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Antitumor and biological investigation of doubly cyclometalated ruthenium(II) organometallics derived from benzimidazolyl derivatives

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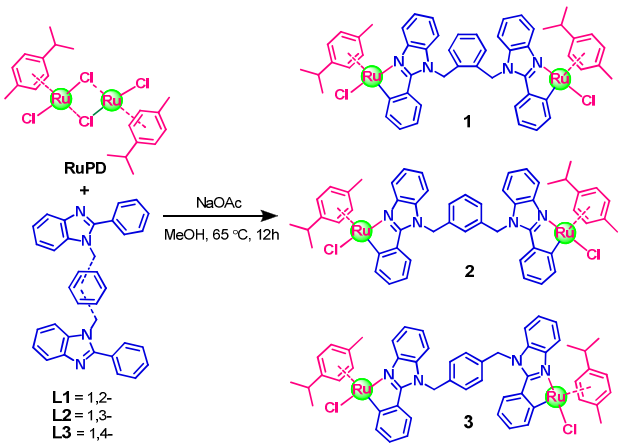
In this study, we report the synthesis, anticancer and biological properties of three doubly cyclometalated phenylbenzimidazole derived ruthenium(II) organometallics (**1–3**) and their corresponding three organic ligands. The structures of **1–3** were fully characterized by various analytical techniques, and the *meso* stereoisomer of the doubly cyclometalated ruthenacycle **3** was unambiguously confirmed by single crystal X-ray diffraction. The anticancer effects of the newly synthesized compounds were tested against selected human cancer cell lines AGS (gastric carcinoma), SK-hep-1 (hepatocellular carcinoma), and HCT-15 (colorectal carcinoma). The growth inhibitory effects of ruthenacycles **1–3** on cancer cells were found to be considerably more effective against the abovementioned cancer cells than the reference drug oxaliplatin. Compound **2** exhibited a more specific effect on the AGS cells. Gene-fishing and ELISA array were performed to analyze the target genes and cytokines secretion by **2**. As a result, a significant reduction was observed in the RPS21 by **2**. Moreover, **2** increased the secretion of cytokine such as IFNγ in macrophages and reduced the release of cytokines such as rantes and IGF-1. These results show that **2** could be a very good anticancer drug through the regulation of RPS21 gene and cytokines.

Introduction

Ruthenium-based anticancer compounds constitute a flourishing area of research, and many novel compounds have been developed[1-2] in attempts to surpass the clinical success of the platinum-based drug cisplatin. In spite of more than five decades of research and investigation of countless clinical candidates, only two more drugs such as carboplatin (in 1993) and oxaliplatin (in 2002) containing Pt have been approved worldwide as the anticancer drugs. Both of them are direct analogs of cisplatin against various cancer types.[3-4] However, it has a deplorable range of side effects, which can include nerve damage, hair loss, and nausea.[5] To overcome these limitations, some compounds based on ruthenium compounds have been developed and tested against cancer cell lines.[6-7] Some of the ruthenium(III) based prodrugs such as imidazolium *trans*-[tetrachlorido(1H-imidazole)(S-dimethylsulfoxide) ruthenate (III)], NAMI-A and imidazolium trans-[tetrachloridobis(1Himidazole)ruthenate(III)], KP1019, with particular promises are under clinical trials.[8] The arene-Ru(II) "*piano stool*" structure of these organometallic species have also proved to be interesting with examples possessing diverse activities so far reported.[9] The considerable scope for structural modification where each ligand may be modulated to allow access to diverse compounds endowed with a wide range of functionality may provide an ideal platform for the development of prospective new chemotherapeutics.

In recent years, interest toward the development of cyclometalated organometallic ruthenacycles is significantly growing as anticancer drugs against several selective cancers.



Scheme 1 Synthesis of doubly cyclometalated ruthenacycles **1–3**.

