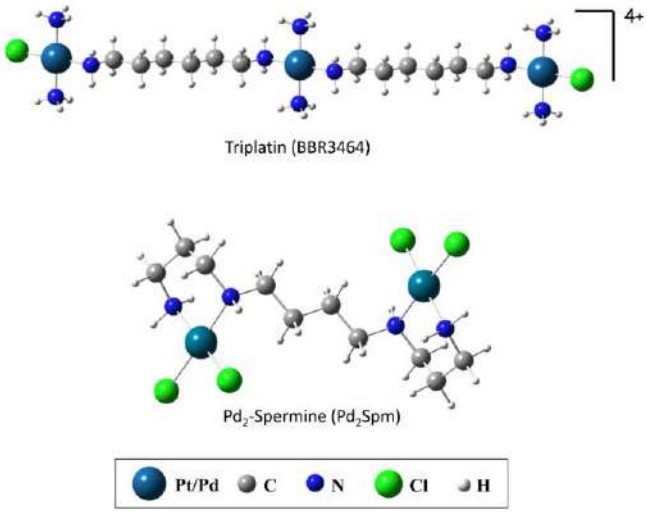

Fig 4. Schematic representation of some poly-homonuclear Pt (II) and Pd (II) complexes with linear polyamines.

Based on their particular chemical properties, polydenticity and biocompatibility, linear polyamines have often been used as coordinating entities in the rational design of Pt (II) and Pd (II) potential anticancer agents, namely those comprising more than one metal center which display a different biochemical pharmacology and a lack of cross- resistance relative to first-generation mononuclear Pt-drugs (e.g. cisplatin-like), thus allowing a broader spectrum of clinical activity (Fig 4). These polynuclear chelates with polyamines as bridging ligands were found to bind covalently to DNA through an interplay not available to conventional drugs, *via* long-range intra- and interstrand adducts (Fig 2 (B)). Moreover, the hydrophobic alkyl linkers (between the metal centers) interact with the DNA minor groove prior to covalent bond formation between the metals and the purine bases, this preassociation favoring crosslink formation. Additionally, the nitrogendonor atoms from the ligand (s) assists the drug´s molecular recognition by DNA´s polyphosphate backbone (due to the formation of (N)H···O interactions), and the amine´s amphiphilic nature may favor the drug´s cellular uptake. This specific interplay prompts a more severe, less-repairable DNA damage (leading to apoptosis) and often circumvents acquired resistance [23,41,62,71,74,79,80-87,109-113]. Nevertheless, the exact nature of the mode of action of this kind of complexes, at a molecular level, is still unknown, thus highlighting the

importance of relating their structural and conformational preferences to biochemical impact and antitumor activity.

Polynuclear platinum and palladium chelates with spermidine and spermine, comprising cisplatin-like moieties (Fig 4), have been investigated at the author´s laboratory following a multidisciplinary approach which provided a comprehensive set of data on: (i) structure and conformational behavior [79,114,115]; (ii) cytotoxicity towards several human cancer cells [76,78,80,84,116]; (iii) specific interactions with proteins, DNA and glutathione [51-53,117]; (iv) impact on cellular metabolism and intracellular water, as well as cellular response to the drugs [50,81,86,87,116,118]. Apart from cellular biology *in vitro* assays for evaluation of anticancer activity, vibrational spectroscopy has been applied – Raman, Fourier-transform infrared (FTIR) and inelastic neutron scattering (INS) – coupled to X-ray absorption spectroscopy (XAS) techniques and theoretical simulations. A Pd-agent, in particular (Pd2Spm), has yielded quite promising results against human metastatic breast cancer [76,87].

Furthermore, in an effort to widen the spectrum of activity and increase patient survival, poly/heterometallic complexes (comprising two or more different metal centres) were introduced in the last decade as promising antitumour agents [119]. These display specific coordination patterns and conformational properties that determine their flexibility, charge and hydrogen-bonding capabilities and consequently their intracellular hydrolysis and activation processes as well as interaction with DNA and other biological targets (which mediate cytotoxicity). These types of heteronuclear metallodrugs were found to have a distinct mode of action relative to homonuclear ones (comprising either one or more metal centers), interacting differently with multiple biological targets (beyond DNA). Among these systems, the dual pharmacophore Pt (II)/As (III)-arsenoplatin (an adduct of the anticancer drugs cisplatin and arsenic trioxide (Fig1(C)) has recently shown a promising activity against acute promyelocytic leukemia, higher than that of the parent agents cisplatin and As_2O_3 in sole administration [120,121]. Within this approach, di- and trinuclear mixed Pt (II)/Pd(II)-agents have also been the object of research (Pt-Pd, Pt-Pd-Pt and Pd-Pt-Pd), based on the selective interactions of Pd (II) and Pt (II) cations with sulfur-and nitrogen-donor atoms from biological nucleophiles (e.g. serum albumin, metallothioneins, amino acids, DNA bases) that may lead to an enhanced cytotoxic efficacy coupled to decreased side effects [119,122-124].

3 Vibrational spectroscopy in drug development

Raman and FTIR vibrational spectroscopy techniques are invaluable tools in pharmaceutical and medicinal research since they are accurate, non-invasive, reproducible and allow rapid measurements with virtually no sample preparation, yielding unique finger print spectra. When coupled to optical microscopy, confocality is attained delivering real-time chemical images at a sub-cellular level and with an unsurpassed signal-to-noise ratio, allowing an accurate mapping of the cellular components without the need for dyes or external probes (unlike numerous other methods that have been developed for studying bio samples, such as fluorescence spectroscopy). Furthermore, coupling FTIR micro-spectroscopy to the brilliance of synchrotron radiation sources (SR-FTIR) has led to a massive increase in the quality of the results (spatial resolution and signal-to-noise ratio), providing sub-cellular sensitivity (including in live cells). Hence, coupling vibrational spectroscopy and optical microscopy techniques to high power radiation sources constitutes an unprecedented approach for high resolution chemical imaging of biospecimens, which is paramount in rational drug development. Additionally, non-ionizing infrared THz radiation allows a unique and non-invasive view into biosystems. Being particularly sensitive to vibrational modes due to collective vibrations and intra/intermolecular interactions (H-bonds and van der Waals), THz spectroscopy can shed light on biomolecules´ conformational changes, closely linked to cellular function [125-133].

