Is matching ruthenium with dithiocarbamato ligands a potent chemotherapeutic weapon in oncology?

In the last years, several metal-based compounds have been designed and biologically investigated worldwide in order to obtain chemotherapeutics with a better toxicological profile and comparable or higher antiblastic activity than the clinically-established platinum-based drugs. In this context, researchers have addressed their attention to alternative nonplatinum derivatives able to maximize the anticancer activity of the new drugs and to minimize the side effects. Among them, a number of ruthenium complexes have been developed, including the compounds NAMI-A and KP1019, now in clinical trials. Here, we report the results collected so far for a particular class of ruthenium complexes – the ruthenium(II/III)-dithiocarbamates – which proved more potent than cisplatin *in vitro*, even at nanomolar concentrations, against a wide panel of human tumor cell lines.

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Inorganic elements are essential in biological processes. To date, 25 elements are thought to be essential for mammalian biochemistry; among them eight are transition metal elements (namely V, Mn, Fe, Co, Ni, Cu, Zn and Mo) [1]. Moreover, it should be underlined that even if most drugs are organic compounds, their metabolism sometimes relies on metalloenzymes, while in other cases they directly or indirectly affect metal ion metabolism (e.g., chelating agents) [2,3]. These findings have paved the way to the design and development of new metallodrugs [4]. In particular, transition metals are endowed with different oxidation states, and their complexes can exhibit a range of geometries and coordination numbers that allow, when designing new drugs, the modulation of their biochemical reactivity, in terms of both kinetics and thermodynamics. Since the casual discovery of cisplatin (*cis*-diaminodichloroplatinum(II), cisDDP, [*cis*-PtCl$_2$(NH$_3$)$_2$], **Figure 1A**) in the early 70's by

Rosenberg and Van Camp [5], a vast library of metal compounds has been synthesized and tested for pharmacological use, especially in the endless fight against cancer [6]. Cisplatin is clinically administered by intravenous injection, and the neutral form of the drug easily enters individual cells. In particular, the drug passes through the cell membrane both by passive diffusion and by active transport, mediated by the copper transporter CTR1 [7]. The relatively high serum chloride ion level (~150 mM) indeed inhibits extracellular hydrolysis, whereas the much lower intracellular concentration of this anion leads to the formation of the mono- (*cis*-[PtCl(NH$_3$)$_2$(H$_2$O)]$^+$) and the di-aquo (*cis*-[Pt(NH$_3$)$_2$(H$_2$O)$_2$]$^{2+}$) *cis*-diammineplatinum(II) complexes. These activated species can in turn react with a variety of intracellular macromolecules including DNA, RNA and proteins. Among these biomolecules, DNA is acknowledged as the main target of cisDDP, whose biological activity

consists in the formation of bifunctional DNA adducts, involving both intrastrand and interstrand crosslinks, and protein–DNA crosslinks. Consequently, binding of cisplatin to DNA causes significant distortion of the helical structure, resulting in inhibition of DNA replication and transcription. In other words, cisDDP kills cancer cells by blocking their ability to synthesize new DNA required for cell division [8]. Nowadays, cisplatin-based therapy can be considered part of a standard treatment regimen against many forms of neoplasia, including malignant mesothelioma, squamous cell carcinoma of the head and neck, testicular, bladder, cervical, ovarian and non-small-cell lung cancer [9]. Unfortunately, its high effectiveness is restricted from dose-limiting severe side effects, such as nausea, alopecia, ototoxicity, neurotoxicity, myelosuppression and nephrotoxicity. Moreover, tumor cells treated with cisplatin, similarly to other drugs, are able to develop resistance during treatment by reducing the uptake/increasing the efflux; exploiting detoxifying intracellular *S*-donor molecules (e.g., glutathione, methionine- and cysteine-containing molecules); improving the processes of lesion-recognition and -adjustment of cisDDP-DNA adducts promoted by the repair enzymes [10].

In the last decades, several metal-based compounds have been designed and biologically investigated worldwide in order to obtain chemotherapeutics with a better toxicological profile along with increased bioavailability, and comparable or higher antiblastic activity than cisplatin. Among the 23 platinum-based drugs that have entered clinical trials, only two gained global approval (carboplatin and oxaliplatin Figure 1B & C), while other three obtained marketing approval in individual countries (nedaplatin in Japan, heptaplatin in Korea and lobaplatin in China Figures 1D–F) [11]. On

the other hand, researchers have also developed new nonplatinum metal derivatives able to maximize the anticancer activity of the metal center (e.g., activity also against tumors resistant to Pt drugs) and to minimize the occurrence of drawbacks in terms of patient's condition. These compounds have been conceived to possess innovative physicochemical properties with respect to platinum drugs, taking advantage of different metal oxidation states, coordination geometries, binding preferences and ligand-exchange rates, that likely lead to unconventional mechanisms of action. Among them, the number of ruthenium complexes investigated as anticancer agents has exponentially increased in last three decades. Interestingly, some of these compounds have shown activity against cisplatin-resistant tumors, with less severe side effects if compared with platinum drugs, and two of them (named NAMI-A and KP1019 Figure 2A & B) have entered clinical trials [12–14].

Our research group has been designing and synthesizing dithiocarbamato (dtc) complexes of different metals (e.g., Pt(II), Pd(II), Au(I)/(III), Ru(II)/(III), Zn(II) and Cu(II)) for some years, with the aim of combining the anticancer properties of the inorganic center with the chemoprotective action of dtc ligands. In fact, dtc involve sulfur-donor atoms, which prevent metal from inactivation by sulfur-containing biomolecules [15,16]. After describing ruthenium metal center from the chemical and biological point of view, followed by a discussion on the chemistry and physiological role of dtc, the next sections are hence focused on their 'combination'. To date, some ruthenium-dtc complexes proved much more potent than cisplatin *in vitro,* even at nanomolar concentrations, against a wide panel of human tumor cell lines.
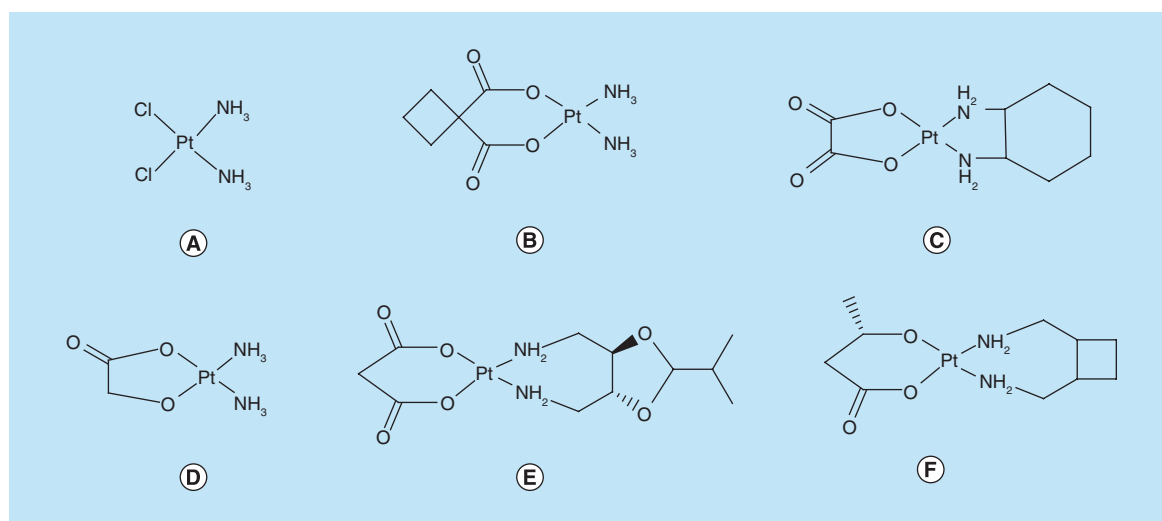