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Herein, we report the synthesis and characterization of three new di-topic *N,C*-donor ligands based on a phenyl-benzimidazole core, and their corresponding three doubly cyclometalated Ru(II)-functionalized ruthenacycles are discussed. All these synthesized six compounds were evaluated as an anticancer drug against using selective cancer cell lines, such as AGS (gastric carcinoma), SK-hep-1 (hepatocellular carcinoma) and HCT-15 (colorectal carcinoma) and comparison with known anti-cancer drugs cisplatin, oxaliplatin and doxorubicin.

Results and discussion

The two equivalents of 2-phenylbenzimidazole were reacted separately with corresponding aryl di-bromide in the presence of excess potassium hydroxide in dim
ethylformamide at room temperature affording bis-benzimidazole ditopic ligands as the colorless crystalline materials in good yield, **L1** (81 %), **L2** (80 %) and **L3** (87 %) (see supporting information, ESI). ^{1}H-NMR spectra indicated the formation of phenylbenzimidazolyl core containing ditopic ligands **L1–L3** (Figs. S1-S3 in ESI). The high-resolution electrospray ionisation mass spectrometry (HR-ESI-MS) data of the ligands showed the molecular ion peaks at m/z 491.10 (**L1**), 491.19 (**L2**) and 491.21 (**L3**) for $[M]^+$, which clearly proved the formation of ligands (Figs. S7-S9 in ESI).

The arene-ruthenium precursor of dichloro(*p*-cymene)ruthenium(II) dimer (**RuPD**) was treated with an equimolar amount of the corresponding key phenylbenzimidazole-based ditopic *N,C*-donor ligands **L1–L3** respectively in the presence of two equivalents of anhydrous sodium acetate in methanol under reflux for 12 h, followed by work-up, affording red-orange crystals of doubly cyclometalated ruthenacycles **1–3** in good yields (Scheme 1). The formation of the doubly cyclometalated ruthenacycles were confirmed by various analytical methods. The ^{1}H NMR chemical shifts of **1–3** apparently observed in all the ruthenacycles downfield shifted compared to their corresponding ligands **L1–L3**, and new peaks appeared as two doublets in the range of δ 0.50–0.83 ppm, a singlet between δ 1.98–2.05 ppm, and one multiplet in the δ range 2.05–2.10 ppm in the alkyl region in each case confirmed the presence of ruthenium *p*-cymene moieties. In addition, eight proton resonances were observed along with ligand proton resonances in the aryl region as well and are attributed to the presence of the two *p*-cymene moieties (Figs. S4-S6 in ESI).

In addition, further structural confirmation of doubly cyclometalated ruthenacycles **1**–**3** were obtained by the HR-ESI-MS data. The mass spectra of the doubly cyclometalated ruthenacycles confirmed their corresponding doubly cyclometalated composition with prominent signals for the loss of one chloride ligand at m/z 995.28, 995.13, and 995.15 for $[\mathbf{1}M-Cl]^+$, $[\mathbf{2}M-Cl]^+$ and $[\mathbf{3}M-Cl]^+$, respectively and the loss of two chloride ligands at m/z 480.17, 479.79 and 480.00 for $[\mathbf{1}M-2Cl]^{2+}$, $[\mathbf{2}M-2Cl]^{2+}$ and $[\mathbf{3}M-2Cl]^{2+}$, respectively.

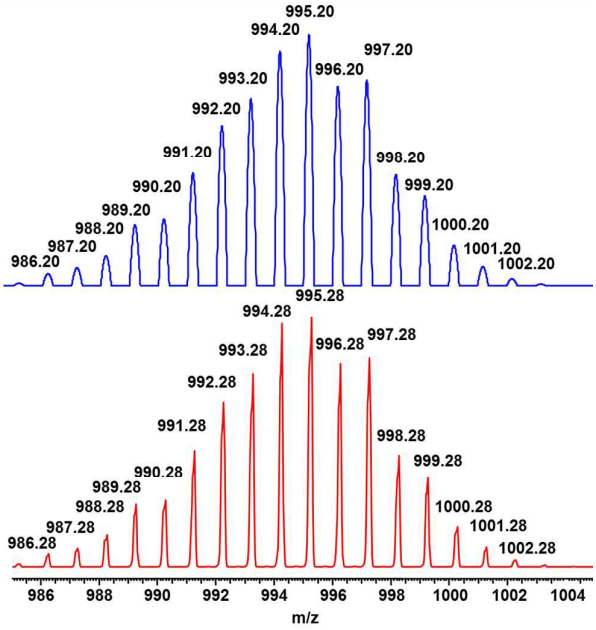


Fig. 1 Calculated (blue) and experimental (red) HR-ESI-MS spectra of ruthenacycle $[\mathbf{1}M-Cl]^+$