Optical vibrational methods (Raman and FTIR) can be reliably applied to *in vitro*, *in vivo* and *in situ* conditions, enabling to: observe distinct cellular states (e.g. viability, apoptosis or necrosis); discriminate

between healthy and diseased biospecimens (cells and tissues); detect specific metabolites in cells/tissues; and monitor drug uptake and cellular impact [51,53,118,134,135]. Therefore, they have been increasingly used in diagnosis [136-154], detection of bacterial pathogens in clinical settings [155,156], and drug design [7,9,116,118,157-162]. Raman spectroscopy, in particular, with hardly any interference from water, is especially suited for the analysis of highly heterogeneous biospecimens – cells, tissues, body fluids and even living organisms – under conditions as similar as possible to the physiological medium (regarding pH, temperature and ionic strength). This constitutes an unmatched approach for routine chemical profiling of biological samples, enabling to accurately probe cellular environments and monitor drug bioavailability, biodistribution and target site accumulation after administration, as well as drug delivery across biological barriers and cellular response to treatment. Since cells are known to yield specific vibrational signatures for distinct physiological conditions (regarding viability, cellcycle, stress or apoptosis), reliable correlations can be attained between a particular vibrational profile and a definite drug effect or mode of action.

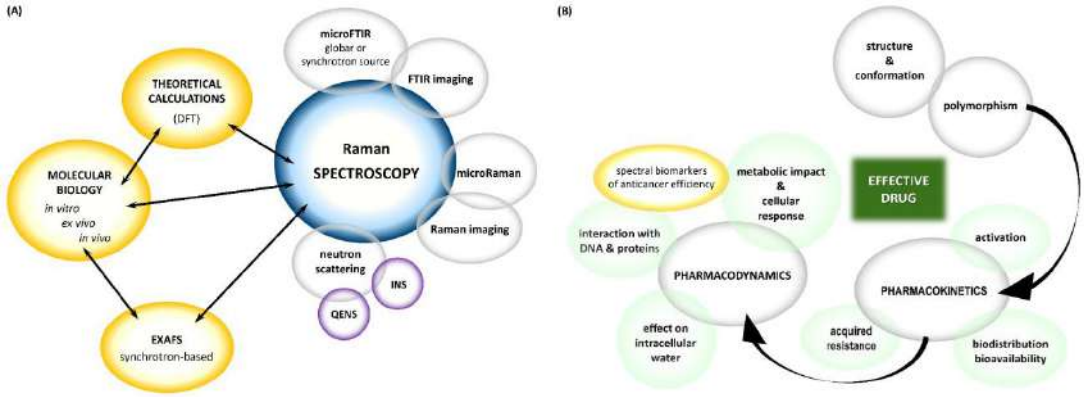

Fig 5. Schematic representation of a multidisciplinary approach based on Raman spectroscopy (A), aiming at the elucidation of the main features that rule the efficacy of novel anticancer drugs (B).

In addition, unique and thorough information can be acquired when combining Raman to theoretical calculations and to complementary vibrational techniques such as infrared or inelastic neutron scattering spectroscopies (Fig 5 (A)).The latter, based on a neutron beam exciting radiation and free of selection rules, allows access, with very high sensitivity, to vibrational modes involving hydrogen atoms as well as to the low frequency spectral region (comprising vibrations characteristic of the lattice and also those associated with H-bond interactions). Moreover, INS and quasi-elastic neutron scattering (QENS) methods are ideal for probing structure and dynamics of water in its various forms, including interfacial water in biological matrices (cells and tissues), at the nanosecond to pico second time scales and on atomic length scales [50-52,56,117,118,163-165]. This was shown to be relevant in drug development studies, since the presence of a prospective chemotherapeutic agent in the highly crowded intracellular medium impacts on the structural and dynamical profiles of intracellular water, which plays a vital role in biochemical processes and may thus mediate cytotoxicity (water being suggested as a secondary therapeutic target) [50,56].

3.1 Structural and conformational characterization of Pt- and Pd-agents

A rational drug design strategy demands the pre-clinical screening of new candidates, for the assessment of their efficiency and safety, the selection criteria for potential candidates being cytotoxic potency and selectivity.Upon the metallodrug´s cellular uptake and metabolization, activation takes place *via* hydrolysis (substitution of leaving ligand (s) by water molecules which are subsequently lost), followed by interaction with DNA and other molecular targets causing deleterious conformational rearrangements leading to functional impairment and cell death. However, details of these pharmacokinetic and pharmacodynamic

processes are still scarcely understood at the molecular level. Since the drug´s mechanism of action is ruled not only by its chemical properties but also by its structural features [62], this information is essential to predict biological activity and chemotherapeutic outcome (SARs) (Fig 5 (B)). In fact, a simple structural modification or conformational rearrangement may drastically affect the drug's ADMET profile (adsorption, distribution, metabolization, excretion and toxicity) and therefore limit its clinical efficacy.

