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COMMUNICATION

Inhibition of 3D Colon Cancer Stem Cell Spheroids by Cytotoxic Ru^{II}-*p*-cymene Complexes of Mesalazine Derivatives

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Ru(II) complex of an imidazole-mesalazine Schiff base is a unique example showing growth inhibition of 3D-colon cancer stem cell spheroids and bulk colon cancer cells at lower dosage than salinomycin or oxaliplatin. Unlike oxaliplatin which increases the expression of stemness genes (SOX2, KLF4, HES1 and Oct4) these complexes maintain a tight regulation.

Cancer stem cells (CSCs) are rare and biologically distinct subset of cells within the total bulk cancer cell population which are long-lived having the potential of tumor initiation and progression by accumulating the necessary mutations.[1, 2] CSCs replicate slowly and have the ability to self-renew.[3, 4] These cells can survive in adverse conditions and evade conventional cancer chemotherapy and radiotherapy treatments. Therefore, after conventional cancer treatment, the CSCs act as the reservoir of cancer cells that may cause cancer relapse.[1, 4] Besides oncogenic transformations can convert non-stem cancer cells to stem-like state.[5] Hence, an ideal new generation chemotherapeutic agent should eliminate rapidly dividing (non-stem) cancer cells as well as CSCs. Metal-based anticancer agents have a major place in chemotherapy; however, no clinically approved metal-based anticancer agents are efficient killer of CSCs. The conventional DNA targeting Pt (II) drugs viz. cisplatin, oxaliplatin (Figure 1) are unable to kill the CSCs effectively mainly due to the elevated level of DNA-repair factors and efflux transporters (ATP binding cassette transporters) in CSCs.[6] Moreover, Pt(II) drugs may enhance stem cells in heterogeneous tumor population and lead to drug resistance and cancer reoccurrence.[2, 7-9]

A recent study showed that trinuclear Pt (II) complexes selectively kills breast CSCs over the bulk breast cancer cells.[10] There are reports of Mn, Ni, Cu, Os, Co, Ir complexes against breast CSCs,[8, 11-16] Ga complexes against osteosarcoma CSCs[17] and a Ru complex against stem cell-enriched colon cancer.[18] Colorectal cancer (CRC) is one of the most aggressive and third most common cancer worldwide.[19] The major impediment in the success of chemotherapy in CRC is the emergence of drug-resistant tumors, which originates from the stem cell compartments.[20] Oxaliplatin is often used to treat the advanced level of CRC either as standalone or in combination with 5-fluorouracil. Due to overexpression of ABCG2 and ATP7B genes in CSCs, oxaliplatin is effluxed out from the cell and hence shows decreased efficacy against CRC.

The binding motifs of the efflux transporters may involve thiol donors.[21, 22] Reduction in thiol binding may lead to ruthenium (II) complexes with potential against Pt-resistant cancers showing a different mechanism of action.[23-28] Two Ru complexes tetrachloridobis(1H-indazole)-ruthenate(III) NKP 1339 and TLD1433 are in the clinical trials (Figure 1). NKP1339/IT139 triggers immunogenic cell death in colorectal 3D cultures, but its effect on colorectal CSCs are unknown.[29] Here, we have employed a 3D-spheroid model of self-renewing colon CSCs to test the efficacy of metal-based chemotherapeutics using 5-amino salicylic acid (5-ASA or mesalazine) based ligands. Mesalazine is a clinically approved to treat inflammatory bowel disease (IBD) including Crohn's disease (CD) and Ulcerative colitis (UC) with a high rate of induction and maintenance of remission.[30-32] In CRC, mesalazine activates PPAR-γ and demonstrates chemoprevention.[33] Mesalazine also activates the AMPK pathway[34] and induces apoptosis in CRC.[35] However, the complete mechanism of action is still unknown. The Ru^{II}-*p*-cymene complexes of imidazole-mesalazine based ligands (**5** and **6**) are effective in inhibition of HT-29 CSCs, without enhancing the stemness. In contrary oxaliplatin treatment of HT-29 CSCs significantly

