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ORIGINAL ARTICLE

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Investigation of the role of Bax, p21/Waf1 and p53 as determinants of cellular responses in HCT116 colorectal cancer cells exposed to the novel cytotoxic ruthenium(II) organometallic agent, RM175

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Abstract Ruthenium(II) organometallic complexes form monofunctional adducts with guanine in DNA in vitro and have a cytotoxic anticancer activity spectrum in preclinical models suggesting lack of cross-resistance with cisplatin. The primary cytotoxic lesion remains to be identified but the downstream mechanism of action is nevertheless of interest. Using isogenic derivatives of the HCT116 colorectal cancer cell line, we investigated the role of p53, p21/WAF1 and Bax in the cellular response to the novel ruthenium(II) organometallic complex RM175, $[(\eta^6\text{-}C_6H_5C_6H_5)RuCl\ (H_2NCH_2CH_2NH_2\text{-}N,N')]^+\ PF_6^-$. Western blotting demonstrated dose-dependent accumulation of p53, Bax and p21/WAF1 within 48 h of the start of RM175 treatment in wild-type HCT116 cells. HCT116 wild-type and Bax-null cells arrested in the G_1 and G_2 phases of the cell cycle. This pattern of cell cycle arrest was not observed in p53-null or in p21/WAF1-null cells. Following RM175 treatment, HCT116 wild-type and p21/WAF1 null cells underwent a dose-dependent induction of apoptosis (Annexin-V and sub-G_1 apoptosis assays). This apoptotic response was not observed in p53-null or Bax-null cells. In short-term sulphorhodamine B assays, the IC_{50} for RM175 was 16 μM for p53-null HCT116, and 8 μM for wild-type cells ($P < 0.05$). However, the sensitivity to RM175 in clonogenic assays at 16 days was independent of p53 status. These results identify determinants of the short-term in vitro response to RM175 demonstrating a role for p53 and p21/WAF1 in the growth arrest and for p53 and Bax in the apoptotic response. The mechanism of p53-independent suppression of long-term clonogenicity remains to be determined.

Keywords Apoptosis · Ruthenium · RM175 · Colorectal cancer

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Introduction

Ruthenium(II) organometallic complexes form monofunctional adducts with guanine when reacted with DNA in vitro and show cytotoxic anticancer activity in preclinical models. These agents have a spectrum of activity suggesting that they do not share cross-resistance mechanisms with cisplatin [1–5]. The primary cytotoxic lesion remains to be identified but the downstream mechanisms of action are nevertheless of interest. Prediction or enhancement of tumoricidal effect, based on knowledge of cellular response determinants, could improve the clinical utility of this class of cytotoxic agent.

Members of the p53 tumour suppressor gene family are implicated in the response of cancer cells to many cytotoxic agents [6–8]. For example, in mismatch repair-deficient HCT116 colorectal cancer cells, disruption of p53 expression confers resistance to oxaliplatin, but increased sensitivity to cisplatin [9]. In general, two competing p53-mediated responses to cytotoxics are observed, namely cell death and cell cycle arrest [10, 11]. Several genes have been identified which regulate these competing responses in a manner dependent on the nature of the cytotoxic insult. Thus abrogation of cell cycle arrest by knockout of p21/WAF1 or 14-3-3σ genes in HCT116 cells can alter the balance of cellular response to doxorubicin from arrest to mitotic death [12–15], via a p53-independent pathway. In contrast, 5-fluorouracil (5FU) induces apoptosis in a p53-dependent manner via the mitochondrial apoptotic pathway, despite intact cell cycle checkpoint machinery [11, 16]. The mitochondrial apoptotic pathway is regu-

lated in turn by members of the Bcl-2 gene family. The effect of p53 disruption on chemosensitivity can therefore depend on the nature of the cytotoxic drug and on the status of cell cycle checkpoints and apoptotic signalling pathways. The role of genetic drug response determinants thus depends on the genetic context [17]. Therefore, the study of such determinants is best performed in cells with a well-defined isogenic background.

Redundant proapoptotic signalling mechanisms transcriptionally regulated by p53 include Bik, Bak, and PUMA, as well as Bax [18]. Each of these proapoptotic Bcl-2 family proteins localize to the mitochondria, and heterodimerize through a BH3 domain with antiapoptotic Bcl-2 family members, such as Bcl-xl [19, 20]. The ratio of pro- to antiapoptotic Bcl-2 family members determines the threshold for induction of mitochondrial dependent apoptosis [21–24]. For example, Bax-null cells are profoundly resistant to apoptosis driven by nonsteroidal antiinflammatory drugs (NSAID), and are partly resistant to 5FU-driven apoptosis [25].

