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RAPID COMMUNICATION

Stabilization of *MYC* G-quadruplex DNA by ruthenium (II) complex overcomes imatinib resistance in chronic myeloid leukemia cells harboring T315I mutation

The key pathogenesis of chronic myeloid leukemia (CML) is the formation of *BCR-ABL* fusion gene, encoding a 210 kDa Bcr-Abl tyrosine kinase, which is crucial for the occurrence and development of CML. Imatinib (IM) is the first targeted anticancer drug approved by FDA for the treatment of CML; however, some patients, especially those in accelerated phase and blastic phase, develop primary or secondary drug resistance to IM. Particularly, the most challenging resistance is caused by T315I mutation of Bcr-Abl, which represents approximately 15%–20% of all acquired mutations and renders cell resistant to a variety of tyrosine kinase inhibitors.[1,2] Thus, there is an urgent need to develop novel strategies to overcome Bcr-Abl T315I-meidated IM resistance.

We have previously reported that arene ruthenium (II) complexes (η6-C6H6)Ru(p-BrPIP)Cl]Cl (referred to as RuBr, Fig. 1A) and its analogue (η6-C6H6)Ru(p-ClPIP)Cl]Cl (referred to as RuCl, Fig. S1A) showed a potent antitumor activity.[3] To demonstrate whether arene ruthenium (II) complexes could overcome IM resistance, we detected their effects on a set of CML cell lines, including IM-sensitive cell lines (e.g., KBM5 and BaF3-p210) and their IM-resistant counterparts (e.g., KBM5-T315I and BaF3-p210-T315I). We found that RuBr and RuCl showed potent cytotoxic activity against these CML cell lines (Fig. 1B; Fig. S1B). Since RuBr is relatively stronger than RuCl, we selected RuBr for the follow-up study. Importantly, the sensitivity of primary monocytes from IM-sensitive and -resistant CML patients to RuBr was higher than that from healthy volunteers (Fig. 1C, D). These findings suggest that RuBr inhibits cell viability of IM-sensitive and -resistant CML cells.

Next, we evaluated the ability of RuBr to induce cell death in CML cells harboring wild-type and T315I mutant Bcr-Abl using annexin V-FITC/PI assay. We observed that RuBr induced apoptosis in IM-sensitive and -resistant CML cell lines, as well as primary CML cells in a dose-dependent manner (Fig. 1E–G; Fig. S1C–G). Consistently, RuBr triggered caspases activation in these CML cells (Fig. 1H, I; Fig. S1H, I). As expected, z-VAD-fmk (a pan-caspase inhibitor) reversed RuBr-induced cleavage of caspase-8 and PARP in KBM5 and KBM5-T315I cells (Fig. S2A). Mitochondria is believed to be the center of cell apoptosis. The release of cytochrome C and AIF from mitochondria to cytoplasm induced by loss of mitochondrial membrane potential constitutes a decisive step of the mitochondrial apoptotic process. The released pro-apoptotic factors cytochrome C and AIF can induce caspase activation by forming apoptosome complex or directly trigger DNA fragmentation. We found that RuBr triggered loss of mitochondrial membrane potential in IM-sensitive and -resistant CML cells (Fig. S2B, C). In addition, an increase in cytosolic cytochrome C and AIF were observed after RuBr treatment in KBM5 and KBM5-T315I cells (Fig. S2D), indicating that RuBr activated the mitochondrial apoptosis pathway. To further clarify the mechanism of RuBr-induced apoptosis, we examined the expression of apoptosis-related proteins. As shown in Figure S2E, RuBr decreased the expression of anti-apoptotic proteins, including Bcl-2, survivin, Mcl-1 and XIAP. These findings suggest that RuBr induces caspase-dependent apoptosis in both IM-sensitive and -resistant CML cells.