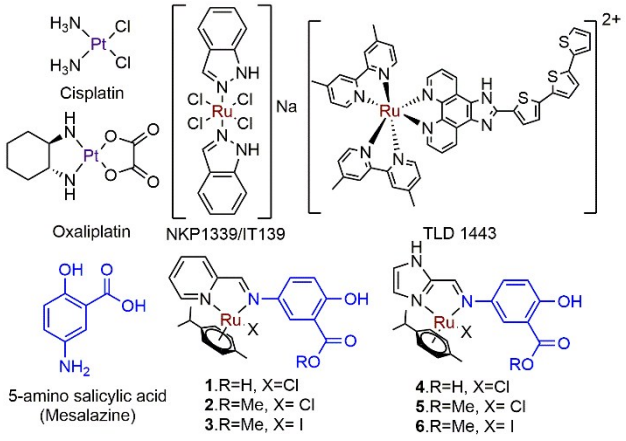

Figure 1. Top Row: Two FDA approved Pt^{II} drugs cisplatin, oxaliplatin (colon cancer), Ru^{III} complex NKP 1339 and Ru^{II} complex TLD1443 under clinical trial. Bottom Row: A FDA approved drug for inflammatory bowel diseases, mesalazine; Mesalazine conjugated pyridine and imidazole Schiff base Ru^{II}-*p*-cymene complexes (**1**-**6**) used in this work.

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increased their stemness. Complexes **5** and **6** inhibit the CSCs, similar to that of salinomycin but at least at a four times lower dosage.

Two sets of chelating ligands were prepared with mesalazine and its methyl ester, one set contained a pyridine (**L1**-**L2**) and another set contained an imidazole (**L3**-**L4**) in the Schiff base moiety. Six new Ru(II) complexes (**1**-**6**) with the respective ligands (Figure 1) were obtained in high yields (60-70%) by reacting the respective ligands with 0.5 mol equivalent of $[Ru^{II}(p\text{-cymene})X_2]_2$ (X = Cl, I) in dry MeOH at 27 °C for 8-12 h (Supporting information, Scheme S1). The complexes were characterized by ^{1}H NMR (nuclear magnetic resonance), ^{13}C-NMR, ESI-HRMS (Electron spray ionization High Resolution Mass Spectrometry), FT-IR (Fourier-transform infrared spectroscopy) and UV-Vis (Ultraviolet-visible spectroscopy) studies. The bulk purity was confirmed by elemental analysis (Supporting information, Figure S1-S24).

The stability of the complexes in the physiological condition was investigated by ESI-HRMS in 1:99 v/v MeOH and phosphate buffer (pH 7.4, containing 4 mM NaCl). All the complexes (**1**-**6**) formed aquated species, to various extents during the 24 h period (Figure S25-S36). The extent of hydrolysis is greater in the case of chlorido coordinated **4** and **5** compared to their respective iodido bound **3** and **6**. The difference is more prominent among the imidazole derivatives **5** and **6**. ^{1}H NMR data shows that hydrolyzed species of **5** starts to appear within 3 h and by 24 h most of the complex convert to its aquated form; **6** showed no hydrolysis even after 24 h (Figure S37-S38), which correlated well with their ESI-MS results. The cellular thiol-based tripeptide glutathione (GSH) binds with metal complexes leading to their deactivation. Therefore, we investigated the binding affinity of the complexes in the presence of 2.5 equivalents of GSH by ESI-MS using the afore-mentioned solution condition. The pyridine analogues (**2** & **3**) completely bound to GSH within 24 h whereas the imidazole derivatives (**5** & **6**) are more reluctant to form GSH adduct (Figure S41-S46).