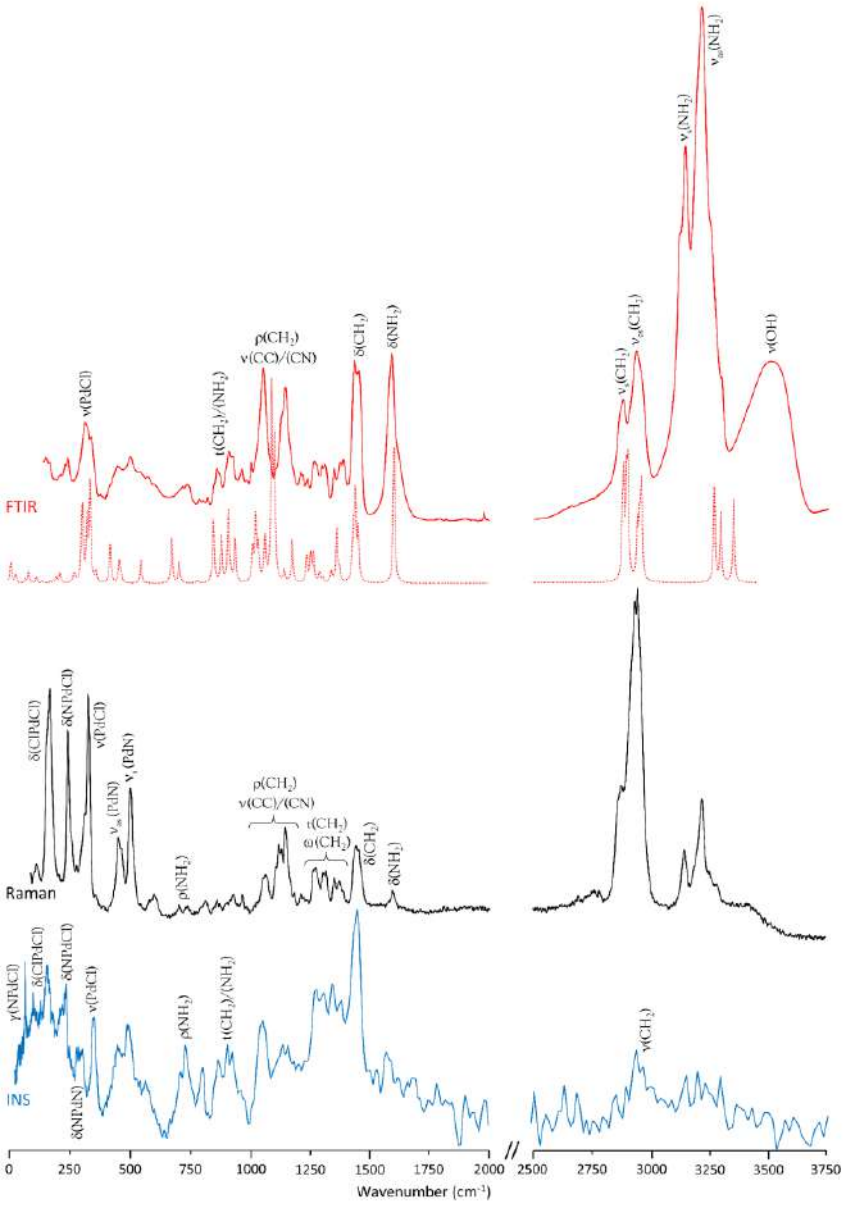

Fig 6. Experimental and calculated Raman, FTIR and INS spectra of Pd2Spm. The INS data was measured at 10 K/-263 ºC (at the ISIS Neutron and Muon Source, UK, https://www.isis.stfc.ac.uk/). The calculated infrared spectrum (at the wB97XD/LANL2DZ/6-31G* level) is represented by a dashed line. ν – stretching; δ – deformation; ω– wagging; t – twisting; ρ – rocking; γ– out-of-plane deformation; as – antisymmetric; a – symmetric.

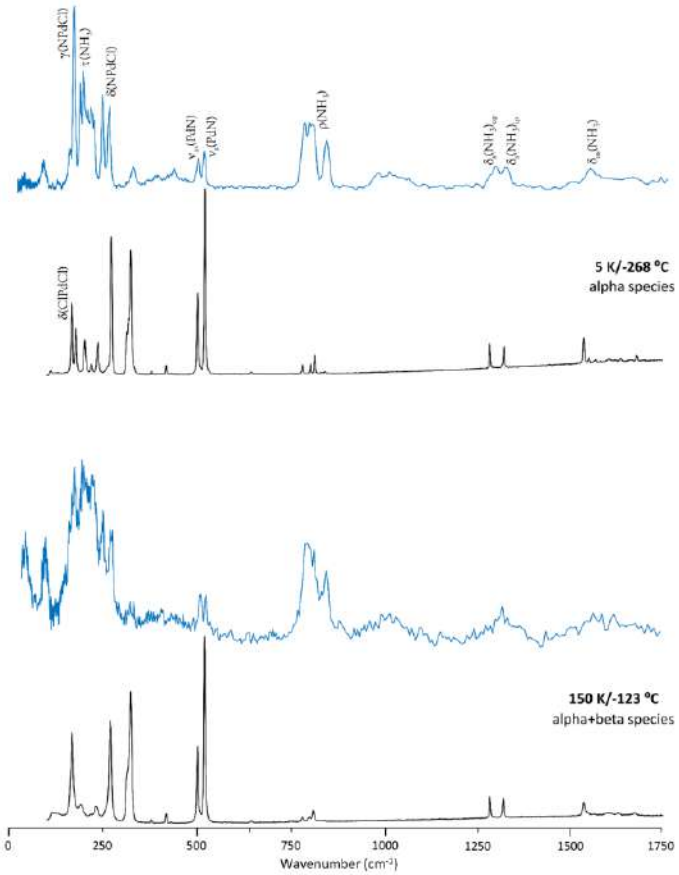

Fig 7. Raman (black) and INS (blue) spectra of the two polymorphic species of cisplatin, measured simultaneously, at 5 K/-268 ºC and 150 K/-123 ºC (at the ISIS Neutron and Muon Source, UK, https://www.isis.stfc.ac.uk/). δ – deformation; ρ – rocking; ν – stretching; τ– torsion; γ– out-of-plane deformation; as – antisymmetric; a – symmetric; ip – in- plane; op – out-of-plane.

Platinum and palladium dinuclear chelates with biogenic polyamines have been synthesised and extensively studied by the author´s team as a promising alternative to the mononuclear Pt-drugs currently used in the clinics. The main goal of these studies has been to attain an accurate understanding, at the molecular level, of the processes underlying antitumor activity (cytotoxicity, antiangiogenic ability and anti-invasive capacity) – i.e. to establish structure-activity relationships, specific for each type of cancer and physiological conditions.This requires a thorough knowledge of the structural and conformational preferences of the metal-polyamine chelates, as well as of their DNA- adducts. Although, this information should be preferably obtained *via* diffraction techniques, the lack of good quality crystals for this kind of inorganic compounds often hinders this approach. Additionally, conventional diffraction methods (e.g. X-ray diffraction) do not provide hydrogen atom positions, which constitutes a major drawback for elucidating biochemical mechanisms that often rely on H-bond interactions. Therefore, a thorough conformational analysis has been performed for dinuclear Pt- and Pd-polyamine complexes by Raman spectroscopy and complementary methods such as FTIR and INS. The latter, particularly well-suited for the study of hydrogenous compounds, allowed the observation of the low frequency vibrations of the complexes that encompass most of the modes associated with the metal (e.g. metal-ligand interactions in the free drugs, or metal-DNA bonds in the adducts), which are extremely difficult

to observe with optical vibrational techniques (either Raman or FTIR). These spectroscopic measurements were coupled to theoretical approaches (at the Density Functional Theory (DFT) level) for calculating the spectra of putative structures and assist interpretation of the experimental data [79,101,114,115,166-168]. Access to the complete range of vibrational spectroscopy techniques (and predicted spectra) allowed us to analyse the full vibrational signature of the novel systems under study (Fig 6). This combined approach yielded the precise conformational profile of each complex, including the intra- and intermolecular network of interactions (e.g. H-bonds) responsible for its structural preferences under physiological conditions.

Apart from polynuclear cisplatin-like complexes, the lead drug cisplatin has been studied by the team as to its conformational preferences and polymorphic equilibrium, mainly by Raman techniques. A combined infrared, Raman and INS analysis has enabled the complete assignment of the vibrational features of the drug, through the observation of the whole spectral window of interest [168]. Three conformers have been identified – only one being stable under physiological conditions [166] – as well as two polymorphic species (alpha and beta, temperature- dependent) [114,169]. Cisplatin´s polymorphic equilibrium was elucidated over a wide temperature range (–268 to 87 ºC), through a cutting-edge simultaneous Raman and INS experiment [114] which allowed us to collect complementary data for the same sample under exactly the same conditions (Fig 7).

In summary, although no systematic SARs pattern is yet to be found for Pt- or Pd-polynuclear agents, the main factors currently considered as determinant for an appropriate ADMET profile and a consequent high chemotherapeutic efficiency are: (i) type, number and oxidation state of the metal center (s); (ii) chain length, flexibility, presence (or absence) of bulky groups and number of coordinating atoms in the nonlabile ligands (bridging moieties) (Fig 4); (iii) nature, number and relative orientation of the labile (leaving) ligands; (iv) total charge.

