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ARTICLE *in* CHEMISTRY & BIODIVERSITY · MAY 2010

Impact Factor: 1.52 · DOI: 10.1002/cbdv.200900288 · Source: PubMed

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In vitro Ruthenation of Human Breast Cancer Suppressor Gene 1 (*BRCA1*) by the Antimetastasis Compound RAPTA-C and Its Analogue CarboRAPTA-C

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The interaction of two ruthenium–arene–1,3,5-triaza-7-phosphaadamantane compounds ([Ru(η^6-*p*-cymene)Cl_2(pta)] and [Ru(η^6-*p*-cymene)($C_6H_6O_4$)(pta)], termed RAPTA-C (**3**) and carboRAPTA-C (**4**), resp.) with the DNA sequence of the human breast-cancer suppressor gene 1 (*BRCA1*) has been studied using a range of techniques that probe conformation, cross-linking, base specificity, restriction analysis, and *in vitro* inhibition of DNA polymerization. The study demonstrates that substitution of the two labile chloride ligands in **3** by the more stable cyclobutane-1,1-dicarboxylate ligand onto the RAPTA framework reduces the rate of reaction with DNA in a similar manner to the analogous Pt-based drug pair cisplatin (**1**) and carboplatin (**2**), suggesting that hydrolysis may be a prerequisite to DNA binding with the Ru compounds. Moreover, the rate of DNA interaction for **3** is in a similar range to that of **2**, despite the fact that these compounds have a different therapeutic profile. The similar rates of reaction contrasting with the different modes of activity suggests that the RAPTA compounds may be clinically useful against cancer cells that have developed resistance to Pt-based therapies, particularly involving excision–repair mechanisms.

Introduction. – Cisplatin (**1**) entered clinical trials in 1971 and within a relatively short time became the most widely used anticancer medicine in the world [1]. Despite the unprecedented success of **1**, it is far from being an ideal drug, as its application is associated with a high level of general toxicity that induces many side effects, and its effectiveness in cancer treatment is somewhat limited due to drug resistance [2][3]. These limitations have fuelled a considerable amount of research activity in the field of metal-based anticancer chemotherapy. However, despite the massive research effort, only a few metal drugs have reached the clinic for cancer treatment, these being cisplatin derivatives.

One of the most successful derivatives of **1**, if not the most successful, is carboplatin (**2**), in which a dicarboxylate ligand replaces the labile chloride ligands in **1**. The cyclobutane-1,1-dicarboxylate ligand has been shown to hydrate more slowly than the chloride ligands in **1**, as this process involves ring-opening, followed by the loss of the ligand [4]. Under physiologically relevant conditions of 150 mM free chloride, as in most extracellular fluids, Pt^{II} complexes can pass the membrane as neutral prodrugs and then become activated at 2–4 mM chloride concentration prevailing in the cytoplasm and the organelles. Close to 50% of the molecules will be in the form of a

monoaqua complex. If the concentration of free chloride is below the millimolar range, the second chloride ligand will be replaced by H_2O, giving rise to a diaqua complex. The rate of drug hydration has been correlated to the toxicity [5][6], with **2** reacting more slowly with DNA [7]. The difference in toxicity seems to be a kinetic one. Due to its bidentate ligand, cyclobutane-1,1-dicarboxylate **2** does not react that rapidly with other ligands, and the rate of binding of Pt to macromolecules, such as plasma proteins, is about tenfold lower with **2** than with **1**. This observation suggests that DNA interactions may not only be central to the mechanism of Pt drug-based apoptosis, but also involved in general toxicity, and, recently, such a link has been established *in vivo* [8].

In the continuing search for improved metal-based anticancer drugs, the introduction of orally active Pt^{IV} carboxylate complexes, such as satraplatin, in the pre-clinical phase has recently been evaluated [9]. In contrast to square-planar Pt^{II} complexes, octahedral Pt^{IV} complexes are kinetically more inert and stable for days in biological media, so that they can reach the tumor site unchanged and then be gradually converted to more labile Pt^{II} species by biological reductants in the hypoxic environment that is necessary to exert their anticancer activity. At the same time, trials have expanded beyond complexes based on Pt to alternative metal centers. Of these, Ru complexes have received much attention [10–12], as they have the ability to form DNA adducts and induce cross-linking, like effective anticancer Pt compounds, but often with significantly lower general toxicity. The lower toxicity of Ru complexes is in part a function of the ligands attached to the metal centre, and in part, it is believed to be a function of the metal itself – Ru can adopt different oxidation states under physiological conditions [13][14] and the more active Ru^{II} oxidation state is promoted in the hypoxic environment of cancer cells, whereas the more inert Ru^{III} state is promoted in healthy tissues. Furthermore, some Ru complexes can mimic Fe in binding to important carrier proteins such as transferrin [9][15][16], which is postulated to be a specific delivery mechanism to rapidly growing cells, including tumor cells, because of their higher Fe requirement.