Bcr-Abl and its downstream signaling pathways have a broad influence on cell survival and apoptosis of cancer cells. For instance, Bcr-Abl and its downstream Stat5 has been reported to be involved in the transcription of apoptosis associated genes (e.g., Bcl-2, survivin, Mcl-1, and XIAP). Thus, we set out to investigate the role of Bcr-Abl in

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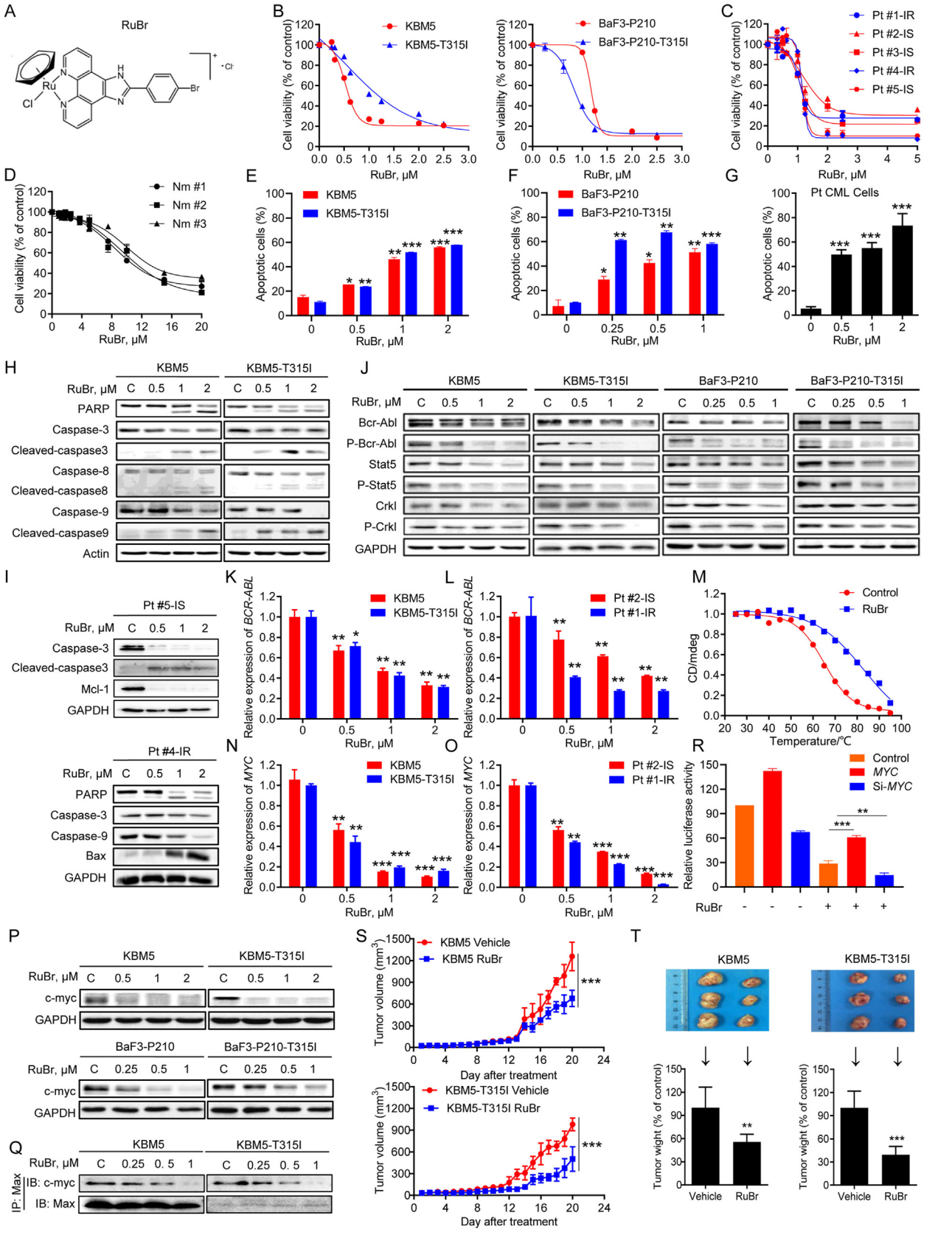

Figure 1 RuBr induces cell apoptosis and downregulation of Bcr-Abl through targeting *MYC* G-quadruplex in IM-sensitive and -resistant CML cells. **(A)** The chemical structure of RuBr. **(B)** KBM5 and KBM5-T315I cells, or BaF3-p210-WT and BaF3-p210-T315I cells were exposed to RuBr in various concentrations for 48 h, followed by MTS assay. **(C, D)** Cells from 5 CML patients (#2/#3/#5: IM-sensitive patients; #1/#4: IM-resistant patients) and 3 healthy volunteers (Nm #1/#2/#3) were exposed to RuBr for 48 hours,