The experimentally observed and theoretically calculated isotopic distributions were in excellent agreement with each other as illustrated in Figs. 1, S10 and S11. Subsequently, to confirm the optical purity of cyclometalated compounds **1–3** conducted a polarimetry experiment in DMSO as well as non-coordinating solvent dichloromethane respectively. The optical rotations of the crude products **1–3** as well as recrystallized samples were all zero in DMSO and dichloromethane. We believe that all the optically active isomers were produced in equal amounts among the possible stereoisomers of *R*/*R* or *S*/*S* (*enantiomers)* and *R*/*S or S*/*R* (*meso* form). Further, to confirm the reactivity of cyclometalated organometallics **1**–**3** with nucleophilic DMSO, cyclometalated compounds **1**–**3** were dissolved in dichloromethane-d_2 along with six equivalents of DMSO, and stirred at ambient temperature and 50 °C in a sealed tube respectively. The solutions were monitored by using ^{1}H NMR spectroscopy in 24 h time intervals and found that their associate NMR resonances were not changed at all comparing with those of pure **1**–**3** and free DMSO even up to 7 days (See Figs. S12–S17 in SI). According to these NMR results, cyclometallated organometallic compounds **1**–**3** are chemically stable in DMSO at room temperature.

In order to understand the structural configuration and coordination geometry around the ruthenium metals, one of the stereoisomers of doubly cyclometalated ruthenacycle **3** was unambiguously confirmed by the single crystal X-ray diffraction (XRD) analysis using the synchrotron radiation. A orange-yellow coloured single crystal suitable for XRD was obtained by slow evaporation of the methanolic solution of **3** at room temperature for a period several days. The doubly cyclometalated ruthenacycle structure was confirmed upon structural refinement, clearly showing the double

cyclometalation. Three independent ruthenacycles **3** were observed in electron density map. Ruthenium atoms and the phenylbenximidazole rings in all three independent molecules are disordered over two positions. In all three molecules the ruthenium(II) metal in the five-membered ruthenacycles is surrounded by a *N,C*-chelation along with a chloride ligand and *p*-cymene moieties as *piano-stool* structure in both sides of the ligand. In molecular structure both the chloride ligands are pointing opposite to each other and ligands are *trans* to each other as illustrated in Fig. 2. The ruthenacycle was strongly stabilized by several strong and weak noncovalent interactions such as C–H•••π, C–H•••Cl, C–H•••N and C–H•••O (see CIF file in ESI).

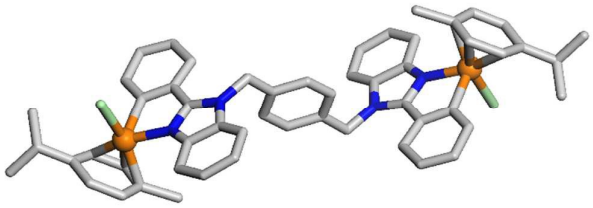


Fig. 2 X-ray crystal structure of the *meso* form of doubly cyclometalated ruthenacycle **3**.