3.2 Effect on cellular metabolism and cellular response

Drug intake and bioavailability, metabolic response to chemotherapy, biochemical and histopathological effects and toxicity profile are crucial subjects for a thorough understanding of the *in vivo* mode of action of a potential anticancer agent. The metabolic profiling of cultured cells and evaluation of their response to exogenous compounds are well-established methods in drug testing and development.

Micro-Raman and micro-FTIR (including SR-micro-FTIR) have been shown to be very for asse ssingtheeffectofbothconventionalandnewlydevelopedPt-andPd-polynuclearpolyaminecompoundsinhuman cell lines (both cancer and non-neoplastic), by monitoring whole cellular systems upon administration of the prospective drug (Fig 8). The results thus obtained contribute to a better understanding of the drug´s mode of action at the molecular level, hopefully helping to predict drug efficacy under specific conditions. These experiments have a twofold objective, and rely on the discrimination of the spectral signatures in different subcellular regions for drug- treated and untreated cells: (i) to assess the drugs´ biodistribution and its variation in real time; (ii) to obtain chemical imagesofthebiochemicaleffectandcellularresponsetoth etestedagents,andassignthisresponsetospecificspectral biomarkers of drug action. In this type of studies on drug-cell interaction, a reliable analysis of the wealth of spectroscopic information gathered on the drug-free and drug-incubated samples demands the use of dedicated multivariate statistical analysis, enabling to unveil consistent information on the biochemical profile of the samples and of the drug-prompted molecular changes: unsupervised principal component analysis (PCA), linear discriminant analysis (LDA) and hierarchical cluster analysis (HCA), as well as partial-least squares discriminant analysis (as a supervised method), amongothers.

Promising cytotoxicity data have been obtained so far by the author´s team for spermine chelates containing two Pt (II) or Pd (II) centers (Pt_2Spm and Pd_2Spm) towards breast cancer (including triple-negative highly metastatic carcinoma) and osteosarcoma [76-78,80,81,99,116], two aggressive cancers with

a poor prognosis. The biochemical impact of these complexes was determined under distinct conditions – type of cell line, sample preparation method (either live cells or dehydrated and formalin-fixed), drug dosage and incubation times – following a multidisciplinary approach: biological screening for assessment of growth-inhibiting and cytotoxic activities, and cytopathological spectral analysis based on micro-Raman and micro-FTIR techniques [7,9,116,118]. Non-malignant cells were also tested, with a view to assess toxicity of these prospective antineoplastic agents. This combined methodology yielded an accurate description (with molecular specificity) of variations in cellular biochemistry in the presence of each of the tested compounds, and led to the identification of vibrational bands assigned to specific drug-elicited effects and to the cell response to this perturbation (spectral biomarkers).

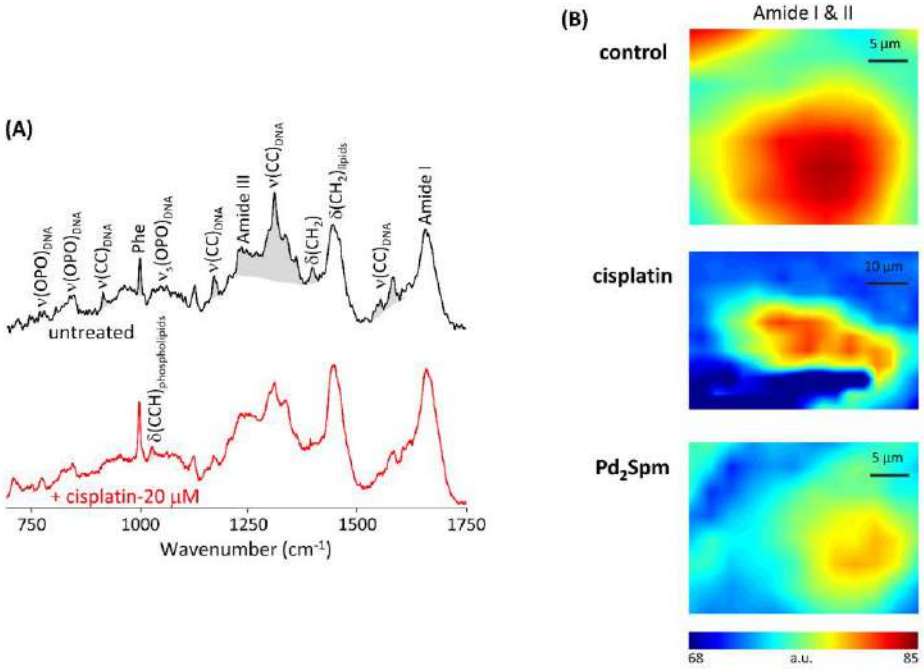

Fig 8. (A) Raman spectra of human breast cancer cells (triple-negative breast cancer, MDA-MB-231) untreated (black) and cisplatin-treated (red). (The shaded bands are those undergoing the most significative drug-induced changes). (B) SR-micro-FTIR integrated area maps for protein bands (Amine I and II) of human osteosarcoma cells (MG-63), untreated and treated with either cisplatin or Pd_2Spm.

Both osteosarcoma and breast cancer cells (triple-negative and estrogen-dependent) showed to be sensitive to the tested compounds, being prompted to apoptosis [116,118]. Drug and dose-dependent effects were unveiled, determined by the nature of the tumorigenic sample and (for the same type of cancer) by the characteristics of the putative antitumor agent – mononuclear (cisplatin) *vs* dinuclear, or Pt- *vs* Pd-based. This suggests the presence of distinct and unconventional pathways of cytotoxicity for these polyamine complexes as compared to mononuclear clinically used Pt-drugs (cisplatin, carboplatin or oxaliplatin). The cellular response was based on specific biomarkers of drug action mainly associated to DNA, proteins and lipids: while the Pd (II)-spermine agent displayed a more significant impact on proteins and DNA´s deoxyribose-phosphate back bone (very sensitive to base stacking and base- pairing interactions), its Pt (II) homologue was found to affect mostly the cellular lipid content (Fig 9). This effect on lipids is noteworthy, in view of the close association between lipid accumulation/unsaturation degree and breast cancer aggressiveness/metastatic spread [170]. Particularly regarding osteosarcoma, a noticeable impact of Pd_2Spm on cellular proteins was

evidenced, significantly more pronounced than for cisplatin-treated cells [118], as shown in the chemical maps generated by integrating across the amide I (1650 cm^{-1}) and amide II (1550 cm^{-1}) infrared regions for the control (drug-free) and drug-exposed cells (Fig 8 (B)). Also, for this type of bone cancer there was a clear effect of the dinuclear Pd-agent on the cellular lipidic content, namely on the membrane phospholipids (including a decreased fatty acid unsaturation degree), as opposed to breast carcinoma, revealing a drug selectivity according to cancer type. This influence of the physiological and histological characteristics of the biological matrix on drug´s activity is of the utmost importance, and their elucidation at the molecular level are therefore, paramount for a successful development of effective drugs. SR-micro-FTIR spectra of human osteosarcoma and triple-negative breast cancer has unveiled clear chemical differences for the former: (i) a lower unsaturation degree of the fatty acids – reflected in the higher CH_2/CH_3 ratio, represented by the bands between 2850 and 2955 cm^{-1} (respectively ascribed to the corresponding symmetric and antisymmetric stretching modes); (ii) a significantly decreased intensity of the $\delta(CH)_{phospholipids}$ and $\nu(CO)_{DNA}$, bands (at *ca.*1030 and 1060 cm^{-1}, respectively); and (iii) a slightly lower water content (ν(OH) at *ca.* 1060 cm^{-1}).