Figure 1. Clinically established platinum drugs. Cisplatin (*cis*-DDP) **(A)**, carboplatin **(B)**, oxalilplatin **(C)**, nedaplatin **(D)**, heptaplatin **(E)**, lobaplatin **(F)**.
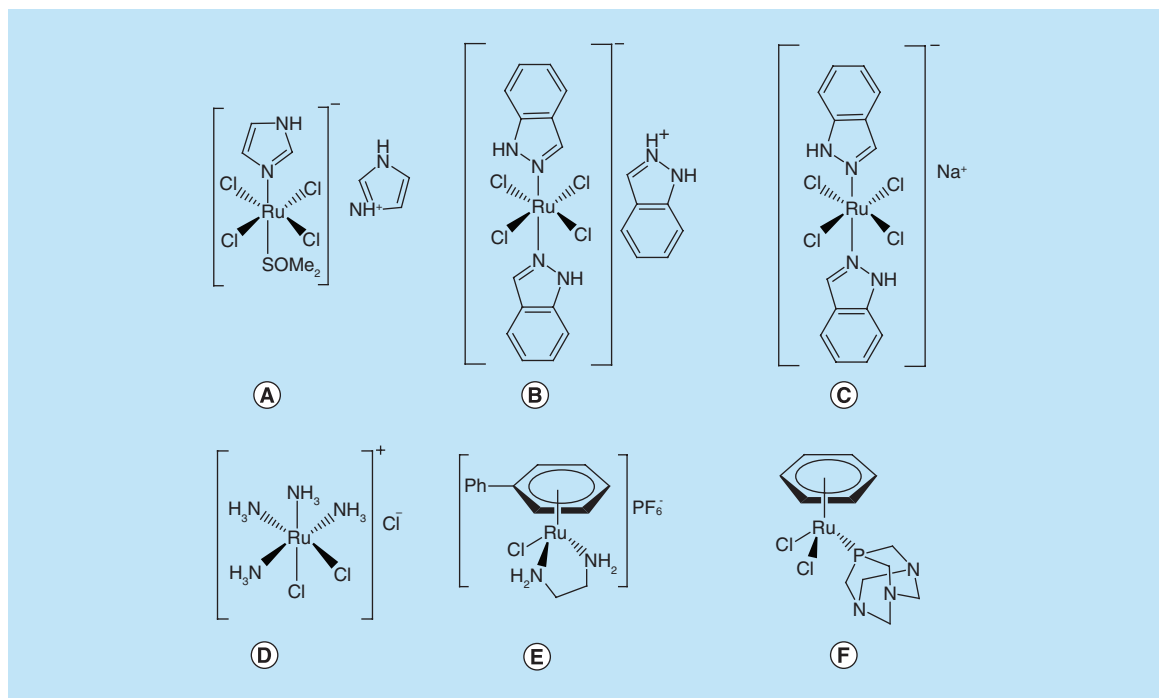
Figure 2. Ruthenium(III) compounds in clinical trials. NAMI-A **(A)**, KP1019 **(B)** and its sodium salt NKP-1339 **(C)**. Some other examples of biologically-active ruthenium derivatives. [*cis*-RuCl$_2$(NH$_3$)$_4$]Cl **(D)**, the RM-type complex [RuCl(η^6-arene)(en)]PF$_6$**(E)** and the RAPTA-type complex RAPTA-C **(F)**.

The bio-inorganic (r)evolution: ruthenium as an intriguing heavy metal

Chemistry of ruthenium

Ruthenium (Ru) is the 44th element of the periodic table, and with iron and osmium belongs to the 6th group of transition metals. With an electronic configuration [Kr]4$d^7$5s^1, this metal was discovered by Berzelius and Osann in 1827 while they were examining the residues left after dissolving crude platinum (from the Ural mountains) in *acqua regia.* Ruthenium is a versatile catalyst and its derivatives show a marked chemical resemblance to those of osmium [17]. Its aqueous chemistry is mainly represented by the Ru(II) and Ru(III) ions. In both oxidation states, the metal is hexa-coordinated, with a distorted octahedral geometry. Due to the high-ligand field energy of stabilization, Ru(III) complexes have the electronic configuration t_{2g}^5, with an unpaired electron that confers a paramagnetic character on the ion. Conversely, Ru(II) complexes are diamagnetic, characterized by a low-spin electronic configuration t_{2g}^6. All complexes of Ru(II) and Ru(III) are usually kinetically inert [18].

Ruthenium as a key player for new metallodrugs

In the 1950s, Dwyer and coworkers first recognized the biological activity (including anticancer properties) of ruthenium complexes [19]. However, only after the unexpected discovery of cisplatin, the interest in new metal-based drugs led researchers to focalize their attention on this element. In 1980, Clarke and his collaborators hypothesized the cancer-selective action of Ru(III) derivatives in terms of 'activation by reduction' [20]. The assumption was based on the possibility that Ru(III) may work as a 'pro drug', converting to Ru(II) by reduction in tumor tissues (E°$_{Ru(III)/Ru(II)}$ = +0.25 V vs SHE at 298.15 K [17]), so to allow a faster coordination to biomolecules. In fact, as the reduction of Ru(III) to Ru(II) leads the d$_\pi$ (t$_{2g}$) orbitals to be completely filled, any π-donor ligand is no longer able to back donate the metal ion, thus reducing the overall stability of the complex that can lose one or more ligands, hence favoring interactions with biological targets. The low O$_2$ content (hypoxic conditions) and the lower pH in tumor cells (that is a consequence of the well-known Warburg effect, explained as a block after glycolysis instead of entering Krebs cycle, with a higher production of lactic acid [21]) favor a strong reducing environment [22]. These evidences were demonstrated with experiments carried out on the complex [*cis*-Ru^{III}Cl$_2$(NH$_3$)$_4$]Cl (E°Ru(III)/Ru(II)= -0.10 V vs SHE at 298.15 K [20]) (Figure 2D), which was able to concentrate in tumor tissues [20]. It is worth noting that glutathione (E°=$_{S-S/S-H}$ = -0.26 vs SHE at pH 7.07, 298.15 K, 0.1 M buffer phosphate) and a large number of redox-active proteins are capable of reducing Ru(III) complexes *in vivo* [23]. On the contrary, if Ru(II) compounds were transported far from tumor microenvironment (e.g., lungs), they would turn

into Ru(III) counterparts by reaction with molecular oxygen, cytochrome oxidase and other oxidants [24,25].

Notably, we should bear in mind that the 'activation by reduction' mechanism of action is only a hypothesis and it cannot account for the cytotoxicity of all Ru(III) complexes. For instance, the aforementioned widely studied Ru(III) complex NAMI-A ([ImH [*trans*-RuCl$_4$(DMSO)(Im)], Im = imidazole) (Figure 2A), first synthetized by Alessio and coworkers, is currently studied in clinical trials (Phase I/II in combination with gemcitabine) as antimetastasizing agent [26]. In particular, contrary to its low cytotoxicity *in vitro*, NAMI-A inhibits lung metastasis formation *in vivo*, without affecting primary tumors [27]. Intriguingly, lung metastases lie in a tissue that can be easily seen as the most oxygenated in the body, thus ruling out a reductive environment [28]. Accordingly, the 'activation by reduction' mechanism is questionable when exploring the anticancer activity of the Ru(III) derivative NAMI-A [28].

The biological activity of ruthenium could be related also to its iron-mimicking properties. In 1983, Som and coworkers labeled transferrin (Tf) with the radioisotopes ^{97}Ru and ^{103}Ru, demonstrating the uptake of these

Figure 3. Dithiocarbamato formation and resonance formulae. (A). Mechanistic pathway for the formation of dtc ligands in protic solvents. **(B).** Limit forms for dithiocarbamato-chelation of transition metal complexes. In the dithiocarbamic limit form **I**, a sulfur electron pair is delocalized within the metal-chelate ring. The multiple character in the metal–sulfur bond is due to the interaction of partially filled *d* orbitals of M with empty low-energy ligand π* orbitals rising from the *d* orbitals of sulfur atoms. In the thioureidic limit form **II,** the nitrogen lone pair is involved in the C–N bond, thus resulting in a formal positive charge on the nitrogen atom and a net shift of electron density toward the sulfur atoms. Accordingly, the sulfur atoms are less able to accept electron density from the metal.

adducts in tumor- and abscess-bearing animals [29]. Ruthenium can indeed mimic iron in binding to serum Tf and human serum albumin (HSA) [30]. The accumulation of ruthenium complexes in tumors might be mediated by the former since neoplastic cells require high levels of iron (the tumor cell membranes are rich in Tf receptors). As a consequence of this transport, *in vivo* studies have shown that there is a 2- to 12-fold increase in ruthenium concentration inside cancer cells compared with healthy ones [31]. In this context, recent investigations have revealed that the reducing properties of tumor-microenvironment can influence the affinity of the abovementioned NAMI-A complex toward albumin and transferrin. These experiments have demonstrated that even small changes in the composition of serum models (e.g., pH) can result in a pronounced effect on binding of ruthenium complexes to proteins [32].