The *in vitro* efficacy of our newly designed complexes (**1**-**6**) were tested against the human CRC HT-29 and also screened for cancer cells derived from other digestive organs viz. Hep G2 (liver carcinoma) and MIA PaCa-2 (pancreatic ductal adenocarcinoma). We have also extended the investigation towards a highly aggressive triple-negative human metastatic breast adenocarcinoma, MDA-MB-231. *In vitro,* cytotoxic data under 2D culture conditions showed that the complexes with free mesalazine groups (**1** & **4**) are non-toxic up to the tested range (IC_{50} > 200 µM) (Table 1). The non-toxicity may be due to the low pK^a of the free -CO_2H group, which leads to its deprotonation at pH 7.4, preventing the complex to traverse the cell membrane. Esterification of the carboxylic group enhanced the membrane traversing capability and therefore increasing the cytotoxicity. IC_{50} of the pyridine derivatives **2** and **3** were in the range 50-100 µM and for imidazole analogues **5** and **6** (with low GSH reactivity) it is around 2-3 µM (Table 1). Complexes **5** and **6** are most potent in the series and are three times more toxic than oxaliplatin in HT 29 cells, a drug used to treat the advanced level of colon cancer. Comparison of **6** with a similar aniline-imidazole Ru^{II}-*p*-cymene complex recently reported shows much poorer IC_{50} (ca. 15 µM) against a similar panel of cancer cells[24]. The use of mesalazine in the ligand seems to induce a positive effect also in enhancing the cytotoxicity of the Ru (II) complexes. **5** & **6** when treated with normal foreskin fibroblast (HFF-1) cells the IC_{50} ranges ca. 5-6 µM, thus the complexes are marginally less toxic to normal cells.

Table 1. *In vitro* cytotoxicity profile of complexes **1**-**6** in various cancer cell lines under normoxic condition in comparison to oxaliplatin.

	IC_{50} (µM) ± SD[a]			
Complexes	HT-29	MIA PaCa-2	HepG2	MDA-MB-231
1	>200	>200	>200	>200
2	82 ± 6	90 ± 6	92 ± 9	97 ± 14
3	56 ± 2	54 ± 6	51 ± 8	42 ± 5
4	>200	>200	>200	>200
5	3.2 ± 0.3	2.8 ± 0.1	2.9 ± 0.3	2.9 ± 0.4
6	2.6 ± 0.3	2.3 ± 0.2	2.4 ± 0.4	2.2 ± 0.2
Oxaliplatin	8.9 ± 0.5	5.7 ± 0.2	9.8 ± 0.3	ND[b]

[a]IC_{50} ± SD are determined by MTT assay in normoxia (~15% O_2). SD = standard deviation. The statistical significance (P) of the data is > 0.001 to <0.05. [b]Not done. Plots are in Figure S47. See experimental section for full detail.

The distribution coefficient between octanol and water (log D) values of the free acid derivatives (**1** & **4**) are in the range of -0.1 to -0.3 whereas esterification enhances the log D to 0.2 - 0.7 (Figure 2A) making the ester-based complexes more lipophilic. Lipophilicity is as an important parameter to optimize passage through the lipophilic cell membrane and promote hydrophobic interaction with protein targets to enhance the cytotoxic efficacy of a compound. The ester derivatives with greater lipophilicity show better cytotoxicity than free acid derivatives. HT-29 cells, even upon treatment with higher concentration of the free acid-based complexes compared to their ester analogues (25 µM for **1**,**4** and 10 µM for **2**-**3**,**5**-**6**) showed multi-fold higher accumulation of the ester derivatives inside the cell (Figure 2B). The imidazole derivatives (**5, 6**) showed higher accumulation than the pyridine analogue (**2**, **3**) besides, iodido analogues accumulates more than their respective chlorido analogues (**3**>**2** & **6**>**5**) thus supporting their toxicity.

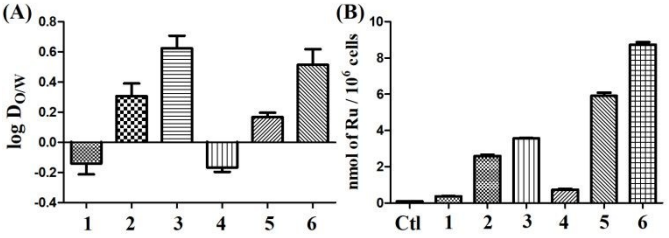

Figure 2. (A) Distribution coefficient of the metal complexes (**1**-**6**) in a 1:1 (v/v) octanol/water mixture at 37°C (B) Total Ru content measured after 6 h of incubation with complexes (**1**-**6**) in HT 29 cells.