Several Ru complexes have already been shown to exhibit excellent *in vivo* antitumor activity, and two such complexes have even entered clinical trials [10][11]. Like **2**, but more pronounced, these active Ru complexes display a much lower *in vitro* DNA-binding activity compared to **1**. Ru complexes with indazole heterocyclic ligands have been shown to be cytostatic and cytotoxic in colorectal tumor cells both *in vivo* and *in vitro* [17–20]. Studies have shown that the drugs are efficiently taken up into the cells, possibly *via* interactions with transferrin [21–25], where they induce apoptosis in a B-cell lymphoma 2 (Bcl-2)-independent fashion [26]. Thus, such compounds are highly valuable for overcoming the limitations of **1** in tumors that overexpress Bcl-2. Bcl-2 is a gene family that regulates mitochondrial permeability and has been linked to drug resistance. Thus, once cancers overexpress members of this protein family, they do not respond well to the current drug treatment. The exact mechanism of the induction of apoptosis remains to be elucidated, although it is postulated to involve DNA interactions [27][28].

A range of Ru^{II} arene complexes containing a 1,3,5-triaza-7-phosphaadamantane (PTA) ligand, termed RAPTA compounds, have been shown to interact with DNA [29] in a pH-dependent manner [30][31]. However, the rate of reaction with DNA is significantly lower than those of **1** or **2**. Despite the lower affinity for DNA, several

RAPTA complexes display excellent *in vivo* activity, reducing the number and weight of solid metastases, although not affecting the primary tumor [32][33]. While many different RAPTA compounds have been developed [34–37], the prototype compound, [Ru(η^6-*p*-cymene)Cl_2(pta)] (**3**), termed RAPTA-C (*Fig. 1*), remains the best characterized compound of the series and the molecular mechanism has been shown to involve mitochondrial-induced apoptosis [38]. Moreover, in addition to DNA binding, interactions with proteins are becoming increasingly implicated as relevant to the mode of action of RAPTA compounds [39].

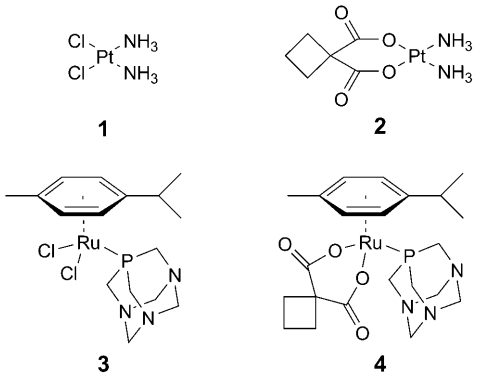

Fig. 1. *Chemical structures of cisplatin* (**1**)*, carboplatin* (**2**)*, RAPTA-C* (**3**)*, and carboRAPTA-C* (**4**)

In the process of elucidating the pathways involved in the response induced by **3**, the second-generation complex carboRAPTA-C (**4**) has been synthesized and characterized [36]. Here, we describe a detailed investigation of the interactions of **3** and **4** with DNA and compare these results with analogous studies involving **1** and its second-generation complex **2**.

Results and Discussion. – *Conformational Study of the* in vitro *Ruthenation of Plasmid pBIND DNA.* Agarose gel electrophoresis was used to probe the Ru complex-mediated conformational changes of plasmid DNA using the pBIND plasmid as a model. Experiments showed that the mobility of the metal complex-treated plasmid DNA is reduced as the molar ratio of Ru per DNA nucleotide increases (*Fig. 2*). This observation can be explained by changes in plasmid supercoiling; supercoiling reduces the three dimensional size of the DNA plasmid such that the rate of DNA plasmid migration during electrophoresis decreases as the number of supercoils decreases. Thus, these data indicate that ruthenation causes progressive unwinding of supercoiled plasmid DNA.

The mean DNA supercoil unwinding angle (Φ), triggered by drug interactions, can be calculated from *Eqn. 1:*

$$\Phi = -18\sigma/r_b(c) \quad (1)$$

where σ is the superhelical density of the DNA plasmid and $r_b(c)$ is the drug concentration at which the supercoiled and open circular forms comigrate. The