Using derivatives of the HCT116 colorectal cancer cell line, we investigated the role of p53, p21/WAF1 and Bax in the cellular response to the novel ruthenium(II) organometallic complex RM175 $[(\eta^6\text{-}C_6H_5C_6H_5)RuCl(H_2NCH_2CH_2NH_2\text{-}N,N')]^+ PF_6^-$. HCT116 colorectal cancer cells carry wild-type p53, p21/WAF1 and Bax, but are deficient for mismatch repair by virtue of silencing of hMLH1 expression. The three derivative cell lines used, referred to here as p53-null, p21/WAF1-null and Bax-null, were each generated by targeted homologous recombination, rendering them null for expression of p53, p21/WAF1 and Bax protein, respectively [25, 26].

Materials and methods

Cell lines and tissue culture

The parental wild-type HCT116 colorectal cancer cell line (HCT116-wt) was obtained from the European Collection of Cell Cultures. This line was selected for study because of the availability of several well-defined derivative cell lines. The p53-null, p21/WAF1-null and Bax-null derivatives of the wild-type HCT116 cell line were gifts from Prof. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, Md.), and immunoblots confirmed the expected lack of expression of p53, p21/WAF1 and Bax protein, respectively [27, 28]. Cell lines were maintained in RPMI 1640 with 10% fetal calf serum and 1% penicillin/streptomycin (all from Life Technologies) and grown at 37°C in a fully humidified incubator under an atmosphere containing 5% CO_2, and passaged once or twice weekly.

RM175 treatment

RM175 (Fig. 1), synthesized as described previously [2], was dissolved in sterile distilled water at 0.5 mg/ml and used fresh. Treatment solutions were made by serial dilution in growth medium. Drug-containing medium was incubated with cells for 24 h then replaced with drug-free medium.

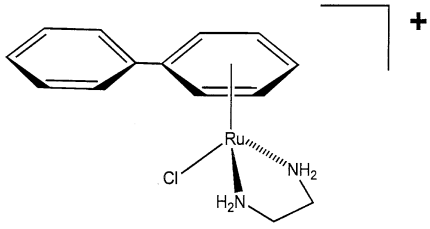

Fig. 1 Structure of the cation in RM175 $[(\eta^6\text{-}C_6H_5C_6H_5)RuCl(H_2NCH_2CH_2NH_2\text{-}N,N')]^+ PF_6^-$

IC_{50} by sulphorhodamine B assay

Drug concentrations that inhibit 50% of cell growth (IC_{50}) were determined using the sulphorhodamine B technique [29]. Cells were plated on day 1 in 96-well plates. The cell density was 2500 cells/well in a volume of 150 µl/well. The cell density was chosen to have a cell growth latency of 24 h and an optical density at the end of the experiment that was greater than 1.5 in the control wells. In each plate, one column contained control cells, and five columns contained increasing concentrations of drugs. All cell lines were treated on day 2. After drug exposure, cells were washed once with cold PBS and placed in 200 µl of drug-free medium for 72 h after the end of drug exposure. The cells were then precipitated with 50 µl of ice-cold 50% trichloroacetic acid for 60 min at 4°C, rinsed six times with water and air-dried. Fixed cells were stained with 50 µl of 0.4% of sulphorhodamine B solution in 0.1% acetic acid, rinsed with 0.1% acetic acid solution and air-dried. Sulphorhodamine B was redissolved with 150 µl of 10 m *M* Tris buffer, pH 10.5. Optical densities were measured at 540 nm with a Biohit BP-800 spectrophotometer (Bio-Hit, Helsinki, Finland). Two independent experiments, each in triplicate, were performed. Growth inhibition curves were plotted as percentage of control cells and IC_{50} values were determined with GraphPad Prism 3 software (San Diego, CA) using sigmoidal curve fitting with variable slope. The goodness of fit determined by r^2 was greater than 0.9 and the Hill coefficient less than −1.