Growth inhibitory effects on cancer cells

The anticancer effect of platinum-based drugs is known in a dose- and time-dependent manner.[10] To determine if these dose-time relationships of ligands **L1–L3,** and doubly cyclometalated ruthenacycles **1–3** could be applied to whole cell cytotoxicity assays, the cytotoxicity of the AGS, SK-hep-1 and HCT-15 human cancer cell lines was measured by varying drug exposure times and concentrations using the colorimetric MTT assay. We have used each stereoisomeric mixture of cyclometalated compounds **1–3** for the biological studies. As indicated in the Table 1, after 72 h exposure, the growth inhibitory effect by ligands **L1–L3** were similar or decreased than those after 48 h in most of the cancer cells (**L1** except in the AGS cells). In contrast, doubly cyclometalated ruthenacycles **1–3** and cisplatin showed the most similar efficacy or increased in proportion to the exposure time. Moreover, doubly cyclometalated ruthenacycles **1–3** (IC_{50} values between 6.1–12.5, 7.6–23.7, and 11.1–26.6 μM against AGS, SK-hep-1, and HCT-15, respectively) showed a more excellent inhibitory effect than ligands **L1–L3** (IC_{50} values between 9.1–67.2, 39.9–82.5, and 61.9–94.2 μM against AGS, Sk-hep-1, and HCT-15, respectively) for 72 h. In particular, most of the samples showed a special effect in the AGS cells. Most of the inhibitory the effect in the AGS cells increased with longer exposure, however, **L2** and **L3** increased the inhibitory effect significantly in 72 h (IC_{50} = 67.2 and 28.6 μM) than those at 48 h (IC_{50} = 16.6 and 7.6 μM). **L2** and **L3** are expected to reduce the stability after 48 h in AGS cells. In addition, doubly cyclometalated ruthenacycles **1–3** showed the highest inhibitory effect in the AGS gastric carcinoma, and of these, **2** exhibited a greater anticancer effect in accordance with the exposure time (IC_{50} was 6.1 μM at 72 h exposure). Even though higher than the cisplatin (IC_{50} = 2.6 μM), the anticancer effect of **2** was much better than that of oxaliplatin (IC_{50} = 25.9 μM).

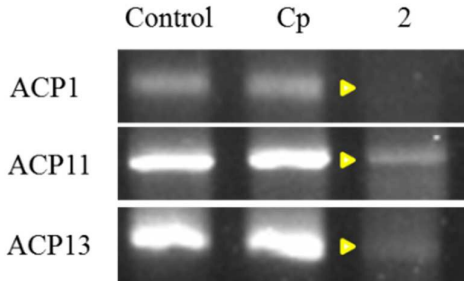


Fig. 3 Differently expressed genes were screened on AGS cells by the RT-PCR method using GeneFishing DEG system. Cells were treated with 20 μM cisplatin (Cp) and 10 μM doubly cyclometalated ruthenacycle **2** for 24 h.

Screening and identification of active genes

Genefishing technique using the annealing control primer (ACP) system has recently been used to screen for differentially expressed gene (DEG) transcripts in many diseases.[11] To investigate the changes of the gene expression associated with the growth inhibition of doubly cyclometalated ruthenacycle **2** in AGS cells, gene expression analysis was performed using the ACP-based differential display RT-PCR technique (GeneFishing DEG screening

Compound	AGS			SK-hep-1			HCT-15		
	24h	48h	72h	24h	48h	72h	24h	48h	72h
L1	67.0±0.77	63.3±2.38	9.1±0.80	66.5±4.06	71.8±0.65	82.5±1.39	75.2±2.64	63.4±1.92	69.2±1.07
L2	52.9±6.40	16.6±0.21	67.2±3.12	>100	40.0±0.45	39.9±1.44	>100	>100	94.2±1.36
L3	63.5±2.16	7.6±0.55	28.6±3.44	54.7±3.46	55.4±2.19	76.0±1.49	68.7±1.60	64.7±0.92	61.9±2.55
1	53.0±2.05	32.9±.3.07	12.5±0.46	26.4±2.66	16.5±0.29	22.8±2.09	54.1±2.18	25.0±1.08	20.2±0.51
2	26.8±4.54	17.5±0.40	6.1±0.04	9.3±0.22	7.3±0.23	7.6±0.65	27.1±2.66	14.2±0.59	11.1±0.80
3	58.9±3.08	35.5±1.76	11.5±0.42	39.0±3.09	26.9±0.24	23.7±1.12	48.3±1.44	23.9±0.92	26.6±0.35
Cisplatin	78.7±1.26	32.1±4.02	2.6±0.22	65.6±6.58	17.2±0.28	16.3±0.38	76.5±5.72	67.2±15.7	4.9±0.14
Oxaliplatin	>100	>100	25.9±0.41	>100	>100	>100	>100	12.2±2.77	31.8±1.30
Doxorubicin	7.7±1.10	3.7±0.06	24.5±2.31	25.0±1.62	3.4±0.09	3.7±0.24	>100	83.5±1.07	>100