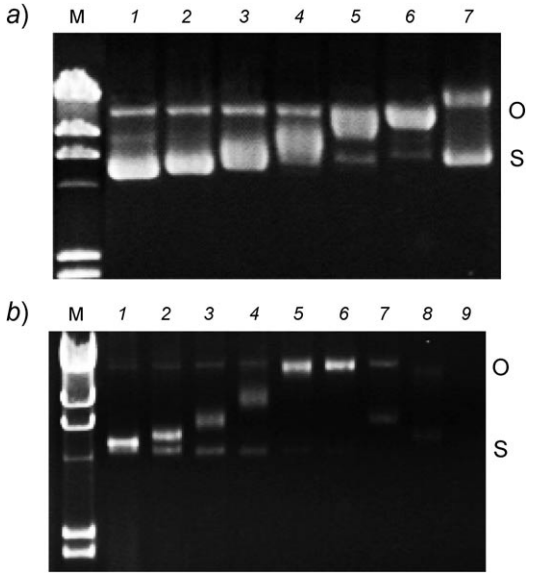

Fig. 2. *Effect of* a) *RAPTA-C* (**3**) *and* b) *carboRAPTA-C* (**4**) *on the conformation of plasmid DNA.* Plasmid pBIND DNA was incubated with the Ru complexes at various molar ratios (r_b = Ru/DNA nucleotide) at 37° for 24 h in the dark. The lanes correspond to r_b values of 0.0000, 0.0026, 0.0065, 0.0130, 0.0160, 0.0190, and 0.0260 (*Lanes 1–7*) for **3** and of 0.0000, 0.0065, 0.0130, 0.0260, 0.0390, 0.0520, 0.0650, 0.1300, and 0.1900 (*Lanes 1–9*) for **4**. The upper bands correspond to the form of open circular DNA (O) and the lower bands to supercoiled plasmid DNA (S). M denotes the λ-*Hind*III-digested marker.

superhelical density was determined as 0.0072 by determining the r_b(c) value for **1** and back calculating the superhelical density based on the published unwinding angle of $\Phi = 13°$ [40] for this metal complex.

The gels shown in *Fig. 2* verify that both **3** (*Fig. 2,a*) and **4** (*Fig. 2,b*) induce different degrees of DNA unwinding. Co-migration of the modified supercoiled and open circular DNA, r_b(c), occurs at 0.019 for **3**, which corresponds to an unwinding angle of 7°, half that of **1**. In contrast, the unwinding angle of **4** is somewhat less; comigration of the open circular and ruthenated supercoiled species occurs at an r_b(c) of 0.039–0.052, a twofold higher molar ratio compared to **3**, and, therefore, the interaction with **4** unwinds the supercoiled plasmid by only 2.5–3°.

Interstrand Crosslink Assay. Alkaline gel electrophoresis was used to study Ru complex-induced DNA crosslinking. *Fig. 3* shows the affect of **3** and **4** on the migration of single stranded DNA through the gels. The initiation of interstrand cross-links can be observed with **3** at an r_b of 0.0016. The intensity of the interstrand cross-link increases as the r_b values increase. The interstrand cross-links are completely formed at $r_b = 0.032$.

For **4**, the initiation of the interstrand crosslink also occurred at an r_b of 0.0016. The intensity of interstrand crosslinks increases as the r_b values increase, but more slowly compared to **3**. Indeed, the reaction does not reach completion at an r_b of 0.1. These results demonstrate that the formation of interstrand crosslinks is more rapid with **3** compared to **4**, as expected from their different rates of hydration [31][32][41].

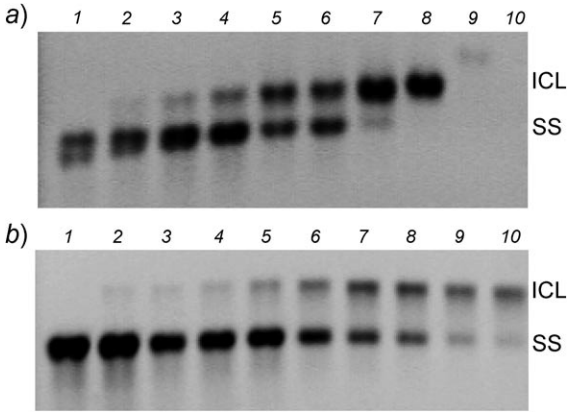

Fig. 3. *Interstrand crosslink formation in the 696-bp* BRCA1 *fragment induced by* a) *RAPTA-C* (**3**) *and* b) *carboRAPTA-C* (**4**). The 696-bp *BRCA1* fragment was incubated with the Ru complexes at various molar ratios (r_b = Ru/DNA nucleotide) at 37° for 24 h in the dark. The lanes correspond to r_b values of 0.0000 (control), 0.0016, 0.0033, 0.0066, 0.0130, 0.0200, 0.0260, 0.0320, 0.0450, and 0.0530 (*Lanes 1–10*; note that multi-crosslinked DNA molecules are formed and thus inhibit the intercalation of EB into the DNA molecules/base stacking) for **3** and of 0.0000 (control), 0.0016, 0.0033, 0.0066, 0.0130, 0.0330, 0.0530, 0.0660, 0.0800 and 0.1000 (*Lanes 1–10*) for **4**. The interstrand crosslinked DNA (ICL) appears as the upper bands on the gel migrating more slowly than the single-stranded DNA (SS) appearing as the lower bands.