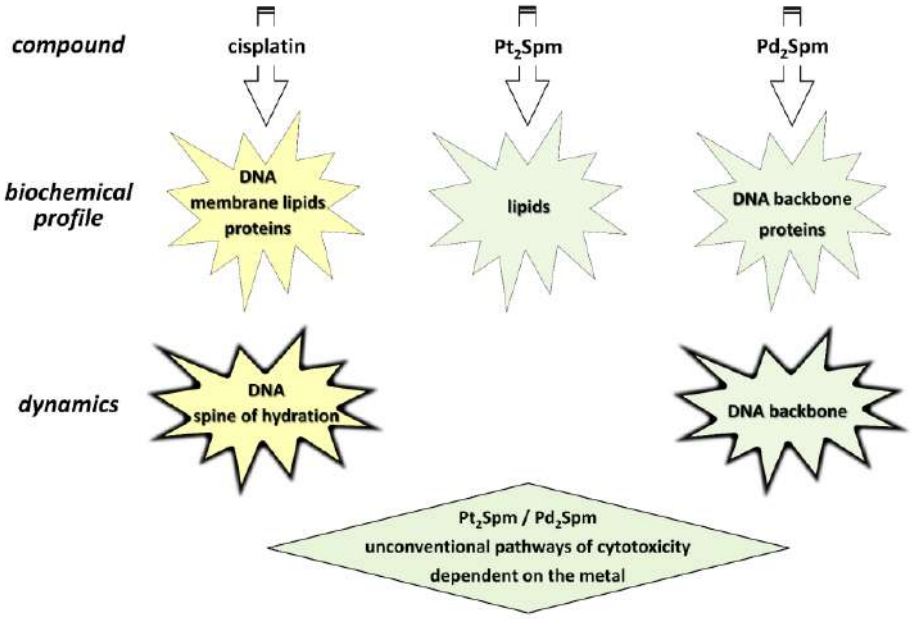

Fig 9. Schematic representation of the impact of cisplatin, Pt_2Spm and Pd_2Spm on the cellular biochemical and dynamical profiles.

It should be highlighted, however, that the direct drug-triggered biochemical effect on the cellular medium and the physiological response from the cell, reflected in the vibrational signatures, were not always clearly distinguished, while changes in DNA´s vibrational features could be unmistakably ascribed to direct drug binding (metal-Npurines), variations in the bands assigned to lipids and proteins were the combined outcome of direct metallodrug interaction with these targets and the cellular reaction to the chemotherapeutic insult (e.g. formation of membrane vesicles for exocytosis, or biochemical events following apoptotic stimuli such as protein degradation). Furthermore, despite the very intense vibrational signals assigned to the free Pt- and Pd-complexes (specifically detected by Raman and INS below 600 cm^{-1}, Fig 6) they were in discernible intracellularly, due to their low concentration (not exceeding 12μM) relative to the cellular components and to their prompt and strong interaction with their biological targets (e.g. DNA and sulphur-containing proteins) upon intracellular chloride hydrolysis.

Since oxidative stress has been recognized as a potential source of carcinogenesis, several types of human neoplasia being oxidative-induced disorders (and therefore largely preventable), an intense research effort has been dedicated to the development of chemopreventive antioxidant agents such as natural-based phytochemical compounds [171,172]. Raman micro-spectroscopy has been applied by this team to assess the biochemical fingerprint of human cancer cells in the presence of some of these systems, namely dietary isoflavones and phenolic acids [7,9]. Figure 10 comprises the Raman spectra of human breast cancer cells (both hormone dependent and hormone-independent) upon incubation with daidzein and *p*-coumaric acid. A clear discrimination was found between the control and cells exposed to daidzein (Fig10(A)), particularly regarding cellular DNA, proteins and lipids, in a cell type dependent way [7]. This strong radical scavenger and estrogen-mimetic isoflavone (Fig 10 (C)) was found to have a higher effect on the estrogen-responsive breast cancer cells, as expected, probably due to an effective interaction with the estrogen receptors of the cancer cells leading to inhibition of estrogen synthesis and cell growth arrest. Hence, a striking cell selectivity was revealed which is suggested to be based on different mechanisms of action: an estrogen-like activity in estrogen-dependent cells *versus* an interference on protein synthesis (leading to cell cycle arrest and apoptosis) in hormone-unresponsive cells. This daidzein-elicited cell viability decrease in human breast cancer is in agreement with its previously reported impact on hepato cellular carcinoma [173]. *p*-coumaric acid (3-(4-hydroxyphenyl)-2-propenoic acid, Fig 10(C)), a phenolic acid present in numerous food products from the daily diet (mainly in a Mediterranean diet), was found to exert a clear effect on the cellular components from human breast cancer (both estrogen-positive and estrogen-negative). Raman analysis of treated and untreated cells unveiled a predominant impact of this hydroxycinnamic acid on proteins and, to a smaller extent, on lipids (Fig 10 (B)) [9].