In 2002 Brabec showed that the cytotoxicity of some ruthenium compounds is associated with their ability to bind DNA in a different manner if compared with cisplatin [33]. The slow rate of ligand exchange of both Ru(II) and Ru(III) centers, suggests that these metal compounds do not undergo ligand dissociation before reaching any of their biological target [34]. On the other hand, even if the initial DNA binding site of several ruthenium derivatives was the same of cisplatin and its analogs, their DNA-binding mode would be diverse, inducing different conformational distortions [35]. In fact, it is worth highlighting that ruthenium is placed in an octahedral environment in both oxidation states +2 and +3, contrary to the square planar coordination geometry of Pt(II) species. This behavior may account for important cytotoxic effects of ruthenium derivatives in tumor cell lines inherently or treatment induced, resistant to cisplatin. The RM- and RAPTA-complexes (Figure 2E & F), developed by Sadler [36] and Dyson [37], respectively, represent another demonstration that DNA is a major target of ruthenium metallodrugs. They are Ru(II) complexes characterized by the presence of metal–carbon bonds, chloride ligands as leaving groups and diamine or phosphaadamantane as nonleaving groups. On passing from the ethylendiamino ligand (RM) to phosphaadamantane (RAPTA), the adduct formation profile switches from primarily DNA-targeting to binding proteins associated with chromatin [38]. Other examples are the well-known Ru(III) drug KP1019 ([IndH] [*trans*-RuCl$_4$(Ind)$_2$], Ind = indazole) (Figure 2B) and its sodium salt NKP-1339 (Na[*trans*-RuCl$_4$(Ind)$_2$]) (Figure 2C), where the latter is the first-in-class ruthenium-based anticancer drug in clinical development (phase I) against solid malignances [39]. These two derivatives efficiently untwist DNA and weakly bend it, showing a preference for N7 of the purine bases guanosine and adenos-
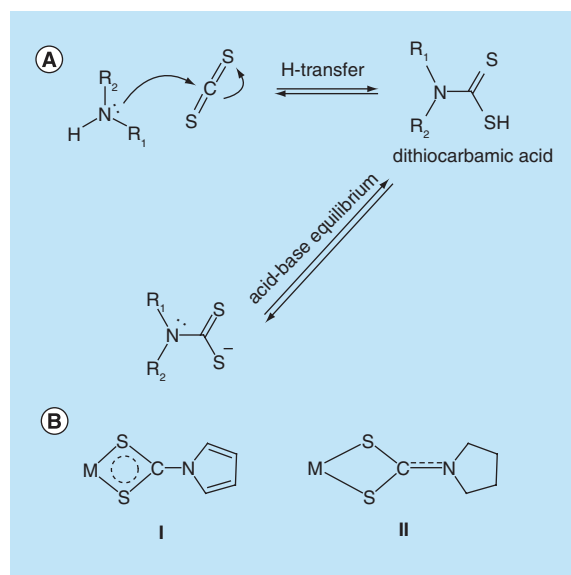
ine and inducing lesions with different biochemical outcomes if compared with cisplatin [40].

The dithiocarbamates
Chemistry of dithiocarbamates

N-substituted carbamodithioates, popularly known as dithiocarbamates (dtc, $R_2NCS_2^-$), form a class of compounds, which find extensive use in chemical practice [41]. They are indeed employed in the industry of polymers [42] or as fungicides [43], pesticides [44], and antioxidants [45]. Moreover, dtc are a versatile class of anionic (LX) sulfur–donor ligands widely used in coordination chemistry, with applications in qualitative inorganic analysis [46] and bio-inorganic medicine [47]. They are generally formed by exothermic reaction between CS_2 and a primary or secondary amine in the presence of a base. The base may be an alkali, such as sodium hydroxide or an excess of the amine itself. In the last case, the ammonium salt of the ligand $([R_2NH_2]^+[R_2NCS_2]^-)$ is formed. According to Miller and Latimer, the reaction of dtc formation occurs via nucleophilic attack of the amine to the electron-poor C atom of CS_2, the crucial step being the hydrogen transfer from nitrogen to sulfur atoms, followed by acid/base reaction giving rise to the corresponding dtc salt [48] (Figure 3A).

Dithiocarbamato derivatives of secondary amines are usually stable in alkaline solution, whereas the monosubstituted counterparts can undergo a reaction with hydroxide ions, yielding isothiocyanates (R-N=C=S) and elemental sulfur. This behavior can account for the paucity of literature data related to monosubstituted dtc ligands [49–51].

Regarding coordination chemistry, dtc salts possess the ability to be, at the same time, strong- and weak-field ligands. As an example, in dtc of aromatic amines, the nitrogen lone pair is involved in the aromaticity of the ring, thus endowing the N–CSS bond with a single-bond character. In this case, the dtc is a π-acceptor ligand, involving π* orbitals arising from the low-energy *d* orbitals of the sulfur atoms, which are characterized by lower electron density if compared with the aliphatic derivatives [52] (Figure 3B, I). On the other hand, in aliphatic-dtc analogs, the lone pair on the nitrogen atom is mostly shifted toward the -CSS moiety, resulting in an increase of the electron density on the sulfur atoms. Therefore, the *d* orbitals of S atoms are partially filled, making the ligand a potential π-donor species [53] (Figure 3B, II). In the light of these two opposite chemical characters, dtc ligands can stabilize transition metals in different oxidation states, coordinating the central ions in a variety of different ways, even if the chelating one is, by far, the simplest and the most common for all *d*-block elements (Figure 4A). Concerning the chelat-
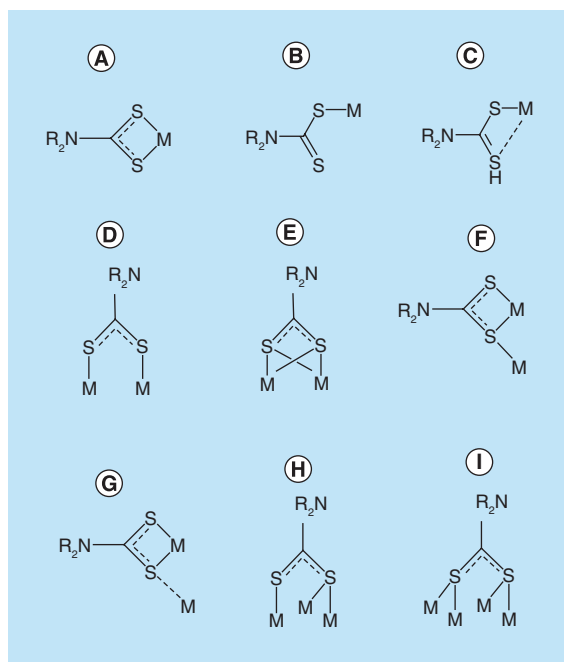

Figure 4. Possible coordination modes of dithiocarbamates [54].

ing coordination, the two metal–sulfur bonds are approximately equal and the ligand can be considered as a four-electron donor (LX), according to the ionic model for the electron counting. In other words, two S atoms in a π-system are coordinated to the metal center and the binding mode is described by η^2 hapticity (also labeled as κ^2 since we are dealing with noncarbon donor ligands) [54]. Moreover, in a number of cases, the dtc may act as a monodentate ligand (Figure 4B). This often results from the presence of other sterically hindered coordinated ligands or it is a consequence of the coordination geometry preferentially adopted by the central atom [55]. Finally, these type of sulfur-based ligands can bridge two metal atoms in a number of other ways (Figure 4D, E, H & I). In fact, a significant group of complexes involving the η^1,η^1-coordination mode (where each S atom binds to a single metal center) has been crystallographically characterized [56,57].