Sequence Preference of Ru-BRCA1 *Adducts.* The inhibition of the activity of restriction enzymes was used to determine the potential affect of DNA damage on the function of the enzyme. In this assay, *Pvu*II (CAG/CTG) and *Eco*O109I (PuG/GNCCPy) enzymes were inhibited in a dose-dependent manner, with **3** demonstrating complete inhibition at a concentration of 600 μM. In contrast, enzymatic activity persists at concentrations of 1000 μM of **4** (*Fig. 4*). Both enzymes demonstrated a similar level of inhibition, suggesting that ruthenation does not show specificity between the two sites. The activity of these two restriction enzymes was affected about twofold less by treatment with **4** compared to treatment with **3**, suggesting that either ruthenation by **3** occurs more rapidly than that by **4** or the former complex is more stable than the latter. Due to the similarity in the nature of the resultant hydration product of both Ru compounds, it is likely that the observed effects are a consequence of the reaction rate rather than the nature of the product.

The sequence specificity of Ru-adduct formation was determined. The chain termination occurred most frequently at A, C, and G in the order as shown schematically in *Fig. 5*. Sequence analysis showed that both **3** and **4** preferentially attack at A, C, and G (and not T), as expected based on their different basicities [42].

Damage of Human Breast-Cancer Suppressor Gene 1 (*BRCA1*) *by Ru Complexes.* The quantitative polymerase chain reaction (QPCR) was used to monitor the progress of *Taq* DNA polymerase utilizing DNA adducts as templates. Previous reports have shown that the QPCR method can be used to study cellular DNA damage and repair after exposure to DNA damaging agents such as **1**, nitrogen mustards [41][43–45], 2-chloro-2-deoxyadenosine [46], chlorambucil [47], alkylbenzylguanine [48], and UV

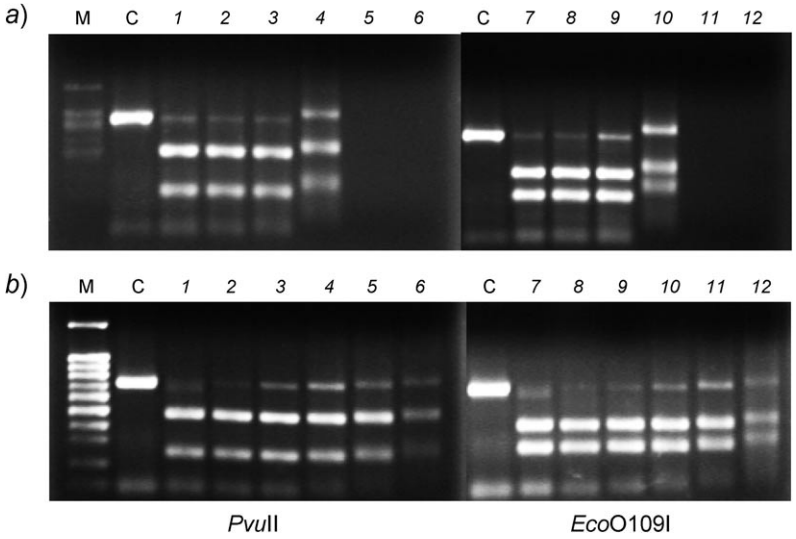

Fig. 4. *Restriction digestion of the 696-bp* BRCA1 *fragment induced by* a) *RAPTA-C* (**3**) *and* b) *carboRAPTA-C* (**4**). The 696-bp *BRCA1* fragment was incubated at 37° for 24 h in the dark with the following concentrations of Ru complexes: 0 (*Lanes 1* and *7*), 100 (*Lanes 2* and *8*), 400 (*Lanes 3* and *9*), 600 (*Lanes 4* and *10*), 800 (*Lanes 5* and *11*), and 1000 μM (*Lanes 6* and *12*). The Ru-treated DNA was precipitated, washed, redissolved in doubly distilled H_2O, and incubated with either *Pvu*II or *Eco*O109I at 37° for 5 and 6 h, respectively. M and C stand for a 100-bp DNA ladder and the control comprising untreated DNA, respectively.

irradiation [44]. Such studies showed that the total amount of amplified PCR product from a given region is inversely proportional to the amount of DNA adducts within the specified DNA fragment, as long as the experimental conditions allow exponential amplification, and the concentration of the DNA template is not a limiting factor [44].

The Ru complexes were found to reduce the amount of amplified DNA compared to the untreated DNA control. Compound **3** was more effective at blocking DNA replication, completely preventing amplification at a concentration of 600 μM. Under the same experimental conditions, DNA replication was still observed with **4** at concentrations exceeding 1000 μM. This observation is consistent with the restriction analysis indicating an approximately twofold higher ruthenation by **3** at the d(GpG) and the d(GpC) sequences compared to **4**.

The ethidium bromide (EB)-stained gels in *Fig. 6* were analyzed using a molecular imager and the amount of DNA amplification in percent, as represented by the units of absorbance of the amplified products, was plotted against the concentration of the Ru complexes (*Fig. 7*). The plots showed that DNA amplification was reduced by half at concentrations of 300 μM for **3** and *ca.* 600 μM for **4**, a twofold difference that fits with the slower rate of reaction observed in all experiments with the latter complex.