Cellular effluxing proteins, and various stemness genes, are highly expressed in CSCs, which help them to survive under drug treatment condition. Promising *in vitro* cytotoxicity profile as well as the GSH resistivity of the imidazole derivatives (**5** & **6**), lead us to investigate the effect of the complexes on HT-29 stem cell-derived 3D-spheroids. The capacity of a compound to

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inhibit sphere formation from the single-cell suspensions is a parameter to demonstrate its ability to target CSCs. HT-29 cells were seeded with stem cell growth supplements in serum-free DMEM-F12 media on non-adherent surfaces to allow growth of CSCs. Concomitantly, **5** & **6** (at 2 & 5 µM concentrations) was added and the spheroid formation monitored for a period of 96 h. At 2 µM, both the complexes significantly reduced the colonosphere formation, and at a higher dosage they completely inhibited the colonosphere generation (Figure S48). Inspired by this observation, we extended the investigation to check if these complexes could inhibit the stemness of the already developed 3D spheroids of HT-29 CSCs. HT 29 CSCs were allowed to grow in self-renewing conditions. Once the sphere diameter reached ca. 50 µm (after 48 h), the complexes **5** or **6** with two different concentrations were added in the culture media. The size of the spheroids was determined each day by images taken using an inverted microscope. While the size of the control spheres (with 0.1% DMSO) was increasing with time, the treated spheres significantly reduced in size, even at sub IC_{50} dose (2 µM), and no growth observed at 5 µM concentration for both the complexes (Figure 3A, 3B and Figure S49). The decrease in growth may be associated with reduced viability of the CSCs. Therefore, we tested the viability of the sphere-forming cells by alamarBlue staining. There was a significant decrease in cell viability with both the complexes at 3.5 and 5 µM concentration (Figure 3C). Whereas the reduced level of viability was also observed at 2 µM, suggesting a lower but significant effect of both the complexes at sub-lethal (2 µM)

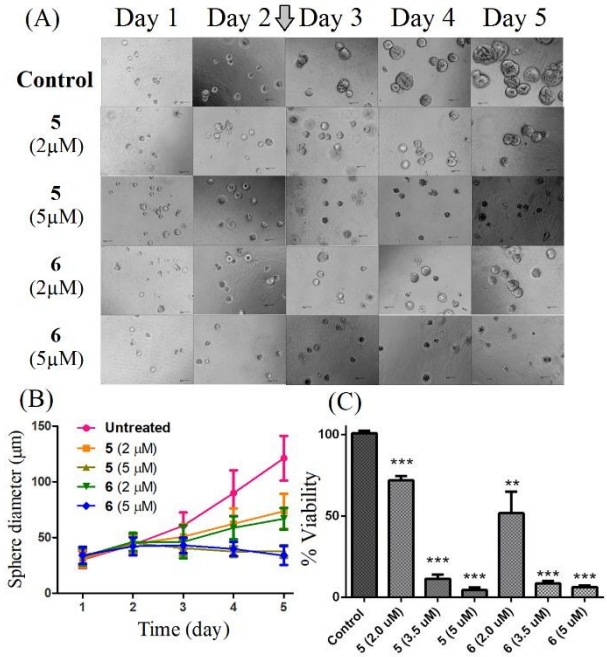

Figure 3. (A) Representative bright-field images of colonospheres of HT-29 cells, in presence of 0.1% DMSO (**control**) and with complexes **5** & **6** treated with 2 & 5 µM concentrations, observed throughout for 3 days after treatment. Complexes were added after 2 days of sphere formation indicated by arrow. Scale bar = 60 µm. (B) The plot of time-dependent change in diameter of HT 29 colonospheres observed over 5 days. (C) Change in viability of HT-29 spheres with respect to untreated cells detected by alamarBlue staining. **indicates p value <0.01; ***indicates p value <0.001.

concentration, responsible for the reduced rate of sphere growth (Figure 3C and Figure S50).

We were next interested in confirming the inhibitory effect of **5** and **6** on CSCs by exploring the expression levels of genes regulating the stemness of the cells.[36, 37] We used oxaliplatin which is a chemotherapeutic agent against colorectal carcinoma and salinomycin which is a CSC-targeting drug, as our controls, to compare the effects. We checked the stemness regulators