Biological overview of dithiocarbamates

The biological profile of dtc was extensively explored from 1930 onward, when they were introduced as pesticides in agriculture. The first dtc derivative to achieve prominence as fungicide was the tetramethylthiuram disulfide (Supplementary Figure 1A), commonly known as Thiram® (Chemtura Italy Srl, Italy) [58]. In 1948, Jacobsen and coworkers discovered the ethanol-sensitizing action of tetraethylthiuram disulfide (Supplementary Figure 1B), sold under the trade names of Antabuse® or Antabus® (Teva Women's Health, PA, USA) as support to the treatment of chronic alcoholism [59]. It should be highlighted

that the cited compounds are disulfides, characterized by a S–S bond (R$_2$NCSS-SSCNR$_2$), and are the oxidation product of the corresponding dtc salts. On the other hand, the dtc anions, in the form of salts (R$_2$NCSS$^-$M$^+$, M = alkali or NH$_4^+$), proved highly reactive toward other molecules containing -SH groups *in vitro* [60]. Moreover, they were investigated as chelating agents for heavy metal intoxications *in vivo*, even if they exert their action also on endogenous metals, thus triggering toxic effects on the organism [61].

In the oncological field, these sulfur-based ligands appeared for the first time in 1979 in the studies of Borch and his coworkers on the chemoprotective effect of diethyldithiocarbamate sodium salt (NaDEDT) (Supplementary Figure 1C) during cisplatin-based chemotherapeutic treatment [62]. In fact, this ligand has got the ability to selectively remove platinum from the thiol groups of different proteins but not from nucleotides involved in Pt-DNA adducts. The administration of NaDEDT to patients 3 h after the drug showed an improvement of the anticancer activity. In this time span, the drug is indeed on its way toward the acknowledged target DNA, and NaDEDT can act as a chemoprotectant by removing Pt(II) from several *S*-donor biomolecules. In fact, DEDT reacts with platinum resulting in the formation of dtc-Pt species, thus decreasing the nephrotoxicity (caused by the formation of Pt-renal enzyme adducts) and, at the same time, enhancing the bioavailability of the metal for triggering additional antitumor activity as a new anticancer complex (between platinum and the DEDT ligand) [63].

Moreover, diethyldithiocarbamate showed a protective effect against cisplatin-induced ototoxicity, associated with a recovery of the antioxidant system in the cochlea [64]. Nevertheless, the overall benefits of NaDEDT are somewhat limited by its acute toxicity. Metabolic studies have shown that this molecular entity undergoes detoxification through *S*-glucuronidation or biodegradation to different metabolites such as carbon disulfide, thiourea and alkylamine. CS$_2$ is a neuropathic and teratogen agent while thiourea interferes with iodine uptake by thyroid and can cause carcinogenic effects [65]. In addition, the sulfur atoms of free dtc can react with different enzymes, by creating S–S bonds (e.g., with) or other covalent interactions, thus inhibiting the biological activity of proteins [66].

Table 1. IC$_{50}$ values recorded after 72 h treatment, expressed as μM concentration ± standard deviation.							
Compound	NCI-4295R	OE-19	OE-21	Igrov	HeLa	Pt-45	MCF-7
Na[*trans*-Ru^{III}Cl$_4$(dmso)$_2$]	–	–	–	–	–	–	–
[Ru^{III}(DMDT)$_3$] (1)	>20	4.0 ± 0.7	18 ± 1	>20	18 ± 1	>20	18 ± 1
[Ru^{III}(MSDT)$_3$] (2)	>20	3.0 ± 0.5	19 ± 1	>20	17 ± 1	>20	19.0 ± 0.9
[Ru^{III}(ESDT)$_3$] (3)	>20	>20	>20	>20	>20	>20	19 ± 1
[Ru^{III}(TSDT)$_3$] (4)	>20	>20	>20	>20	>20	>20	>20
[Ru^{III}(PDT)$_3$] (5)	–	–	–	>20	>20	–	–
[Ru$^{III}_2$(DMDT)$_5$]Cl † (6)	4.5 ± 0.5	0.8 ± 0.2	3.0 ± 0.6	19 ± 1	3.0 ± 0.4	0.7 ± 0.5	0.8 ± 0.1
α-[Ru$^{III}_2$(DMDT)$_5$]Cl (6a)	–	–	–	–	–	–	–
β-[Ru$^{III}_2$(DMDT)$_5$]Cl (6b)	–	–	–	–	–	–	–
[Ru$^{III}_2$(MSDT)$_5$]Cl † (7)	>20	>20	>20	>20	>20	>20	>20
[Ru$^{III}_2$(ESDT)$_5$]Cl † (8)	>20	2.5 ± 0.4	3.5 ± 0.9	>20	15 ± 1	15 ± 1	3.0 ± 0.8
[Ru$^{III}_2$(TSDT)$_5$]Cl † (9)	3.0 ± 0.2	0.8 ± 0.1	0.9 ± 0.2	>20	0.7 ± 0.2	0.7 ± 0.1	2.0 ± 0.4
α-[Ru$^{III}_2$(PDT)$_5$]Cl (10a)	–	–	–	19 ± 1	14 ± 1	–	11.3 ± 0.8
β-[Ru$^{III}_2$(PDT)$_5$]Cl (10b)	–	–	–	–	–	–	–
Cisplatin	5.2 ± 0.8	>20	>20	3.8 ± 0.5	14.1 ± 0.8	16.6 ± 0.9	18 ± 1
[*cis*-Ru^{II}Cl$_2$(dmso)$_4$]	–	–	–	–	–	–	–
[Ru^{II}(DMDT)$_2$(DMSO)$_2$] (1)	– ‡	– ‡	– ‡	– ‡	– ‡	– ‡	– ‡
[Ru^{II}(PDT)$_2$(DMSO)$_2$] (2)	– ‡	– ‡	– ‡	– ‡	– ‡	– ‡	– ‡

Each value represents the average of more sets of independent experiments (cytotoxicity was measured by MTT assay). Human tumor cell lines: adrenocortical carcinoma (NCI-4295R); esophageal squamous carcinoma (OE-19 and OE-21); ovary adenocarcinoma (Igrov and Ovcar-3); uteri cervix carcinoma (Hela), pancreas adenocarcinoma (PT-45); breast cancer (MCF-7); lung carcinoma (A-549); leukemia wild type (Cem WT); leukemia resistant vimblastin (Cem Vim); colon carcinoma grade II (HT-29); non-small-cell lung cancer (NCI-H1975).
†[Ru$_2$(dtc)$_5$]Cl tested as a mixture of α and β isomers.
–: Compound not tested.

Ruthenium dithiocarbamates as potential chemotherapeutics in the oncological field

As dtc have led to encouraging results as chemoprotectants against the nonspecific coordination of a metal center under physiological conditions, a large number of metal-dtc complexes have been designed in order to combine the protective action of the ligand with the anticancer effectiveness of the metal center [15]. In this context, the antiproliferative activity of different Ru(II)/(III)-dithiocarbamato derivatives was worldwide investigated. The next three sections will describe the syntheses and the biological evaluation of these complexes. For clarity reasons, the discussed compounds are divided into homoleptic complexes, where the ruthenium center bears only dtc ligands, and the heteroleptic derivatives, involving one or more dtc ligand and at least another type of coordinating group.

Homoleptic Ru(III)-dithiocarbamato complexes

Ruthenium(III)-dtc derivatives of the type $[Ru(S_2CNR_2)_3]$ (R= Me, Et, Bu) (Figure 5A, I) were first synthesized by the Italian chemist Lamberto Malatesta in 1938, by mixing the corresponding salt NaS_2CNR_2 with $K_2[Ru^{IV}Cl_6]$ [67]. The crystal structures of both mono- and di-nuclear species have been resolved (Supplementary Figure 2A & B). Moreover, in the early 1970s, Hendrickson and Pignolet reported the synthesis and the chemical/electrochemical interrelations of a number of tris- and pentakis(dialkyldithiocarbamato) complexes of Ru(III), the latter with the general formula $[Ru_2(S_2CNR_2)_5]X$ (X= Cl$^-$, BF$_4^-$, PF$_6^-$) (Figure 5A, II & III) [56,68–71]. These were obtained from the reaction between $RuCl_3$ and NaS_2CNR_2, followed by purification in gravity column chromatography. In addition, $[Ru_2(S_2CNR_2)_5]BF_4$ could be obtained by oxidation of $[Ru(S_2CNR_2)_3]$ with BF_3 (Figure 5B). In both the mono- and di-nuclear Ru(III) derivatives, the metal centers are characterized by a distorted octahedral coordination attained by six sulfur-donating atoms. Interestingly, in the dinuclear species, each metal ion is heptacoordinated, if counting the Ru–Ru bond (~2.7 Å) with two bridged sulfurs between them. Furthermore, the $[Ru_2(S_2CNR_2)_5]Cl$ is present as two isomers (α and β) (Figure 5A, II & III) and the conversion to the thermodynamically favored β-$[Ru_2(S_2CNR_2)_5]Cl$ is carried out in methanol under

Table 1. IC$_{50}$ values recorded after 72 h treatment, expressed as μM concentration ± standard deviation (cont.).