Growth assays by Coulter counting

HCT116-wt or p53-null cells were plated in triplicate in six-well trays for 2 days and then exposed to RM175-containing medium. Wells were replenished with fresh drug-free medium after 24 h of drug exposure. Adherent cells were washed with PBS 48 h after the start of treatment and harvested in trypsin/EDTA (Life Technologies) for 3 min. Growth medium was then added, and single-cell suspensions were made by three passages through a

22-gauge needle. Aliquots of 200 μl were added to 9.8 ml 0.9% saline and 0.5-ml samples counted in a Z2 Coulter counter. Growth curves were defined by the proportion of treated cells surviving relative to untreated control cells.

Clonogenic assays

For each cell line between 300 and 600 cells were plated overnight in 25-cm^2 flasks, then incubated for 24 h with a range of RM175 concentrations. Drug-containing medium was then replaced with fresh medium and the flasks were incubated for a further 16–18 days without disturbance. Visible colonies were washed, fixed (2 min in 2 ml of 2:1 acetone/methanol), stained with haematoxylin and counted.

Immunoblots for actin, p53, p21/WAF1, Bax

Adherent cells were harvested and counted, pelleted at 13,000 rpm at 4°C, washed with cold PBS and stored at −20°C until use. Cell pellets were resuspended in 100 μl lysis buffer (50 m *M* Hepes, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 150 m *M* sodium chloride, 5 m *M* EDTA with protease inhibitors pepstatin A 2 μg/ml, aprotinin 10 μg/ml, leupeptin 10 μg/ml and PMSF 100 μg/ml) per million cells and mixed for 25 min at 4°C. After centrifugation at 13,000 rpm at 4°C, lysate supernatants were subjected to a Bradford assay for protein concentration. Aliquots of total protein (20 μg) were denatured in loading buffer at 95°C for 5 min, electrophoresed (35 min 60 mA, then about 180 min 35 mA) on a 10% SDS-PAGE gel and transferred overnight (30 V cold) to a polyvinylidene fluoride (PVDF) Immobilon-P transfer membrane (Millipore). Protein loadings were checked by Ponceau staining. Membranes were blocked in 5% fat-free milk powder (Marvel, Premier Brands) in Tris-buffered saline (pH 7.5) with 0.1% Tween-20 (TBS-T) for 1 h at room temperature (RT) and cut as appropriate, incubated with primary antibody in 5% Marvel in TBS-T (1 h at RT for actin, p53 and Bax, or overnight at 4°C for p21/WAF1), washed again three times in TBS-T and twice in 5% Marvel/TBS-T, incubated with secondary antibody conjugated to horseradish peroxidase (1 h at RT), and washed five times in TBS-T and twice in TBS. Blots were visualized by chemiluminescence (Santa Cruz Biotechnology) and photographed (exposures determined by strength of signal). Actin immunoblots were used to confirm equal protein loading per lane.

Antibodies and concentrations used were: mouse ascites monoclonal anti-actin IgM CP01 at 1:120,000; mouse monoclonal anti-p53 IgG OP43 at 1:200; mouse monoclonal anti-p21/WAF1 IgG OP64 at 1:100 (all Oncogene Research Products); and rabbit polyclonal anti-Bax IgG sc-493 at 1:500 (Santa Cruz Biotechnology). Secondary antibodies were goat anti-mouse IgM 401225 at 1:4000 (Calbiochem); goat anti-rabbit IgG sc-2030 at 1:2000, and goat anti-mouse IgG sc-2031 at 1:2000 (both Santa Cruz Biotechnology).

Cell cycle analysis

One million cells were washed in cold PBS, fixed in 70% ethanol in PBS for at least 1 h on ice, washed, resuspended in PBS containing 25 μg/ml propidium iodide (PI) and 100 μg/ml ribonuclease A (Sigma) and incubated for 30 min at 37°C. Fluorescence was measured on a Becton Dickinson FACSCalibur flow cytometer (excitation 488 nm, measurement 564–607 nm) within 1 h. Data were analysed using the MODFIT 2.0 program (Verity Software). For the demonstration of growth arrest, adherent cells only were harvested, and the mean and standard errors for the percentage of cells in each phase of the cell cycle were derived from at least three independent experiments, each in duplicate. For demonstration of induction of a sub-G_1 peak during apoptosis, adherent and floating cells were pooled.