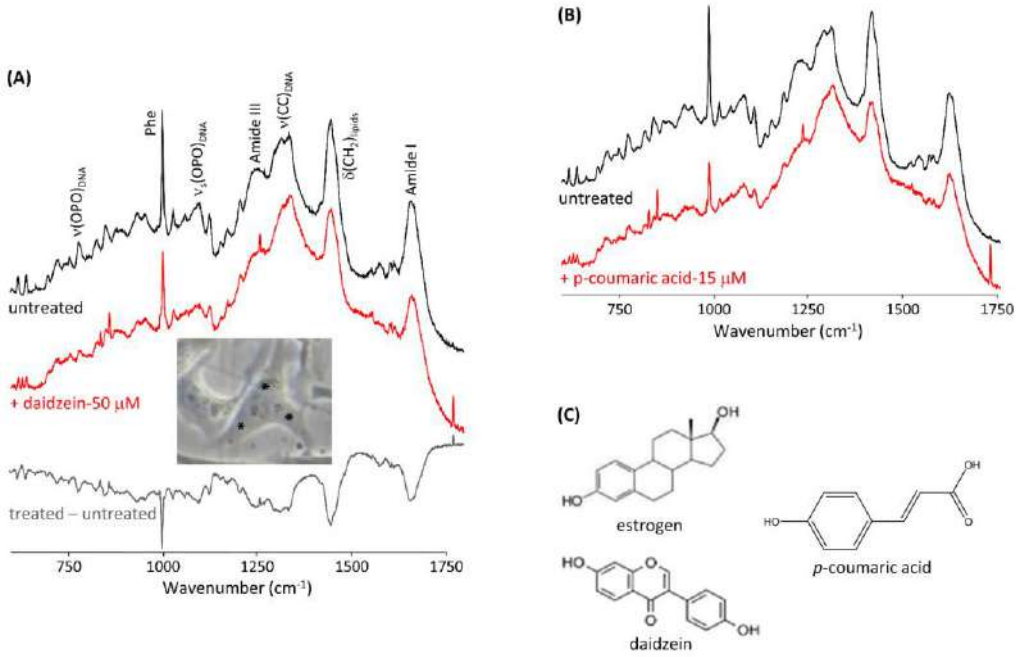

Fig 10. (A) Raman spectra of estrogen-dependent human breast cancer cells (MCF-7) untreated (black) and daidzein-treated (red), as well as [treated-untreated cells] difference spectrum (grey). A microscopic image of a cell (x100 magnification), showing some of the points at which data were captured, is also shown. (B) Raman spectra of triple-negative human breast cancer cells (MDA-MB-231) untreated (black) and *p*-coumaric acid-treated (red). (C) Structures of estrogen, daidzein and *p*-coumaric acid.

3.3 Interplay with cellular components

Raman and FTIR (including synchrotron radiation FTIR) have been applied for a thorough characterization of the adducts formed between Pt- and Pd-polyamine agents and several biomolecules (DNA, glutathione and proteins), since they allow to distinguish even the smallest changes in these biological targets upon drug binding. INS nicely complemented the data provided by these optical techniques, by enabling the observation of the low frequency vibrations of the complexes (< 600 cm^{-1}), encompassing most of the modes associated to the metal(s) (not detectable by either Raman or FTIR). These experiments led to the establishment of a reliable correlation between the drugs´ structural/conformational features and their cytotoxic activity, unveiling some of their preferred pharmacological targets (e.g. DNA purine bases) as well as the molecular mechanisms associated to acquired resistance (e.g. binding to glutathione, metallothioneins and serum albumin) [51,53,117]. Complementary Raman, FTIR and INS data delivered the full vibrational signature of the systems and provided an accurate molecular picture of drug-target interaction and of glutathione-mediated resistance. A thorough understanding of the covalent binding between the metallodrug and DNA bases was attained, which is the basis for the drug´s growth-inhibitory and cytotoxic activities. Also, drug scavenging and deactivation by glutathione (an ubiquitous endogenous antioxidant) prior to reaching the biological target was clearly evidenced. In addition, a clear preference of cisplatin for guanine over adenine during its interplay with DNA was evidenced. Figure 11 depicts the Raman and INS spectra of cisplatin and its adducts with adenine and guanine, clearly revealing chloride hydrolysis of the drug and subsequent coordination to the purine´s nitrogen atoms.

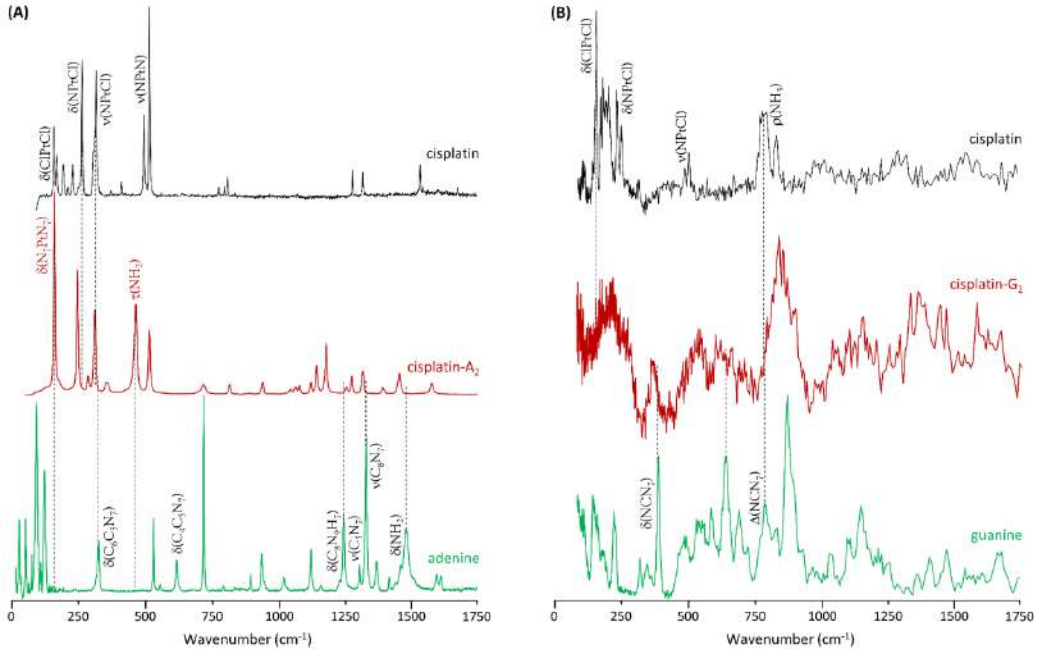

Fig 11. Raman (A) and INS (B) spectra of cisplatin and its adducts with adenine (cisplatin-A2) and guanine (cisplatin-G2). The spectra of free adenine (A) and guanine (G) are also shown. (The most characteristic bands of the adducts are assigned in red. The main drug-elicited changes are highlighted by dashed lines).