A-549	Ovcar-3	Cem WT	Cem Vim	HT-29	NCI-H1975	Ref.
–	–	–	–	–	>20	[57]
>20	>20	3.0 ± 0.1	3.0 ± 0.7	–	0.6 ± 0.2	[57,72]
>20	>20	5.0 ± 0.2	18 ± 2	–	–	[72]
>20	3.0 ± 0.1	3.0 ± 0.2	3.0 ± 0.6	–	–	[72]
19 ± 1	>20	3.0 ± 0.2	19 ± 1	–	–	[72]
>20	>20	–	–	>20	0.5 ± 0.1	[57,73]
5.0 ± 0.4	0.5 ± 0.1	0.7 ± 0.2	>20	–	–	[72]
–	–	–	–	–	0.7 ± 0.2	[57]
–	–	–	–	–	0.5 ± 0.2	[57]
>20	>20	>20	>20	–	–	[72]
8 ± 1	3.0 ± 0.2	3.0 ± 0.1	>20	–	–	[72]
3.0 ± 0.5	0.7 ± 0.1	0.6 ± 0.2	15 ± 2	–	–	[72]
–	9±1	–	–	15.3 ± 0.9	0.057 ± 0.001	[57,73]
–	–	–	–	–	0.067 ± 0.002	[57]
5.2 ± 0.9	13 ± 1	17 ± 2	10 ± 2	18 ± 2	1.0 ± 0.1	[57,72,73]
–	–	–	–	–	>20	
–‡	–‡	–‡	–‡	–‡	>20	
–‡	–‡	–‡	–‡	–‡	12 ± 3	

Each value represents the average of more sets of independent experiments (cytotoxicity was measured by MTT assay). Human tumor cell lines: adrenocortical carcinoma (NCI-4295R); esophageal squamous carcinoma (OE-19 and OE-21); ovary adenocarcinoma (Igrov and Ovcar-3); uteri cervix carcinoma (Hela), pancreas adenocarcinoma (PT-45); breast cancer (MCF-7); lung carcinoma (A-549); leukemia wild type (Cem WT); leukemia resistant vimblastin (Cem Vim); colon carcinoma grade II (HT-29); non-small-cell lung cancer (NCI-H1975).
†$[Ru_2(dtc)_5]Cl$ tested as a mixture of α and β isomers.
‡Compound not tested

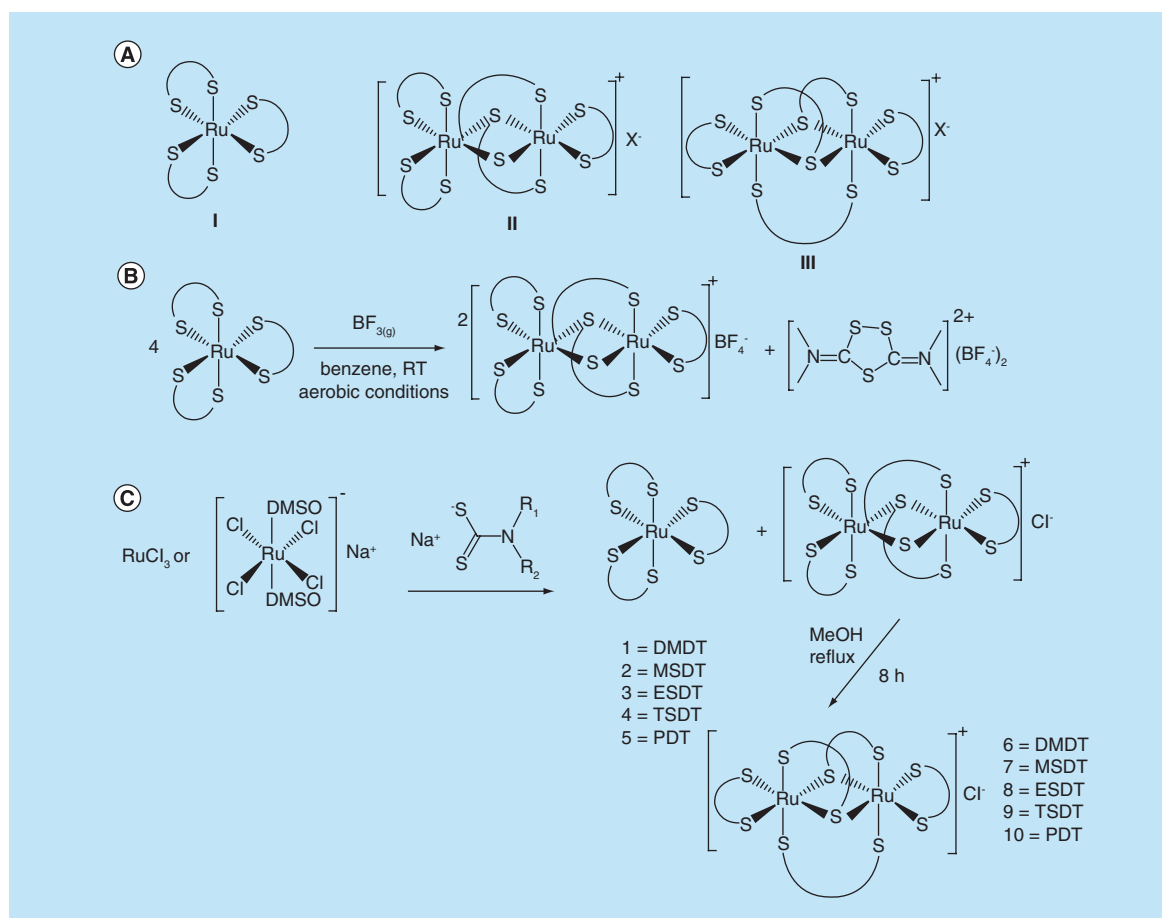**Figure 5. Ru(III)-dithiocarbamato homoleptic complexes. (A)** Mononuclear complexes with general formula [Ru(S$_2$CNR$_2$)$_3$] I, and the ionic dinuclear isomers α-[Ru$_2$(S$_2$CNR$_2$)$_5$]X (X= Cl$^-$, BF4$^-$, PF$_6$$^-$) II and β-[Ru$_2$(S$_2CNR_2$)$_5$] X (X= Cl$^-$, BF4$^-$, PF6$^-$) III. **(B & C)** Synthetic strategies for the synthesis of homoleptic Ru(III)-dithiocarbamato complexes. The formation of [Ru$_2$(dtc)$_5$]BF$_4$ species occurs via addition of gaseous BF$_3$ to a benzene solution of the mononuclear derivative [Ru(dtc)$_3$] under aerobic conditions, with the oxidation of two dtc ligands to the correspondent oxidized disulfide **(B)**. Direct synthesis of mono- and di-nuclear mixture and, after gravity column chromatography, isomerization of α isomer to the thermodynamically stable β-[Ru$_2$(dtc)$_5$]Cl compound in methanol at reflux **(C)**.