Annexin-V assays

One million adherent cells were harvested, washed in cold PBS and resuspended in 100 μl 1× Annexin-V binding buffer containing 5 μg/ml PI and 0.5 μg/ml Annexin-V-FITC, incubated for 15 min at RT in the dark and diluted in 400 μl 1× Annexin-V binding buffer (R&D Systems). Fluorescence was measured on a Becton Dickinson FACSCalibur flow cytometer (excitation 488 nm, measurement 515–545 nm) within 1 h. The collected events were gated on the forward and side scatter plots to exclude cellular debris. Three discrete cell populations were identified, using standard cut-offs in each experiment, namely viable (unstained), early apoptotic (Annexin-V but no PI staining) and late apoptotic/necrotic (Annexin-V and PI staining). The mean and standard errors for the proportion of cells in the early stages of apoptosis were thus derived from at least three independent experiments, each in duplicate.

Statistical analysis

The significance of differences between experimental conditions was determined using the two-tailed Student's *t*-test.

Results

The impact of p53 status on short-term cytotoxicity and clonogenicity following treatment with RM175

The p53-null HCT116 cells were twofold more resistant than HCT116-wt cells to short-term RM175-mediated growth suppression whether assayed by direct cell

counts or by sulphorhodamine B assay (Figs. 2 and 3, $P < 0.05$ at every point across the dose range in both figures). The IC_{50} at 96 h (from the start of RM175 treatment) by the sulphorhodamine B assay for p53-null cells was 16 μM (95% confidence interval 13–20 μM), whereas for wild-type HCT116 cells it was 8 μM (95% confidence interval 6–10 μM). Direct counts of cell numbers at 48 h also demonstrated twofold resistance in the p53-null cells (Fig. 3). We attribute the difference in the absolute sensitivity measured by these two methods (counts at 48 h compared to sulphorhodamine B assay at 96 h) to the fact that the assay at 48 h is more sensitive to the continuing survival of growth-arrested cells.

In contrast to these short-term results, in longer-term clonogenic assays (colonies counted on day 16–18) no statistically significant differences in RM175-induced loss of clonogenicity were apparent between the HCT116-wt and the p53-null cell lines (Fig. 4).

RM175 induces a G_1 and G_2 growth arrest in a p53- and p21/WAF1-dependent manner

Experimental time-points quoted hereafter are relative to time 0, the start of drug treatment. Immunoblots demonstrated that RM175 induced accumulation of p53 and the cell cycle inhibitor p21/WAF1 at 24 and 48 h (Fig. 5a, b) and Bax at 48 h (Fig. 5c). Cell cycle analysis demonstrated a mixed G_1 and G_2 growth arrest by 48 h in HCT116-wt ($P = 0.007$ and $P = 0.029$ for G_1 and G_2, respectively) and Bax-null cells ($P = 0.043$ and $P = 0.001$ for G_1 and G_2, respectively). The G_1 and G_2 growth arrest was not observed in p53-null or in p21/WAF1-null cells (all G_1 and G_2 changes not significant). Therefore, the mixed growth arrest was p53- and p21/WAF1-dependent, but independent of Bax (Fig. 6).

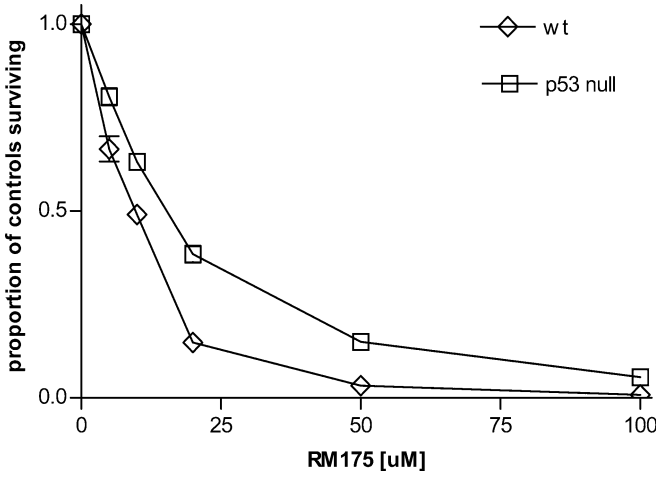

Fig. 2 RM175 induced p53-dependent cytotoxicity in short-term growth assays assessed by the sulphorhodamine B assay. HCT116-wt or p53-null cells were treated in triplicate with RM175 as indicated. Wells were replenished with fresh drug-free medium at 24 h. Surviving adherent cell numbers were assayed 72 h after the end of treatment by sulphorhodamine B staining. The number of cells surviving in drug-treated wells was expressed as a fraction of the average number of cells surviving in wells untreated with drug and IC_{50} values were determined using GraphPad Prism 3 software (San Diego, Calif.) using sigmoidal curve fitting with variable slope