Further, structural and dynamical information of drug-DNA interplay was obtained by synchrotron-based FTIR-ATR (Fourier-Transform Infrared Spectroscopy in Attenuated Total Reflectance mode), EXAFS (Extended X- ray Absorption Fine Structure) and QENS, which constituted an innovative way of tackling a drug´s mode of action through a multidisciplinary approach [51]. DNA extracted from drug-exposed human

triple negative breast cancer cells was used, in order to evaluate the effect of the unconventional Pt2Spm and Pd2Spm dinuclear agents on this low prognosis type of cancer. Raman measurements, combined with far-and mid-FTIR, yielded the vibrational fingerprint of drug-exposed DNA, allowing the first simultaneous detection of the drug and its primary target (DNA) as well as the observation of the drug-induced variations in the conformation of the nucleic acid (Fig 12). Specific spectral biomarkers of drug impact were identified reflecting changes in both the drug and DNA, prompted by drug-target interaction. In particular, variations in the DNA breathing modes were clearly unveiled by far-IR, as a consequence of drug-elicited disruption of the H-bonded base-pairs within the double helix. To the best of the author´s knowledge, this was the first observation of the DNA breathing process in the presence of a chemotherapeutic compound. Moreover, changes detected in the characteristic vibrational bands of the metallodrugs provided evidence of structural and conformational variations upon cellular accumulation and interaction with the target. The use of coherent synchrotron radiation in these FTIR experiments greatly contributed to the results, due to the broadband spectral coverage and very high signal quality achieved. In turn, QENS data provided the drug impact on DNA's dynamical profile *via* its hydration layer, a drug-triggered enhanced mobility having been observed. The stiffness of native DNA was apparently disrupted by the drug, highlighting the influence of the water molecules from the first hydration sheath on the nucleic acid functionality. Finally, EXAFS delivered an accurate picture of the local environment of the Pt(II) or Pd(II) centers within the drug-DNA adducts, thus yielding a precise molecular view of the drug interaction with the target (pharmacodynamics), and allowed to identify metal-glutathione binding which is the basis of glutathione- mediated drug resistance (pharmacokinetics) occurring *in vivo* for these type of metal-based chemotherapeutics.

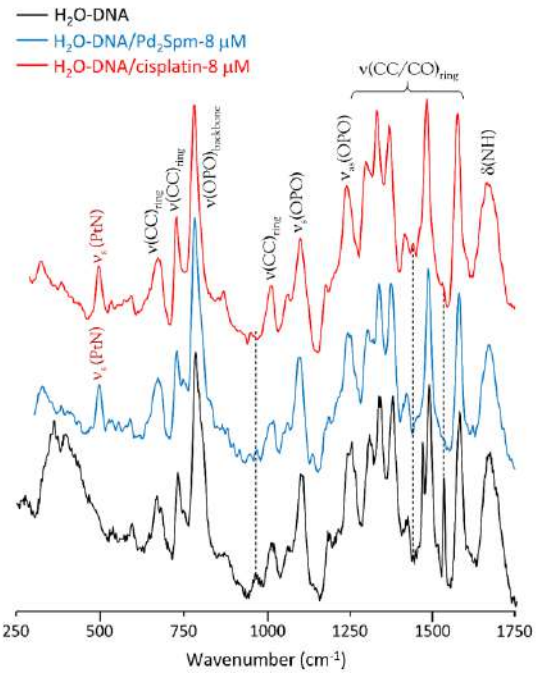

Fig 12. Raman spectra of untreated, cisplatin-treated and Pd2Spm-treated hydrated DNA. (The bands from the drugs in the DNA adducts are assigned in red. The main drug-elicited changes are highlighted by dashed lines).

Recently, two complementary cutting-edge techniques were applied by this team to investigate the metalation of proteins in the presence of a metal-based drug [53] – synchrotron radiation THz spectroscopy

and inelastic neutron scattering, unique molecular fingerprint tools for probing the low energy region of the vibrational spectrum.Two processes were addressed, that can severely decrease the effectiveness of metallo drugs and undermine chemotherapy: (i) impaired drug transport, often due to drug binding to human serum albumin (HSA) upon intravenous administration, and (ii) acquired drug resistance, associated to intracellular drug coordination to thiol-containing biomolecules (*e.g.* glutathione and metallothioneins). Spectroscopic evidence of protein metalation was found for the adducts of either cisplatin or Pd_2Spm with HSA, through S- and N-donor ligands (from albumin´s cysteine, methionine and histidine residues), leading to changes in the conformation and flexibility of the native protein (Fig 13). In particular, a noticeable variation was observed for the stretching signals from SH and SS protein groups, as a consequence of drug binding to sulphur atoms (Fig 13 (A)). In addition, the bands ascribed to (M–Cl) vibrations within the drugswere found to disappear in the drug-protein adducts, as expected upon intracellular chloride hydrolysis followed by Pt- or Pd-coordination to the protein (Fig 13(B)).

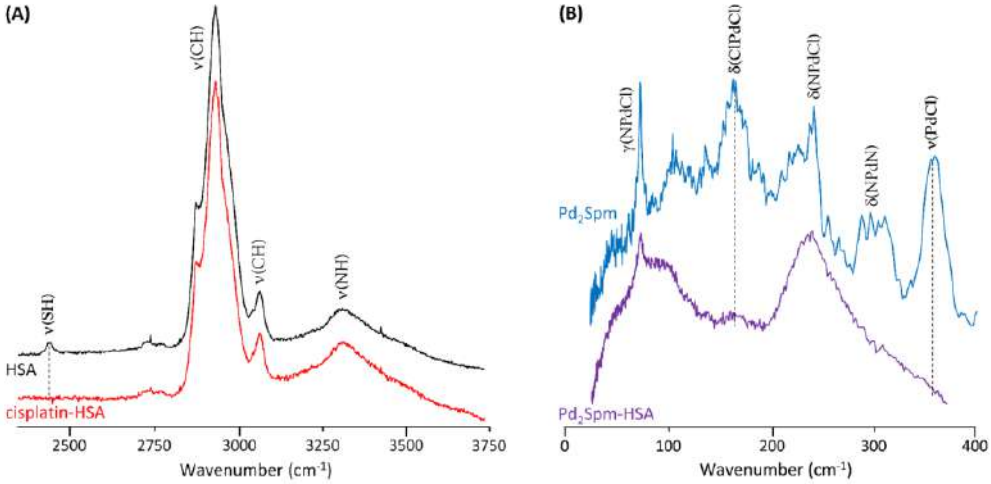

Fig 13. Raman (A) and INS (B) spectra of cisplatin and Pd_2Spm adducts with human serum albumin (HSA). (The main drug- elicited changes are highlighted by dashed lines).

3.4 Impact on cellular dynamics – Intracellular water as a new drug target

Recent studies have focused on the biophysics of the cancer state, shedding a new light on carcinogenesis beyond the recognized biochemical and genetic variations associated to malignancy. A strong correlation between neoplasia and cellular plasticity has been identified, cancer cells displaying anenhanced deformability relative to non-malignant ones [165,174-176], which seems to favor uncontrollable growth. In addition, apart from the biochemical/metabolic changes associated to malignancy carcinogenesis is recognized to be intimately related to the cell´s biomechanical profile, in particular to intracellular water dynamics [177]. This interfacial water is a conserved structural element with distinctive features, that plays a fundamental role in normal cell activity through maintenance of the functional conformation of biopolymers thus regulating vital biological processes [178-180]. Any alterations in its properties can be the driving force to disrupt homeostasis and initiate a series of events leading to biomolecular disfunction, that can facilitate neoplastic growth [181]. Elucidation of water properties in biological systems is, therefore, of the utmost importance in drug development, once a drug´s interaction with its pharmacological receptor may take place *via* the water molecules both in the intracellular medium (cytoplasm) and in the close vicinity of biopolymers (hydration layers).