reflux. Inspired by these studies, our research group has developed novel homoleptic Ru(III) compounds with different dtc ligands in order to evaluate their cytotoxic activity *in vitro* against different human tumor cell lines [57,72,73]. In particular, we focused on the methyl-dtc (DMDT) (**1,6**), the sarcosine ester-dtc (RSDT, R= M (methyl), E (ethyl), T (*tert*-buthyl)) (**2–4, 7–9**) and the pyrrolidine-dtc (PDT) (**5,10**). These complexes were prepared by mixing RuCl$_3$ or the Ru(III) precursor Na[*trans*-Ru^{III}Cl$_4$(dmso)$_2$] [74], with the dtc salts in a 1:3 metal-to-ligand stoichiometry, and purifying the crude product by column chromatography to obtain the neutral mononuclear complex [Ru(dtc)$_3$] and the ionic dinuclear derivative [Ru$_2$(dtc)$_5$]Cl (as a α/β mixture). The latter can be completely converted to β-[Ru$_2$(dtc)$_5$]Cl by isomerization (Figure 5C). We have deeply characterized the compounds also obtaining the single crystal structures for some of them. In

particular, Supplementary Figure 2C & D report the structure solved for [Ru(ESDT)$_3$] and [Ru(PDT)$_3$], respectively. The mononuclear derivatives are low-spin paramagnetic species, as confirmed by electron paramagnetic resonance (EPR) analysis and ^{1}H-NMR spectroscopy (the presence of a paramagnetic center significantly increases the spectral width and dramatically broadens the NMR resonance signal due to a short relaxation time). On the contrary, the compounds of the type β-[Ru$_2$(dtc)$_5$]Cl are diamagnetic, with the ^{1}H-NMR resonances occurring in the expected range 0–12 ppm. The complexes have been studied for their stability (over 24 h) in dimethyl sulfoxide (used as solubilizing agent for *in vitro* antitumor screening), phosphate-buffered saline and DMEM growth medium (Dulbecco's Modified Eagle's Medium) by means of UV-Vis spectrophotometry [75]. Both the neutral and the ionic species proved stable in the organic solvent

without any significant spectral change. Moreover, they showed a good stability also in phosphate-buffered saline, where a precipitation occurred over the time yet. Finally, no significant interactions were found between the Ru(III)-dtc derivatives and the components of the culture medium used for the *in vitro* assays [75].

As a final point, the cytotoxicity assays were performed on 13 human tumor cell lines. The compounds were first solubilized in DMSO and the solutions were added to the cell medium at a maximum ratio of 1:1000 (at this concentration, the solvent is nontoxic). Then, cell culture was exposed for 72 h to increasing concentrations of the complexes, and growth inhibition was evaluated by measuring cell viability by MTT assay. The obtained results, expressed as IC_{50} values ± SD (μM), are reported in Table 1 (cisplatin was used as a reference drug).

The ionic dinuclear Ru(III)-dithiocarbamato complexes (i.e., compounds **6–10** in Figure 5C) exert significant antiproliferative effects, being much higher than cisplatin with IC_{50} values, in some cases, up to tenfold lower than the reference drug (i.e., DMDT and TSDT derivatives in PT-45 and Cem wild-type cell

lines, PDT in NCI-H1975 cell line). Moreover, a comparison between the corresponding α and β isomers of the dinuclear derivatives **6** and **10** has shown a similar antiproliferative effect, pointing out that the isomerism does not influence the biological activity of these complexes. On the other hand, $[Ru(dtc)_3]$ derivatives are generally less active than the dinuclear counterparts, except for the two CEM lines (not-solid tumors), thus highlighting the possibility of a selective action of the latter against leukemic cells. In this context, it has to be underlined that new polynuclear complexes of different metals (i.e., Pt(II), Au(I/III), Os(0), Ru(II/III)) are also reported in literature involving novel modes of action. In some cases, even if no extensive work on structure-activity relationships has been reported so far, new ideas could be developed for promising anticancer chemotherapy on the basis of some chemical features, which are often not accessible with mononuclear complexes (e.g., improved redox activity, steric hindrance, rigidity of the structure, cooperative action of two or more metal centers) [76–78].

In the light of these considerations, the hypothesis of the electronic cooperation between two Ru(III) metal
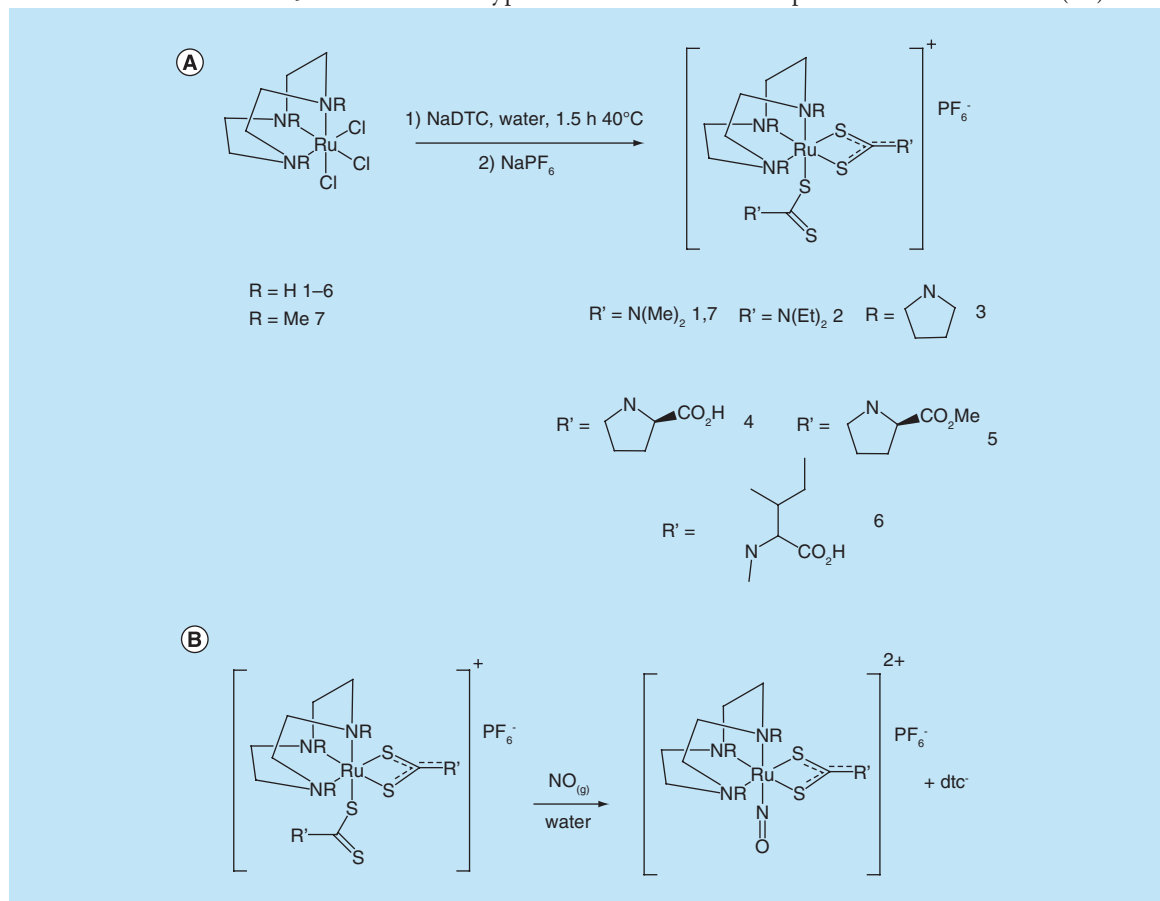

Figure 6. Ru(III)-dithiocarbamato heteroleptic complexes. (A) Synthetic strategy for the synthesis of heteroleptic Ru(III) triazacyclononane dithiocarbamato complexes. **(B)** Nitric oxide substitution reaction on [Ru^{III}(tacn)(η²-dtc) (η¹-dtc)][PF$_6$] type complexes.

centers mediated by the bridged dtc ligands, may be the keystone for the great activity of our dinuclear complexes.

Heteroleptic Ru(III)-dithiocarbamato complexes

In literature, a number of heteroleptic Ru(III) complexes containing at least one dtc ligand are reported. In general, the synthesis of this kind of compounds occurs via substitution of a good leaving group (e.g., Cl⁻, DMSO, CH₃CN) by the dtc ligand. The reported Ru(III) precursors contain also ligands with great affinity for ruthenium (e.g., phosphines PR₃, thiolates, chelating amines or O-donor anionic ligands), thus prompting the dtc anion to replace other more labile ligands [79–82].