RuBr-mediated toxicity in CML cells. First, we examined the expression and activation of Bcr-Abl kinase after RuBr treatment. We found that RuBr downregulated the total protein level and phosphorylated protein level of Bcr-Abl in IM-sensitive and -resistant CML cells in a dose- and time-dependent manner (Fig. 1J; Fig. S3A, B). Meanwhile, RuBr inhibited the phosphorylation of downstream target proteins of Bcr-Abl, including Stat5 and Crkl (Fig. 1J; Fig. S3A, B). Next, we tested the effect of RuBr on the transcription activity of *BCR-ABL* gene by using HeLa cells transfected with plasmids encoding *BCR-ABL* gene promoter-Luc and Rellina-Luc. The results indicated that RuBr significantly inhibited the transcription activity of *BCR-ABL* gene in a dose-dependent manner (Fig. S3C). We questioned whether RuBr-induced Bcr-Abl downregulation through transcriptional repression of *BCR-ABL*. RuBr-treated CML cells were subjected to RT-qPCR analysis. We found that RuBr downregulated Bcr-Abl expression at the transcriptional level in IM-sensitive and -resistant CML cell lines (Fig. 1K; Fig. S3D) and primary CML cells (Fig. 1L). Collectively, these findings demonstrate that RuBr induces the downregulation of Bcr-Abl and its downstream events.

Ruthenium (II) complexes have been reported to stabilize G-quadruplex of *MYC* (encoding c-MYC protein), a well-known transcription of *BCR-ABL* gene.[4] The G-quadruplex always function as a transcriptional repressor element.[5] Thus, we wondered whether RuBr was capable to directly stabilize *MYC* or *BCR-ABL* G-quadruplex. CD spectra were used to monitor the stabilizing behavior of RuBr with G-quadruplex DNA. We found that RuBr was able to bind with the G-quadruplex of *MYC* DNA (Fig. 1M), but not that of *BCR-ABL* DNA (Fig. S4A). In addition, we performed molecular docking study to investigate the binding information between the complex RuBr and 6 typical G-quadruplex DNAs (*MYC*, *KRAS*, *VEGF*, *BCL2*, *KIT*, and *RET*). This assay showed that the predicted binding capacity of RuBr to *MYC* G-quadruplex DNA was the strongest among the 6 docking modes, with the lowest binding energy of −7.99 kcal/mol (Table S1). Accordingly, RuBr significantly inhibited the mRNA of *MYC* (Fig. 1N, O; Fig. S4B) and protein levels of c-Myc (Fig. 1P; Fig. S4C, D). Similarly, RuCl also inhibited the mRNA levels of *MYC* and *BCR-ABL* (Fig. S4E, F). c-Myc binds with Max to form a heterodimer, which is critical for the transcription of Bcr-Abl.[4] As expected, we observed a decrease in c-Myc and Max interaction in KBM5-T315I cells treated with RuBr (Fig. 1Q). In addition, the transcription levels of two c-Myc target genes *ODC1* and *CDK4* were decreased by RuBr treatment in KBM5 and KBM5-T315I cells (Fig. S4G). Importantly, overexpression of *MYC* inhibited, whereas knockdown of *MYC* enhanced RuBr-mediated effect on *BCR-ABL* transcriptional activity (Fig. 1R). Likewise, 10053-F4, a c-Myc/Max inhibitor, enhanced RuBr-mediated downregulation of Bcr-Abl (Fig. S4H). Thus, these findings confirm that RuBr suppresses Bcr-Abl expression through downregulating *MYC* in IM-sensitive and -resistant CML cells.

We also constructed a xenograft tumor model in nude mice to clarified whether RuBr inhibited the growth of CML cells *in vivo*. RuBr was intraperitoneally given to nude mice bearing KBM5 and KBM5-T315I xenografts at a dose of 10 mg/kg/day. The results showed that, compared with the control group, RuBr significantly inhibited the tumor growth (Fig. 1S) and decreased the tumor weight (Fig. 1T) of KBM5 and KBM5-T315I xenografts. However, mice weight did not change significantly (Fig. S5A), indicating RuBr is well tolerated. The results of mice blood test showed that RuBr had no obvious hepatotoxicity (as indicated by the ALT and AST levels) and nephrotoxicity (as indicated by the creatinine [Cr] levels) in nude mice (Fig. S5B—D). Similar to the *in vitro* study, c-Myc, Bcr-Abl and p-Bcr-Abl, as well as its downstream p-Stat5 and p-Crkl were downregulated by RuBr treatment in tumor tissues (Fig. S5E). Moreover, immunohistochemical analysis of tumor tissues confirmed that Ki67, Bcr-Abl, and c-Myc were decreased by RuBr treatment, while cleaved caspase-3 was significantly increased by RuBr addition (Fig. S5F). These data illustrate that RuBr is effective in overcoming IM resistance of CML cells *in vivo*.