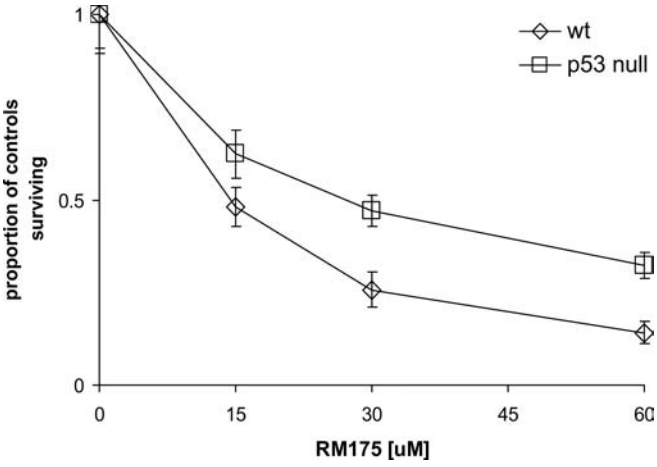

Fig. 3 RM175 induced p53-dependent cytotoxicity in short-term growth assays assessed by Coulter counting. HCT116-wt or p53-null cells were treated in triplicate with RM175 as indicated. Wells were replenished with fresh drug-free medium at 24 h. Adherent cells were harvested 48 h after the start of treatment and counted. The number of cells surviving in drug-treated wells was expressed as a fraction of the average number of cells surviving in wells untreated with drug

RM175 induces apoptosis in a p53- and Bax-dependent manner

Immunoblots demonstrated that RM175 had induced accumulation of p53 and Bax by 48 h (Fig. 5a, c).

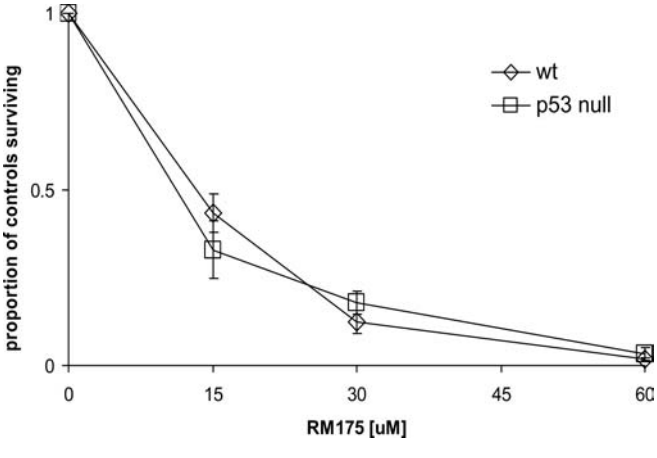

Fig. 4 p53-independent RM175 cytotoxicity in clonogenic assays. Cells plated overnight at low density (300–600 cells per 25-cm^2 flask) were treated in triplicate with the indicated concentration of RM175. Flasks were replenished with drug-free medium after 24 h. Visible colonies were fixed 16 to 18 days later, stained and counted. The surviving proportion of colonies was expressed relative to the untreated controls. The graph displays mean ± standard error for three independent experiments