The Ru(III) triazacyclononane dithiocarbamate [Ru^{III}(tacn)(η²-dtc)(η¹-dtc)][PF₆] (tacn = 1,4,7-triazacyclononane), described by Cameron and coworkers in 2003, is an interesting example of these heteroleptic derivatives [83]. Its synthesis takes place in water starting from the precursor [RuCl₃(tacn)] via substitution of the chloride ligands by dtc ligands (used as sodium salt). A series of different complexes were obtained wherein the tacn ligand coordinates the Ru(III) center with a facial geometry (Figure 6A). In the reported complexes, the remaining coordination sites were taken up by a bidentate and a monodentate dtc ligand, in particular, DMDT (**1,7**), DEDT (**2**), PDT (**3**), L-proline dithiocarbamate (**4**), L-proline methyl ester dithiocarbamate (**5**) and L-N-methylisoleucine dithiocarbamate (**6**). Although unlikely for a dtc molecule (inherently being a chelating ligand), it can act also as a monodentate ligand in some Ru(III) complexes [55,83]. Its replacement by nitric oxide (NO) via reaction with gaseous NO (Figure 6B) is a strong evidence for a monodentate dtc coordination. This process is favored by the formation of an extremely strong Ru–NO bond, being stable under a variety of both redox and substitution conditions. It is worth nothing that, at the biological level, this phenomenon could be exploited in terms of NO scavenging properties. In fact, the radicals NO and O₂⁻ are known to be key mediators in a number of diseases, including inflammations [84]. In the light of these considerations, Cameron and collaborators exploited RAW264 murine macrophage cells to *in situ* produce NO [83]. In aqueous solution, the produced NO can react with O₂ to form nitrite and nitrate. Therefore, the quantification of these byproducts in the presence or absence of a potential NO scavenger is useful to evaluate the NO scavenging ability. The Ru(III)-tacn-dtc complexes **1–7** demonstrated a good scavenging ability, with **5** being the most effective ($\Delta[NO_2]^- = -49.5$; value calculated between treated and nontreated RAW264 cells at a complex concentra-

tion of 50 μM). These findings are important as there are many diseases in which an overproduction of NO is implicated (i.e., atherosclerosis, neurodegenerative diseases, autoimmune diseases and cancer). In fact, NO plays a pivotal role in the physiology of the different tissues, including cells of the immune system, and its levels must be carefully regulated to maintain the cellular homeostasis [85]. With respect to the oncological field, the upregulation of NOS (NO synthase, a heme-containing metalloenzyme responsible for NO generation) has been demonstrated in a variety of human and murine tumors (e.g., glioblastomas, gynecological cancers, neoplasia of breast, head, neck, prostate, bladder and colon). This suggests NO could promote tumor growth by regulating the blood flow or by acting as a mediator in angiogenesis [86].

Concerning other heteroleptic Ru(III)-dithiocarbamato complexes, Ali and collaborators synthetized some thalidomide-based dithiocarbamato-derivatives of Cu(II), Ni(II) and Ru(III) (Supplementary Figure 3) [87]. The physicochemical analyses of these compounds led them to define a structure with two metal centers, the first one being coordinated by two dithiocarbamato ligands in a bidentate manner, while the second metal center was proposed to be chelated by four oxygen atoms of the thalidomide moiety.

These three compounds were tested for their cytotoxic activity *in vitro* against MCF-7 cells (human breast cancer), in the concentration range 1.0–0.0001 μg/ml, using DMSO as a vehicle (all species proved stable in this medium over 24 h). None of them showed significant tumor cell growth inhibition after 24 h treatment (cell viability around 100% evaluated by an MTT assay). The highest inhibition (24%) was observed with the Cu^{II}-thalidomide-dithiocarbamato derivative at 1 μg/ml, whereas the treatment with the Ru(III) complex resulted in 91% cell viability (at the same concentration). These complexes were investigated also for their ability to bind calf thymus DNA. UV-Vis spectral changes of the metal species in the presence of different calf thymus DNA concentrations indicated that the complexes are able either to bind DNA via noncovalent interactions (e.g., H-bonds occurring between the base pairs and the nitrogen and oxygen atoms of the complexes), or to simply uncoil the DNA double helix, leading to a higher number of water-exposed DNA bases. Another scenario may foresee the electrostatic attraction between the positively-charged compounds and the negative sugar-phosphate backbone of DNA. The calculated binding constant (K$_b$) for the Ru(III)-thalidomide-dithiocarbamato complex is 4.5 10⁴ M⁻¹, while the copper(II) derivative has got the highest K$_b$, evaluated as 1.4 10⁵ M⁻¹ [87].

Our research group worked on heteroleptic Ru(III)-dithiocarbamato compounds as well. In particular, we reported on the anticancer activity of methylated dithiocarbamates of PDT and DMDT [73]. Briefly, the dithiocarbamato salts of these ligands, NH_4PDT and NaDMDT, were reacted with CH_3I to obtain the neutral ligand forms PDTM and DMDTM, respectively (Figure 7A). It should be highlighted that the methylation occurs only on one of the two sulfur atoms. The resulting ligands possess a lower nucleophilic character than their starting salts, so the reaction with the Ru(III) precursor Na[$trans$-RuCl$_4$(DMSO)$_2$] in a 1:1 metal-to-ligand stoichiometry yields the neutral paramagnetic complex [mer-RuCl$_3$(DMSO)(dtcm)] (dtcm = PDTM and DMDTM) (Figure 7B).

Interestingly, contrary to our homoleptic Ru(III)-dtc derivatives (see previous section), these syntheses did not yield the dinuclear Ru(III)-dtc species, likely due to the lower nucleophilic character and the higher steric hindrance of the methylated ligand. The *in vitro* screening for cytotoxicity evaluation on five human tumor cell lines (Ovcar-3, HT-29, Igrov, MCF-7 and HeLa) did not afford any result, since all the collected IC$_{50}$ values were higher than 100 μM.

Heteroleptic Ru(II)-dithiocarbamato complexes

Various Ru(II) mono- and bis-dithiocarbamates have been reported whereas no homoleptic derivative has been described so far [54]. It should be highlighted that the charge (+2) of the metal center along with its coordination geometry (octahedral) likely prevent the coordination of more than two dtc ligands so to avoid the formation of an anionic complex. According to the Hard/Soft Acid/Base Theory, Ru(II) is less hard and has a less electrophilic character than Ru(III). +2 is the most common oxidation state for ruthenium, and it can be stabilized by phosphines and *C*-donor ligands, in particular by carbon monoxide (CO) [54,88]. Examples of mono-dithiocarbamato complexes of Ru(II) are hydrides of the type [RuH(CO)(PPh$_3$)L(dtc)] (L= PPh$_3$, pyridine, piperidine) [89,90]. A number of bis-dithiocarbamato derivatives have been characterized as well, and among them the compound *cis*-[Ru(CO)(PPh$_3$)(dtc)$_2$] was prepared substituting two coordinated NO$_3^-$ groups (counting total three coordination sites) and a phosphine of the precursor with two dtc ligands [91].

Although the oxidation state +2 is overall the most common for dithiocarbamato complexes of ruthenium [54] and Ru(II) derivatives are object of intense studies in the research for new anticancer drugs [92,93], the only example of application in the oncological field was described by our research group in 2012 [57]. In fact, we reported the synthesis and the *in vitro* cytotoxic activity on NCI-H1975 cells (non-small-cell lung cancer) of the

neutral complexes [Ru(dtc)$_2$(DMSO)$_2$] (dtc = DMDT and PDT whose structures are shown in Supplementary Figure 2 E&F, respectively), obtained by mixing the ruthenium(II) precursor [*cis*-RuCl$_2$(DMSO)$_4$] (first synthetized by Evans [94]) with the dithiocarbamato salt in a 1:2 metal-to-ligand stoichiometry (Figure 7C).