Lesion introduction within the 696-bp *BRCA1* fragment can be semi-quantified by assuming a random (*Poisson*) distribution of damage [41]. The number of Ru atoms in the DNA adducts may be calculated using the *Poisson* equation (see *Exper. Part*). The relationship between the concentration of the Ru complexes and Ru ions in the 696-bp

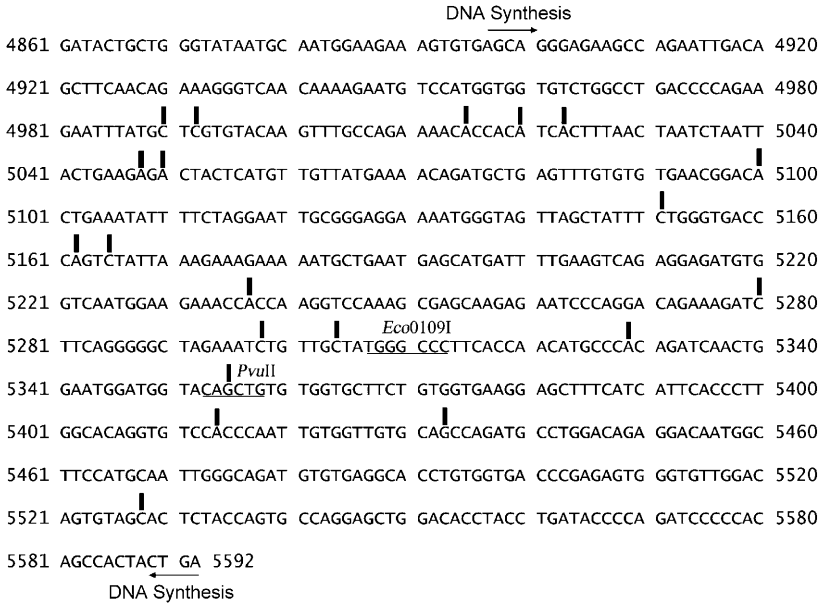

Fig. 5. *Schematic diagram showing the portion of the base sequence of the 696-bp* BRCA1 *fragment used to monitor the inhibition of DNA synthesis on the template modified by* **3** *and* **4**. The arrow indicates the start site and the direction of DNA synthesis. The bar represents monofunctional adducts. TGGGCCC is a recognition sequence of *Eco*O109I and CAGCTG is a recognition sequence of *Pvu*II.

BRCA1 fragment is illustrated in *Fig. 8*. The results show an approximately three to fourfold higher rate of ruthenation by **3** compared to **4** under equivalent experimental conditions.

Ru Complexes have demonstrated anticancer activities that do not always correlate with DNA damage. However, RAPTA complexes have been shown to damage DNA in a pH-dependent manner, with the degree of DNA damage increasing as the pH is lowered from 7.5 to 6.5 [30]. This pH-dependent activity may allow tumor cell targeting, as healthy cells have a pH > 7, whereas the more rapid tumor growth creates a hypoxic environment with a lower pH of < 7. Indeed, studies show that RAPTA compounds exhibit limited damage to healthy cells [32]. Consequently, RAPTA compounds may have tumor cell-targeting properties *in vivo* that reduce the general toxicity of the drug treatment. Indeed, drug toxicity is a current limitation to Pt-based therapies. The substitution of the labile chloride ligands in the parent complex **1** by the more slowly hydrating cyclobutane-1,1-dicarboxylate ligand present in the second generation derivative **2** reduces the rate by which the Pt^{II} center hydrolyzes, a prerequisite to DNA damage, and also reduces the general toxicity of this compound. Thus, a similar change in activity may be observed in the RAPTA series of compounds when the two labile chloride ligands are replaced by a dicarboxylate ligand, and indeed, a comparable affect of ligand substitution on the DNA-binding screens between the Pt and Ru pairs suggests that the substitution results in a parallel change in properties.

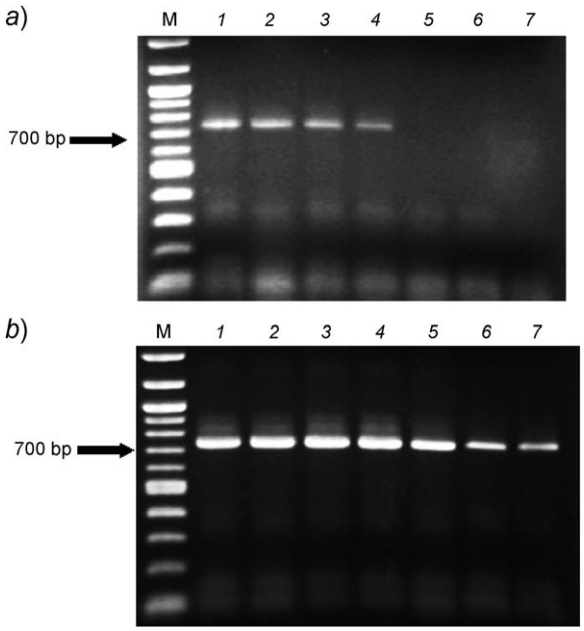

Fig. 6. *DNA Amplification of the 696-bp* BRCA1 *fragment induced by* a) *RAPTA-C* (**3**) *and* b) *carboRAPTA-C* (**4**). The 696-bp *BRCA1* fragment was incubated at 37° for 24 h in the dark with the following concentrations of Ru complexes: 0, 100, 200, 400, 600, 800, and 1000 μM (*Lanes 1–7*, resp.). The 696-bp *BRCA1* fragment was then amplified by semi-QPCR. Semi-QPCR products were separated by electrophoresis on a 1.5% agarose gel, stained with EB and visualized with UV light. M denotes a 100-bp DNA ladder.