Table 1 IC_{50} values of ligands **L1–L3** and doubly cyclometalated ruthenacycles **1–3** with the exposure time against AGS, SK-hep-1 and HCT-15 human cancer cell lines.

[a] IC_{50}: the drug concentration necessary for 50 % inhibition of cell viability; data given are mean ± standard error from at least three independent experiments, as obtained by the MTT assay using exposure times of 24, 48, and 72 h.

technology). The differential expression of the mRNA fragments observed on agarose gels indicates three down-regulated genes from the non-treated and AGS cells-treated with **2** using the GeneFishing DEG system (Fig. 3). The result of the analysis using BLAST (NCBI GenBank) indicates that the gene expressed by ACP1 was ribosomal protein S21 (RPS21, CR542132.1), which was reduced because of the treatment of doubly cyclometalated ruthenacycle **2** in the AGS cells. In addition, the gene expressed by ACP11 and 13 were identified as H36 mitochondrion (KJ994345.1) and ZAM115 mitochondrion (KJ185427.1), respectively. Of the down-regulated genes, the RPS21 is known as a component of eukaryotic ribosomes and is strongly associated with ribosomal protein SA (RPSA) protein in human cells.[12] RPSA is known as a nonintegrin laminin receptor, overexpressed on the cell surface of various cancer cells. Thus, RPSA was considered to play a significant role in the tumor progression.[13] To confirm the expression level of the selected gene RPS21, qRT-PCR experiments were performed using the mRNA of the AGS cell streated with doubly cyclometalated ruthenacycle **2**. As a result, **2** significantly reduced the expression of RPS21 mRNA in a dose-dependent manner, and the exposure to 2.5 μM **2** reduced the expression of RPS21 to 50% relative to the control (Fig. 4). In particular, 20 μM treatment significantly inhibited the expression by 88% compared to the control ($p < 0.01$). However, interestingly, cisplatin rather increased the expression of RPS21, and this was similar to the genefishing results.

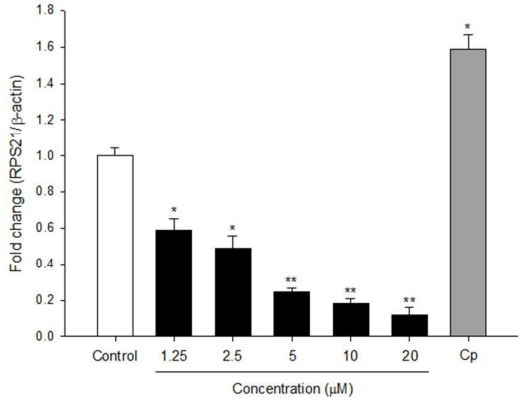
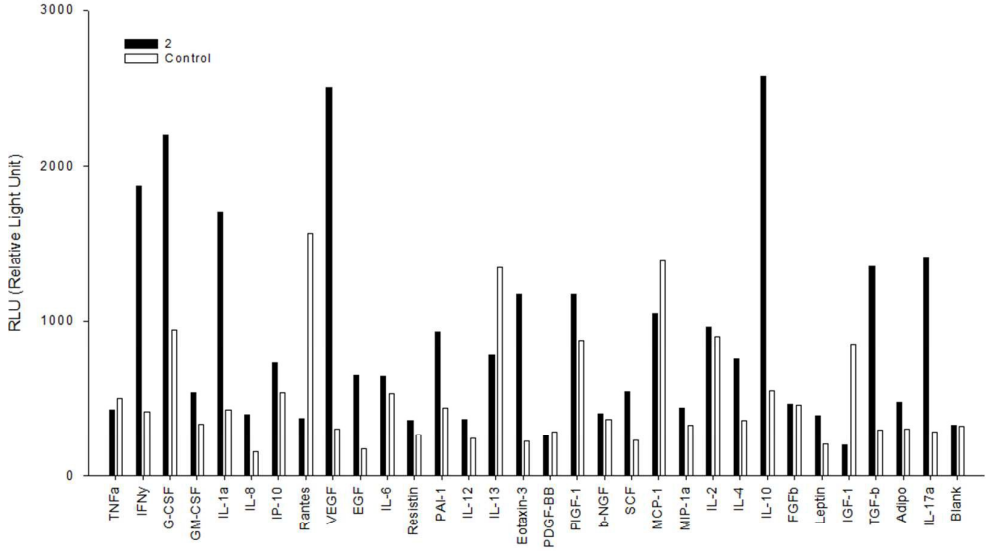