Since neutron techniques are able to distinguish between different diffusion processes (at atomic length scales and from nano- to picosecond timescale), extending the available conventional methods (such

as Magnetic Resonance Imaging), QENS has been applied by the author´s team to interrogate the dynamics of water confined in biological matrices (cells and tissues). This is a pioneer approach for monitoring perturbations to water´s behavior, allowing to disclose biomarkers of malignancy [165] and of drug activity [50-52,56,118]. This type of studies aim at a better understanding of the molecular basis of -bility as well as at disclosing new targets for chemotherapy. Actually, apart from the conventional drug targets (e.g. DNA, proteins) there may be other receptors such as intracellular water – both within the cytoplasm and the hydration layers of biomolecules..

The first study on human nucleated cells by INS (to probe structure) and QENS (to monitor dynamics), as a complement to Raman data, was reported by Marques *et al* regarding the impact of cisplatin on intracellular water in breast cancer cells [50]. This approach was further extended to Pd2 Spm towards osteosarcoma cells [118] and allowed to attain an accurate representation of the distinct dynamical components that take place in the highly heterogeneous cellular systems and the way they are affected by exposure to a chemotherapeutic agent. Drug and cell-dependent effects were unveiled, evidencing variations in the cellular biochemical profile concurrent with a progressive mobility reduction within the cytoplasmic medium in treated cells (associated with changes in the native organization of the intracellular water molecules) (Fig14).

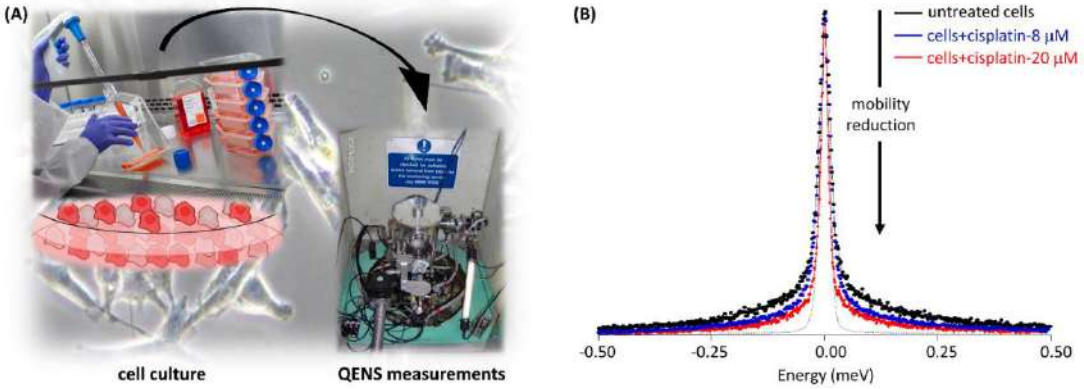

Fig 14. (A) Schematic representation of the experimental procedure for quasi-elastic neutron scattering measurements in cultured cells (A). (B) QENS profiles of human breast cancer cells (MDA-MB-231), untreated (black) and cisplatin-treated at different concentrations (blue and red).

Furthermore, Raman studies coupled to FTIR, INS and QENS measurements were used to tackle the impact of cisplatin, Pt_2Spm and Pd_2Spm on DNA *via* the biopolymer´s first hydration layer, which is known to be closely linked to DNA function [51,52]. A drug-triggered increased flexibility of water molecules within these hydration layers was clearly evidenced, dependent on the drug type (e.g. nature and number of metal centers), dosage and exposure time, and specific for each type of cancer (e.g.breast cancer or osteosarcoma). Interestingly enough, distinct modes of action were unveiled for Pt- *versus* Pd-agents: cisplatin was found to have a predominant effect on DNA´s spine of hydration, while the dinuclear Pd-agent (Pd_2Spm) had a higher influence on the nucleic acid´s backbone dynamics (Fig 9). The Raman spectra revealed drug-prompted changes mainly through the bands assigned to the phosphates (ν(OPO) at 830 cm^{-1}) and base rings (ν(CC), ν(CO) at *ca.* 756, 970, 1450 and 1530 cm^{-1}), due to direct metal coordination to the double helix and to interference with the surrounding hydration waters.

Clear plasticity changes were also identified for non-malignant *versus* malignant human cells [165], this normal-to-cancer transition being still a poorly understood process: cancer cells showed a significantly higher flexibility than their healthy counterparts, and different types of neoplasia revealed distinct dynamical profiles. This constitutes further evidence of the recognized dissimilarities between normal and neoplastic states regarding morphological, biochemical and functional properties. These results can help to understand

how these dynamical changes arise and accumulate during carcinogenesis and how they may contribute to tumor invasiveness and metastasis.

4 Conclusions

Raman spectroscopy was confirmed as a powerful non-invasive molecular probe of biosamples, which can greatly contribute for a successful drug design through an improved understanding of a drug´s pharmacokinetic and pharmacodynamic behavior. Biochemical profiling by Raman micro-spectroscopic analysis of human cells, coupled to complementary techniques such as FTIR, INS and QENS, has been shown to deliver unique data, namely regarding cisplatin and cisplatin-like anticancer agents such as polynuclear Pt-and Pd-complexes that have been investigated by the author´s team in the last two decades.

Combined vibrational spectroscopy and neutron scattering techniques have provided a complementary and comprehensive set of data that allowed us to elucidate the impact of these metal-based unconventional agents on both cellular biochemistry and water dynamics, in distinct types of human cancer cells. In particular, the application of quasi-elastic neutron scattering studies to complement data from Raman and infrared optical vibrational spectroscopies is a pioneer way of tackling the cell´s biomechanical properties. The data thus gathered constitutes the first experimental proof of a drug impact on the cytomatrix and should disclose novel chemotherapeutic intervention paths– in particular, intracellular water as a mediator of drug action (i.e. a promising novel therapeutic target). Specific spectral biomarkers of drug activity and selectivity have been unveiled, and they were interpreted in the light of the structural and conformational preferences of the compounds.

Coupled with biological assays for evaluation of antitumor activity, these results should help to gain a more thorough understanding of drug-induced cytotoxicity and contribute to the elucidation of novel pathways of chemotherapeutic activity for tailored Pt(II)- and Pd(II)-complexes, leading to enhanced pharmacological properties and selectivity as well as to minimal acquired resistance and deleterious side effects, which are the main goals of a rational drug design. This may take place *via*: (i) direct binding to biomolecules (specific receptors) and interference with its hydration shell – causing disruption of the native biomolecule´s structure/conformation and triggering functional disability; (ii) impact on intracellular water (within the cytosol), with an expected overall effect on essential cellular components thus hindering normal cell function. In addition, research on natural-based antioxidants (e.g. dietary phytochemicals) is underway, aiming at their application as chemopreventive agents and/or adjuvants in conventional chemotherapy, particularly regarding oxidative-related neoplasia.

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