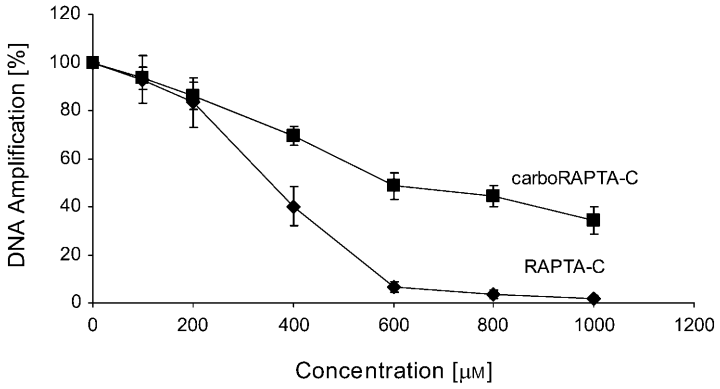

Fig. 7. *DNA Amplification of the 696-bp* BRCA1 *fragment after* in vitro *treatment with* **3** *and* **4**. The gels shown in *Fig. 6* were quantified using a *Bio-Rad* molecular imager, and the amount of DNA amplification [%] was plotted as a function of the concentration of the Ru complexes.

The two Ru complexes reported herein were found to reduce the amount of amplified DNA, with **3** blocking DNA replication and completely preventing

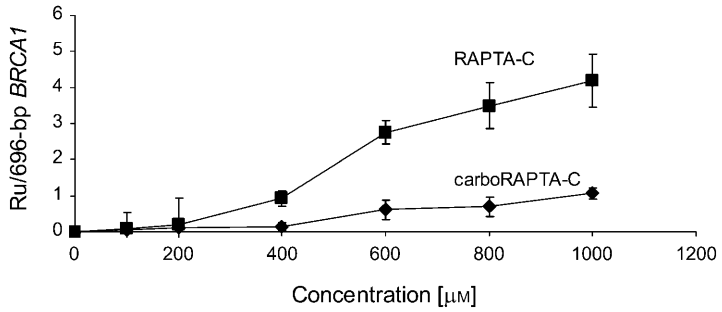

Fig. 8. *Dose responses of ruthenation of the 696-bp* BRCA1 *fragment by* **3** *and* **4**. Absorbance units from the amplification products were scanned to determine the relative amplified 696-bp *BRCA1* band intensities without and with **3** or **4**. Absorbance units were applied to the *Poisson* equation, and the ratio Ru atoms/696-bp *BRCA1* was plotted as a function of the concentration of the Ru complexes. The data were derived from four independent experiments ±S.D.

amplification at a concentration of 600 μM and **4** only showing the same effects at a concentration exceeding 1000 μM. Complete inhibition of DNA amplification by **1** and **2** using this method is observed at 50 and 400 μM, respectively [7]. Thus, the inhibition concentrations of **2** and **3** are in the same range. The ruthenation of DNA by **3** is also very similar to the platination value observed for **2**. Nevertheless, **3** and **2** show very different *in vitro* cytotoxicity and *in vivo* profiles. Both **3** and **4** form different Ru-DNA adducts with predominantly monofunctional adducts at A and C and to a lesser extent at G, which contrasts with the behavior of **1** [49]. This observation agrees with the hydration study of **3**, demonstrating that the mono-aqua species of **3** was found to be the most abundant hydration product [50][51]. It should be noted, however, that mass spectrometry studies used to analyze single-strand oligonucleotides indicate that both chloride ligands in **3** are lost on binding to DNA and under certain conditions the arene ligand may also be lost [34][35]. This difference may be attributed to both the different experimental conditions of the mass spectrometry experiment and to the more flexible nature of the oligonucleotide, which can more easily adapt to occupy the coordination sites of the Ru center.

Conclusions. – The present study demonstrates that substitution of two chloride ligands by the more stable cyclobutane-1,1-dicarboxylate ligand onto the RAPTA framework reduces the rate of DNA ruthenation with a similar effect to that observed with the analogous cisplatin-carboplatin pair. This observation has important implications in the clinical evaluation of the RAPTA series of compounds, in that if it proves necessary to reduce the general toxicity of the complexes, substitution of the labile chlorides for the cyclobutane-1,1-dicarboxylate anion may achieve the desired effect. The degree of DNA metalation observed for **3** and **2** are in a similar range, suggesting that if DNA damage is central to the mechanism of both compounds, the complexes would be active at similar doses. The reduced rate of DNA damage resulting from substituting the chloride ligands for the dicarboxylate ligand on the RAPTA framework may result in the need for an increased dose; however, with a lower general toxicity such higher doses may prove a more effective cancer treatment. Furthermore,

the two series of metal complexes exhibit different base-pair specificity and unwinding angles, opening the possibility of the Ru complexes overcoming certain mechanisms of drug resistance.