Fig. 4 qRT-PCR analysis of RPS21 expression in the AGS cells treated with doubly cyclometalated ruthenacycle **2**. Cells were treated with **2** (1.25–20 μM) and 20 μM cisplatin (Cp) for 24 h. Values represent the mean ± standard error (n = 3). * $p < 0.05$, ** $p < 0.01$: significantly different from control.



Cytokines	Fold change[a]	Cytokines	Fold change	Cytokines	Fold change
TNFα	0.9	Resistin	1.3	MIP-1α	1.3
IFNγ	4.5	PAI-1	2.1	IL-2	1.1
G-CSF	2.3	IL-12	1.5	IL-4	2.1
GM-CSF	1.6	IL-13	0.6	IL-10	4.7
IL-1α	4.0	Eotaxin-3	5.3	FGFβ	1.0
IL-8	2.6	PDGF-BB	0.9	Leptin	1.9
IP-10	1.4	PIGF-1	1.3	IGF-1	0.2
Rantes	0.2	β-NGF	1.1	TGF-β	4.6
VEGF	8.2	SCF	2.4	Adipo	1.6
EGF	3.7	MCP-1	0.8	IL-17α	5.0
IL-6	1.2				

Fig. 5 Change in cytokine secretion of THP-1 human monocytes with doubly cyclometalated ruthenacycle **2**. Cells were treated with **2** (10 μM) for 24 h. [a]Fold change: The relative ratio of the control.

Secreted cytokine analysis

Cytokines are signaling molecules that have decisive roles in many biological processes including the cell growth, differentiation, gene expression, migration, inflammation, and immunity. Macrophages release cytokines to activate and recruit other cells during inflammation or as direct killing agents.[14] Co-cultivation of the activated macrophages with cancer cells either up- or down regulate the production of different cytokines to defend against cancer cells.[15]

The arene-Ru derivatives are used for controlling the direct cancer cell cycle;[16] however, the cytokines produced by these derivatives may have an indirect effect on the cancer cells. Therefore, the amount of 31 cytokine secretion in macrophage was measured by the doubly cyclometalated ruthenacycle **2** using Human Cytokine ELISA Plate Array. As shown in Fig. 5, eight cytokines (IFNγ, IL-1α, VEGF, EGF, Eotaxin-3, IL-10, TGF-β and IL-17α) increased the AGS cells-treated with 10 μM of **2**, and the secretion of vascular endothelial growth factor (VEGF) increased 8.2-folds compared to the untreated control, which is known as the central mediator of angiogenesis more than three folds in the AGS cells-treated with 10 μM of **2**, and the secretion of may rather help the growth of cancer cells.[17] In the release of cytokines from macrophages by **2**, cytokine showing the characteristics of the anticancer was interferon-γ (IFNγ, 4.5-fold increased). IFNγ mediates the antitumorigenic effects indirectly by modulating immunomodulatory responses or directly by regulating proliferation and differentiation of cancer cells.[18]

In addition, doubly cyclometalated ruthenacycle **2** reduced rantes and insulin-like growth factor 1 (IGF-1) secretion from the macrophage (0.2-fold). Rantes is highly expressed in various cancers and promotes the tumor growth and metastasis by inducing cancer cell proliferation and angiogenesis.[19] IGF-1 shows powerful effects on each of the key stages of cancer development, cancer cell proliferation, apoptosis, angiogenesis, and metastasis.[20] In other words, the macrophage treated by doubly cyclometalated ruthenacycle **2** can suppress the growth of cancer cells, because of decreasing rantes and IGF-1 secretion.