followed by MTS assay. **(E—G)** RuBr induces cell apoptosis in CML cells. KBM5 and KBM5-T315I cells (E), BaF3-p210-WT and BaF3-p210-T315I cells (F), or primary CML cells (G) were treated with different concentrations of RuBr for 24 h, and apoptotic cells were detected by Annexin V-FITC/PI double staining with flow cytometry. Mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus control group. **(H, I)** RuBr induces the activation of caspase-3, -8, and -9. KBM5 and KBM5-T315I cells (H) or primary CML cells (I) were exposed to the indicated doses of RuBr for 24 h, and then the cleavage of PARP and/or caspases were detected with Western blot. Pt #4-IR: IM-resistant CML patient; Pt #5-IS: IM-sensitive CML patient. **(J)** CML cells were exposed to RuBr and then harvested for Western blot analyses for the indicated proteins. **(K—L)** KBM5 and KBM5-T315I cells (K) or primary CML cells (L) were exposed to various concentration RuBr for 6 h, and then the relative mRNA level of *BCR-ABL* was detected with qRT-PCR. Mean ± SD ($n = 3$). $^{*}P<0.05$, $^{**}P<0.01$, versus control group. Pt #1-IR: IM-resistant CML patient; Pt #2-IS: IM-sensitive CML patient. **(M)** Typical CD melting curves of c-Myc promoter element Pu22 DNA (2 μM) with or without RuBr (10 μM) in 10 mM Tris-HCl and 2 mM KCl buffer (pH 7.2), which was conducted at 263 nm. **(N, O)** KBM5 and KBM5-T315I cells (N) or primary CML cells (O) were exposed to various concentration RuBr for 6 h, then the relative mRNA level of *MYC* was detected with qRT-PCR. Mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$, versus control group. Pt #1-IR: IM-resistant CML patient; Pt #2-IS: IM-sensitive CML patient. **(P)** KBM5 and KBM5-T315I cells or BaF3-p210-WT and BaF3-p210-T315I cells were exposed to RuBr and then harvested for Western blot analyses for c-Myc. **(Q)** Endogenous Max were immunoprecipitated from CML cells using an anti-Max antibody, and co-immunoprecipitated c-Myc proteins were detected using Western blot. **(R)** The transcriptional activity of *BCR-ABL* was analyzed by dual-luciferase activity assay. HeLa cells were transfected with Bcr-Abl gene promoter-Luc, Rellina-Luc, when *MYC* was overexpressed or knocked down, and then exposed to 2 μM RuBr for 24 h. Mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control. **(S, T)** *In vivo* effect of RuBr on KBM5 and KBM5-T315I cell-derived mouse xenograft models. Nude mice bearing KBM5 and KBM5-T315I cells were exposed to vehicle or RuBr (10 mg/kg/d) for 15 days. Tumor volume curves (S) were plotted against days post CML cell inoculation. On day 20 after inoculation, the mice were sacrificed and the tumor weights were assessed (T). Mean ± SD ($n = 6$). $^{**}P < 0.01$, $^{***}P < 0.001$, versus control group.

In summary, this work shows that ruthenium (II) complex RuBr has potent inhibitory effect on IM-resistant CML cells harboring Bcr-Abl-T315I through binding and stabilizing G-quadruplex of *MYC* (Fig. S6). These findings provide insights into a novel therapeutic strategy for overcoming IM resistance through targeting G-quadruplex of *MYC* and broaden the application of ruthenium complexes.

Ethics declaration

The study was approved by the Ethics Committee of Guangzhou Medical University. All healthy volunteers and patients who provided clinical specimens signed the written informed consent form.

Author contributions

YS performed the experiments and analyzed the data. XC, SL, AL, LJ, HZ, and WD performed the experiments. XC and WM interpreted the data and revised the manuscript. XS and JL designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.08.019.

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