This work was supported in part by the research grants from the *National Research Council of Thailand* (to *A. R.*; grant no. 02011420-0003) and the *Thailand Research Fund* under the *Royal Golden Jubilee Ph.D. Program* (to *P. T.*). The authors are indebted to Dr. *Wee Han Ang* for providing the sample of carboRAPTA-C.

Experimental Part

Materials. RAPTA-C (**3**) and carboRAPTA-C (**4**) were prepared using literature protocols [30][36]. *Taq* DNA Polymerase, dNTP, and *Tris*-HCl were purchased from *New England Biolabs.* The nucleotide sequences of forward and reverse primers were obtained from *Invitrogen*; RT-PCR: forward primer, 5′-AGCAGGGAGAAGCCAGAATTG-3′ and reverse primer, 5′-TCAGTAGTGGCTGTGGGGGAT-3′; 696-bp *BRCA1:* forward primer, 5′-GGAATTCCATATGAGCAGGGAGAAG-3′ and reverse primer, 5′-ATTGGTTCTGCAGTCAGTAGTGGCT-3′. All other reagents were of highest purity grade.

Preparation of the 696-Base Pair (bp) *Breast Cancer Suppressor Gene 1* (*BRCA1*) *Fragment.* Messenger RNA (mRNA) was extracted from white blood cells using an mRNA isolation kit and biotinylated oligo-dT (*Qiagen*). The purified mRNA was used for complementary DNA (cDNA) synthesis and the amplification of the 696-bp *BRCA1* fragment (nucleotide 4897–5592) was performed with the *Qiagen OneStep RT-PCR Kit* (*Qiagen*). The RT-PCR mixture was prepared in a 1.5-ml microcentrifuge tube with a final volume of 50 µl, containing 5 µl reaction buffer, 400 µM of each dNTP, 0.6 µM of forward primer, 0.6 µM of reverse primer, *OneStep* RT-PCR enzyme mix (*Qiagen*), and RNase-free H_2O. The template RNA was finally added in order to initiate the PCR reaction using two-step thermal cycling. The first step comprised one cycle at 48° for 45 min, allowing the synthesis of the first strand cDNA by the action of reverse transcriptase. The reverse transcriptase was inactivated at 94° for 2 min. The second step included 40 cycles of denaturation at 94° for 30 s, annealing at 60° for 1 min, and extension at 68° for 2 min. The extension of the final cycle was expanded to 7 min. The amplified product was applied to a 1% agarose gel and subjected to electrophoresis at 80 V for 60 min. The gel was stained with ethidium bromide (EB), visualized under ultraviolet light (UV), and the DNA extracted with a gel extraction kit (*Qiagen*). The sequence of the purified 696-bp *BRCA1* fragment was subsequently verified using an automated DNA sequencer (*ABI PRISM™ 377 DNA Sequencer*) with a *PRISM™ Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit* (*Applied Biosystem*, USA).

In vitro *Ruthenation of the 696-bp BRCA1 Fragment.* The 696-bp *BRCA1* fragment (4.25 µg) was incubated with various concentrations of the Ru complexes (0–1000 µM) in 20 µl of double dist. H_2O at 37° for 24 h in the dark. Ru complex-treated DNA was precipitated with EtOH and centrifuged at 13,500*g* for 30 min at 4°. After drying, the DNA pellet was washed with 70% EtOH, dried in vacuum, and redissolved in 20 µl of double dist. H_2O. DNA Adducts were electrophoresed on a 1.5% agarose gel. The gel was stained with EB and visualized under UV light.

Restriction Analysis of Ruthenated-BRCA1 Adducts. The 696-bp *BRCA1* fragment (4.25 µg) was incubated with various concentrations of the Ru complexes (0–1000 µM) in 20 µl of double dist. H_2O at 37° for 24 h in the dark. Ruthenated DNA was EtOH precipitated and centrifuged at 13,500*g* for 30 min at 4°. After drying, the DNA pellet was redissolved in 20 µl of double dist. H_2O and incubated at 37° with *Eco*O109I and *Pvu*II for 5 and 6 h, resp. Restricted samples were electrophoresed on a 1% agarose gel. The gel was stained with EB and visualized under UV light.

Sequence Specificity of Ru-BRCA1 Adducts in vitro. Localization of Ru-*BRCA1* adducts was determined based on premature termination of DNA synthesis on a Ru-modified *BRCA1* template. The use of thermostable *Taq* DNA polymerase and repetitive cycling in a PCR machine provides the amplification of the signal from the damaged template [43]. The 696-bp *BRCA1* fragment (4.25 µg) was treated with **3** or **4** at a concentration of 500 µM at 37° for 24 h in the dark. The Ru complex-treated DNA or control (non-ruthenated) was precipitated with EtOH and centrifuged at 13,500*g* for 30 min at 4°.