Stability of doubly cyclometalated ruthenacycle 2

To determine the stability of doubly cyclometalated ruthenacycle **2**, a 20 μM solution of **2** was pre-incubated in a cell culture medium and DMSO for 0, 12, 24, and 48 h at 37 °C. The cell growth was examined as described in the methods (Table 1) for the AGS cancer cells. As shown in Fig. 6, 50% loss in the growth inhibitory activity was observed for the doubly cyclometalated ruthenacycle **2** after ~32 h of pre-incubation in the cell culture medium. In contrast, in the presence of DMSO, the growth inhibitory activities were stable until about 24 h of pre-incubation. These results indicate that the doubly cyclometalated ruthenacycle **2** is stable in DMSO and cell culture medium for 24 h. The stability of the ruthenacycles **1**-**3** in

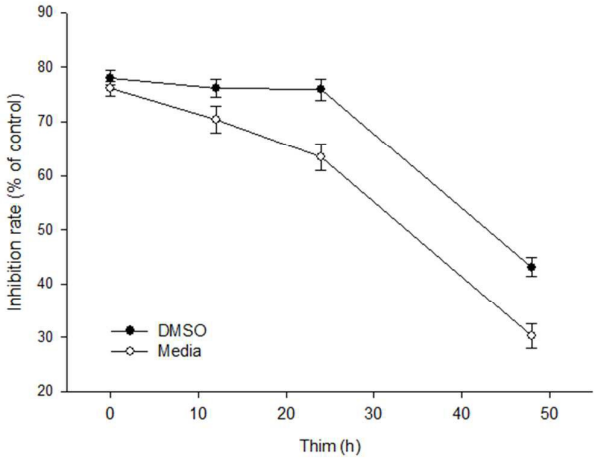


Fig. 6 Loss of growth inhibitory activity of the doubly cyclometalated ruthenacycle **2** on AGS cells. The cells were treated with 20 μM **2** for 0, 12, 24, and 48 h.

DMSO-d_6 was also investigated by time-dependent ^{1}H NMR spectroscopy. Time-dependent ^{1}H NMR data revealed that the associated proton NMR signals of the ruthenacycles **1**–**3** remained unchanged even after 72 h (Figs. S18–S20 in SI).

Conclusions

In conclusion, three new phenylbenzimidazole-based *N,C*-donor ligands and their corresponding three novel doubly cyclometalated ruthenacycles (**1**–**3**) were synthesized and characterized. The antitumor action of these compounds was evaluated, indicating that bis-benzimidazole-based organometallics have the potential to act as potent anticancer agents, particularly in cell lines, which are resistant to Pt-based drugs. The growth inhibitory effects in the preliminary studies of ruthenacycles **1**–**3** showed better results than that of the reference drug oxaliplatin. Among these three doubly cylcometalated ruthenacycles, **2** showed anticancer potency against selected human cancer cell lines AGS, SK-hep-1, and HCT-15. In particular, **2** exhibited a more specific effect on the AGS cells, by decreasing the expression of RPS21 with the potential anti-cancer marker. In addition, **2** increased the secretion of cytokine such as IFNγ in macrophages and decreased the release of cytokines such as rantes and IGF-1. Therefore, the anticancer effect of **2** is likely to appear through the complex operation by direct action to cancer growth via the reduction of RPS21, and indirect anticancer action mechanism of cytokines secreted by macrophages. Based on these results, we will further study the mechanism by which the RPS21 is modulated by major changed cytokines among experimented in this study.

Acknowledgements

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Notes and references

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