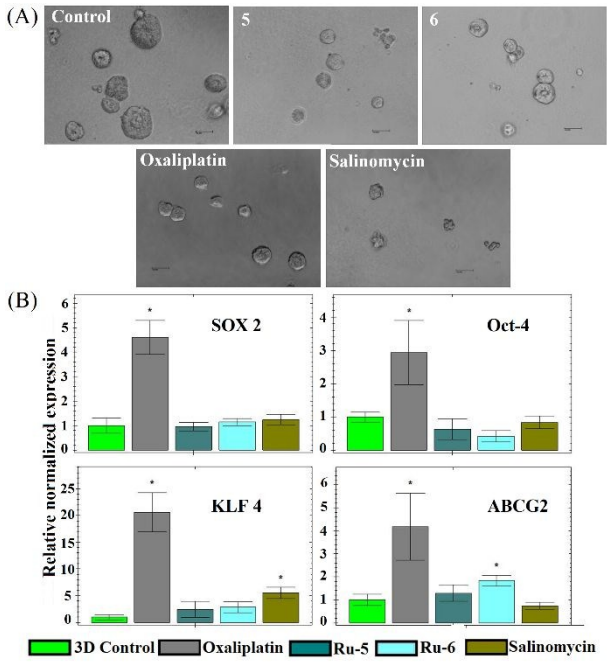

Figure 4. (A) Representative brightfield images (20x magnification) of the HT-29 colonospheres treated with Ru complexes **5** & **6** (2 µM), oxaliplatin (15 µM) and salinomycin (20 µM) observed after 48h of treatment on day 4.(B) Bar diagram of relative normalized gene expression level of the stemness markers SOX2, Oct4, KLF4 and efflux transporter ABCG2 obtained by RT-PCR data. *indicates p value <0.05.

SOX2, KLF4, Oct4, HES1 and the efflux transporter ABCG2 by real-time PCR technique. HT 29 stem cell spheroids were treated with 2 µM concentration of **5** and **6,** 15 µM of oxaliplatin and 20 µM of salinomycin for 48 h to achieve the equivalent reduction in sphere size. Gene expressions in the stem cells was determined at this time point. Interestingly, even though oxaliplatin treatment suppressed sphere sizes compared to untreated control (Figure 4A), this treatment was found to increase the stem cell regulators and drug transporter in sphere-forming HT 29 cells (Figure 4B), suggesting drug-induced stemness in these cells. This may be one of the reasons for low efficacy of oxaliplatin as a single-agent against colorectal cancers. Importantly, under similar conditions, **5** and **6** suppressed the growth of stem-like cancer cells with minor or no increase in the expression of multidrug efflux gene ABCG2 and other tested stemness genes Oct4, SOX2 and KLF4 (Figure 4B). Thus, the complexes demonstrated similar efficacy against stem-like cancer cells as Salinomycin; conversely at 10 times lower dose than Salinomycin (Figure 4B). Moreover, unlike oxaliplatin and salinomycin, **5** & **6** do not enhance the expression of HES-1 gene (Figure S51), a crucial downstream gene in the Notch signalling pathway which is involved in the

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self-renewal and tumourigenicity in CRCs.[38] Hence, unlike salinomycin, Notch signalling may be one of the mechanisms by which our complexes exert inhibitory effects on the stemness of CRCs.

Initial studies with another efflux transporter protein ATP7B showed that **5** is more resistant to sequestration by ATP7B compared to oxaliplatin (Figure S52), emphasizing its thiol resistivity over Pt drugs. Both **5** & **6** bind with the model nucleobase 9-EtG and complex **6** shows moderate binding affinity (K_b~1.4 x $10^4 M^{-1}$) with CT-DNA, implying DNA as one of the targets (Figure S53-S57). Complexes **5** and **6** induce apoptosis in a dose-dependent manner and arrest the cell cycle in G2/M phase (Figure S58 and S59).

To summarize, the Ru^{II} complexes **5** and **6** showed excellent efficacy in inhibiting the growth of colon CSC spheroids at a much lower dose than oxaliplatin or salinomycin. Complexes **5** and **6** are more resistant than oxaliplatin against efflux by different transporters and do not enhance the expression of stemness regulating genes (SOX2, KLF4, Oct4). Unlike salinomycin **5** & **6** inhibit the colon CSCs without enhancing the expression of HES-1 which warrants future studies with our complexes to gain further insight on the mechanistic pathway. The results widen the horizon of Ru^{II} complexes and open new avenues for their investigation as anti-CSC agents.

Conflicts of interest

The authors have no conflicts to declare.

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