After drying, the DNA pellet was resuspended in 20 µl of double dist. H_2O and the concentration of DNA was determined spectrophotometrically. Ru-treated DNA (200 ng) was mixed with *BigDye* terminator (*Applied Biosystem*, USA) in a PCR tube (20 µl) containing 5 pmol of forward primer (5′-GGAATTCCATATGAGCAGGGAGAAG-3′) or reverse primer (5′-ATTGGTTCTGCAGTCAG-TAGTGGCT-3′), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1 mM *Tris*-HCl (pH 8.3), and 1 unit of *Taq* DNA polymerase. The reactions were subjected to temp. cycling using a *Perkin-Elmer Model 9600* cycler. The DNA denaturation was initiated by rapid temp. rise up to 96° for 1 min, and three steps of PCR temp. for 25 cycles were then set as follows: 96° for 10 s, 50° for 5 s, and 60° for 4 min. The unincorporated *BigDye* terminator was removed by adding 80 µl of 70% ^{i}PrOH. The tube was centrifuged at 14,000*g* for 20 min at r.t. The supernatant was aspirated, 250 µl of 70% EtOH was added to the tube, and the tube was centrifuged at 14,000*g* for 5 min. The supernatant was aspirated, and the sample was dried in a heat block at 90° for 1 min. The sample was resuspended in 6 µl of loading buffer (deionized formamide (50 mM EDTA, pH 8.0)/blue dextran 1:5) and was heated at 90° for 2 min before incubation on ice. Aliquots of sample (1 µl) were loaded onto a 6% polyacrylamide/8M urea DNA-sequencing gel using an automated DNA sequencer (*ABI PRISM™ 377 DNA Sequencer*, *Applied Biosystem*, USA). The DNA synthesis on the template containing the Ru adducts produced DNA fragments migrating on the sequencing gel as intense bands, which corresponded to the termination sites of DNA synthesis.

Semi-Quantitative PCR (QPCR). Semi-quantitative PCR was used to assess the polymerase inhibiting effect of DNA ruthenation. The 696-bp *BRCA1* fragment (4.25 µg) was incubated with 10 µl of various concentrations of the appropriate Ru complex (0–1000 µM) at 37° for 24 h in the dark. The Ru complex-treated DNA was precipitated with EtOH and centrifuged at 13,500*g* for 30 min at 4°. After drying, the DNA pellet was resuspended in 20 µl of double dist. H_2O, and the concentration of DNA was determined spectrophotometrically. The PCR mixture (50 µl) contained 100 ng of Ru complex-treated DNA, 5 pmol of each forward and reverse primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1 mM *Tris*-HCl (pH 8.3), and 1 unit of *Taq* DNA polymerase. The PCR conditions were as follows: 3 min at 94°, 25 cycles of 30 s at 60°, 45 s at 72°, and a final extension for 10 min at 72°. The PCR product (20 µl) was separated by electrophoresis on a 1.5% agarose gel at 80 V for 100 min. The gel was stained with EB and the bands visualized with UV light.

Semi-Quantification of DNA Lesions. Product amplification was measured directly from agarose gels using a *Bio-Rad* molecular dynamics densitometer. The degree of amplification was represented by the units of absorbance of the products. Amplification inhibition data were converted to the lesion frequency using the *Poisson* distribution formula (*Eqn. 2*) with the assumption that one lesion in the target area was sufficient to eliminate the amplification of that template [41]:

$$S = -\ln(A_d/A) \quad (2)$$

where S is the lesion frequency/strand, A the absorbance units produced from a given amount of non-damaged DNA template, and A_d the absorbance unit produced from a given amount of damaged DNA template (damaged by a particular dose of Ru complex). Therefore, A_d/A is the fraction of non-damaged template at a given dose.

Ru-Treatment of pBIND DNA. Plasmid pBIND DNA (6360 bp) (*Promega*, USA) was transformed into *Escherichia coli* DH5*α*. Transformed *E. coli* cells were grown in *Luria* broth medium with 50 µg/ml ampicillin at 37° for 16 h. The cells were harvested and lyzed. The plasmid DNA was purified as described previously [52]. Samples of pBIND plasmid (4 µg) were incubated with the Ru complex in 20 µl of double dist. H_2O at various molar ratios of Ru per DNA nucleotide (r_b) at 37° for 24 h in the dark. Ru-treated DNA was precipitated with EtOH and centrifuged at 13,500*g* for 30 min at 4°. After drying, the DNA pellet was washed with 70% EtOH, dried in vacuum, and redissolved in 20 µl of double dist. H_2O. The DNA adducts were electrophoresed on a 0.8% agarose gel. The gel was stained with EB and visualized under UV light.

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Received September 17, 2009