These Ru(II)-dithiocarbamato species showed none or very low cytotoxicity compared with the reference drug cisplatin (Table 1). In the light of these findings, we hypothesized that the cytotoxic activity of our Ru(III)-dithiocarbamato complexes could occur via a Ru(III)/Ru(II) reduction. Moreover, the *in vitro* biological tests put in evidence that the antiproliferative activity does not depend only on the oxidation state of ruthenium, but it is strongly affected by the chemical structure of the dithiocarbamato ligand as well. In fact, comparing the data collected in 1, both the Ru(II) and Ru(III) complexes containing PDT ligands are much more active than the DMDT counterparts. The higher rigidity of the pyrrolidinic ring may account for this
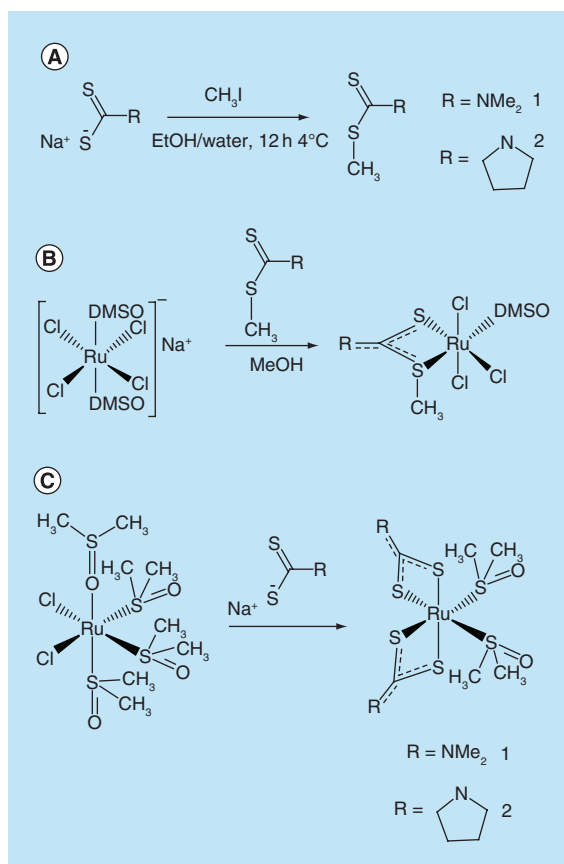

Figure 7. Preparation approaches. Syntheses of the methylated dtc ligands **(A)** and of the related heteroleptic Ru(III) complexes **(B)**. Synthetic strategy for the synthesis of Ru(II)-dithiocarbamato complexes of the type [Ru(dtc)$_2$(DMSO)$_2$] (dtc = DMDT **1** and PDT **2**) **(C)**.

dtc: Dithiocarbamate.

behavior. Besides, both the Ru(II) and Ru(III) precursors used in our syntheses (*cis*-[RuCl$_2$(DMSO)$_4$] and Na[*trans*-RuCl$_4$(DMSO)$_2$], respectively) lack in an antitumor activity, thus underlining the biologically relevant role of the ligand moiety for the efficacy of the metallodrugs.

Conclusion

In this review, after a general introduction on the chemical features of ruthenium and its main oxidation states, we have described the very interesting properties of dithiocarbamates (dtc). Among the latter, we have discussed their use either as drugs (Antabuse) or chemoprotectants (in platinum-based anticancer chemotherapy). Then, for clarity reasons, we have divided this manuscript into three sections on the basis of the two principal ruthenium oxidation states (+2 and +3) and of the range of the coordinated ligands. In particular, we have described homoleptic complexes where the ligands are only dithiocarbamates, and heteroleptic derivatives whose coordination sphere consists of other ligands, such as CO and phosphines, in addition to at least one dithiocarbamato moiety.

For all the ruthenium dithiocarbamato compounds, we have reported the synthetic procedures and the *in vitro* biological studies, mainly designed to elucidate their antineoplastic properties against human tumor cell lines. Taken together, only the homoleptic Ru(III)-dithiocarbamato complexes proved active as anticancer agents, detecting for both the mono- and the di-nuclear DMDT and PDT derivatives as very promising antiproliferative activity, chiefly toward the non-small-cell lung cancer cells NCI-H1975. In particular, the homoleptic α-[Ru$^{III}_2$(PDT)$_5$]Cl was the most active with a recorded IC$_{50}$ of 0.059 ± μM. Such a low value represents a promising starting point for the development of new chemotherapeutics able to fight against tumors still largely incurable, including the non-small-cell lung cancer.

Future perspective

In the light of all our findings and taking into consideration that our Ru(II) compounds are not active, the

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Executive summary

- Transition metals are endowed with different oxidation states, and their complexes can exhibit a range of geometries and coordination numbers that allow, when designing new drugs, the modulation of their biochemical reactivity, in terms of both kinetics and thermodynamics.
- Cisplatin is clinically administered by intravenous injection, and the neutral form of the drug easily enters individual cells. In particular, the drug passes through the cell membrane by both passive diffusion and active transport, mediated by the copper transporter CTR1.
- Tumor cells treated with cisplatin are able to develop resistance during treatment by different mechanisms that are first associated with the affinity of platinum for intracellular *S*-donor molecules, such as glutathione, methionine- and cysteine-containing proteins.
- The number of ruthenium complexes investigated as anticancer agents has exponentially increased in last three decades. Interestingly, some of these compounds have shown activity against cisplatin-resistant tumors, with less severe side effects if compared with platinum drugs.
- The potential drug NAMI-A ([ImH] [*trans*-RuCl$_4$(DMSO)(Im)], Im = imidazole) was tested in clinical trials (Phase I/II) in combination with gemcitabine. NAMI-A inhibits lung metastasis formation *in vivo*, without affecting primary tumors.
- The Ru(III) derivative KP1019 ([IndH] [trans-RuCl4(Ind)2], Ind = indazole) is the first-in-class ruthenium-based anticancer drug in clinical development against solid malignances. This coordination compound efficiently untwists DNA and weakly bends it, showing a preference for N7 of the purine bases guanosine and adenosine.
- Activation by reduction: mechanism of action hypothesized for some ruthenium derivatives such as NAMI-A.
- Dithiocarbamates are a versatile class of anionic sulfur-donor chelating agents widely used in coordination chemistry, with applications in qualitative inorganic analysis and bio-inorganic medicine.
- Dithiocarbamates were used in the past as chemoprotectants against the nonspecific coordination of a metal center under physiological conditions. Some metal-dithiocarbamato complexes have been designed in order to combine the protective action of the ligand with the anticancer effectiveness of the metal center.
- Homoleptic Ru(III)-dithiocarbamato complexes are mono- and di-nuclear Ru(III) derivatives with the same dithiocarbamato ligands in the coordination sphere. The metal centers are characterized by a distorted octahedral coordination attained by six sulfur-donating atoms.
- Heteroleptic Ru(III)-dithiocarbamato complexes are ruthenium derivatives containing at least one dithiocarbamato ligand in the coordination sphere; the other ligands being different entities of the type: phosphines, thiolates, chelate amines or *O*-donor anionic molecules with a great affinity for the metal.
- Both the mono- and the di-nuclear DMDT and PDT homoleptic Ru(III)-dithiocarbamato derivatives proved very active toward the non-small-cell lung cancer cells NCI-H1975.

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abovementioned 'activation by reduction' mechanism, already hypothesized for other ruthenium derivatives such as NAMI-A, is not conceivable for our dithiocarbamato derivatives. In particular, we have hypothesized that the cytotoxic activity of Ru(III)-dithiocarbamato complexes could be directly associated with the Ru(III)/Ru(II) reduction reaction that ultimately may trigger some cell death cascades. In this context, it should be noted that not all the dtc ligands confer the same anticancer activity to the corresponding ruthenium complexes, and that the rigidity of the PDT ligand seems to play a crucial role in the antiproliferative ability of its 1:3 and 2:5 derivatives. In the light of these considerations, it appears of particular importance to design a library of new ruthenium agents with different steric hindrance and electronic- and cancer-targeting properties. Likewise, the presence of a rigid PDT ligand in our Au(III) dithiocarbamato derivatives recently resulted in a remarkable and fast antiproliferative activity *in vitro* [95].

Another important account is the inherent ability of some monodentate dtc derivatives to act as NO scavengers. Such a reaction, driven by the formation of an extremely strong Ru–NO bond, is indeed stable under a variety of both redox and substitution conditions. It is nowadays accepted that NO plays a pivotal role in the physiology of the different tissues, and its levels must be carefully regulated to maintain the cellular homeostasis. Moreover, in the oncological field, the upregulation of NO synthase was demonstrated in a variety of human cancers, thus suggesting NO could promote tumor growth by regulating the blood flow or by acting as a mediator in angiogenesis.

In conclusion, the *in vitro* data recorded so far for a number of different Ru(II)/(III) dithiocarbamato complexes, may be of significance with respect to the possibility to obtain new chemotherapeutics providing hope to patients, especially in the case of orphans tumors and of malignancies resistant to standard chemotherapies. However, the *in vitro* data are only indicative if not accompanied by in depth *in vivo* tests on different human tumor xenografts and acute and chronic toxicological studies.

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