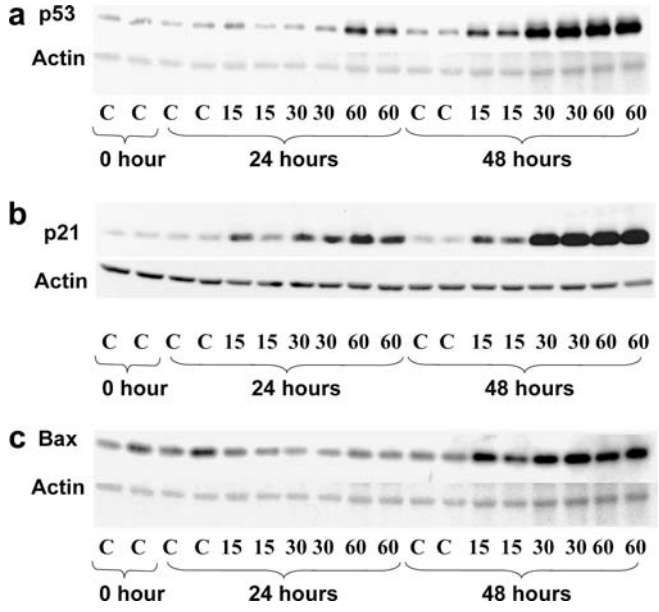

Fig. 5 Induction of p53, p21/WAF1 and Bax following RM175 treatment. Lysates from HCT116-wt cells treated with 0, 15, 30 or 60 μM RM175 were subjected to Western immunoblot analysis for actin, p53, p21/WAF1 or Bax. Blots were visualized using a chemiluminescence assay and photographed. Ponceau staining and the results for actin chemiluminescence confirmed near-equal protein loadings

Annexin-V apoptosis assays demonstrated the induction of increased apoptosis (relative to untreated cells) within 24 h of the start of treatment in HCT116-wt cells ($P < 0.05$ at 30 and at 60 μM) and HCT116 p21/WAF1-null cells ($P < 0.05$ across the dose range). On the other hand, no significant increase in apoptosis was induced in p53-null or Bax-null cells (Fig. 7). A sub-G_1 peak appeared in HCT116-wt and in p21/WAF1 null cells treated with RM175, consistent with the onset of internucleosomal DNA cleavage in late apoptosis [30]. No sub-G_1 peak appeared in p53-null or Bax-null cells (Fig. 8). Thus p53 and Bax are required to mediate RM175-induced apoptosis within the first 48 h of treatment.

Discussion

Using the wild-type HCT116 cell line and its p53-null, p21/WAF1-null and Bax-null derivatives, we identified genetic determinants of the early (within 48 h) cellular response to the novel cytotoxic ruthenium complex RM175. As the derivative lines were each generated in a specific and well-characterized fashion from the parental HCT116-wt line, they allowed the study of genetic determinants of the effect of RM175 on an otherwise isogenic background, and therefore in a well-controlled fashion.

We demonstrated that RM175 induced the accumulation of p53, the cell cycle inhibitor p21/WAF1 and the proapoptotic Bcl-2 family member Bax. The cell cycle response to RM175 in HCT116-wt cells was a mixed G_1 and G_2 growth arrest which was fully developed by 48 h. As expected, the G_1 component of growth arrest was p53- and p21/WAF1-dependent. RM175 also induced apoptosis in a p53- and Bax-dependent manner, indicating the involvement of p53 and Bax in the relevant apoptotic pathway. Despite these short-term p53-dependent effects, longer-term clonogenic assays demonstrated no dependence of RM175 cytotoxicity on p53

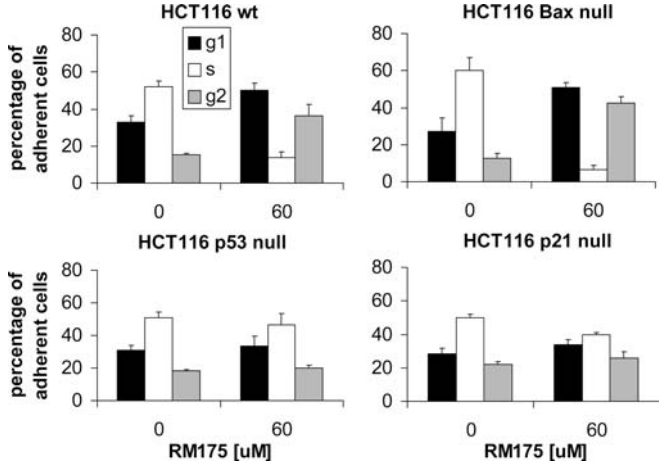

Fig. 6 RM175 induced a mixed G_1 and G_2 growth arrest in HCT116-wt. The G_1 component was p53- and p21/WAF1-dependent. Cells were treated in triplicate with RM175 as indicated. Wells were replenished with fresh drug-free medium at 24 h. Adherent cells were harvested 48 h after the start of treatment and cell cycle distribution determined by flow cytometry following ethanol fixation, ribonuclease A treatment and PI staining. The mean and standard errors for the proportion of cells in the G_1, S or G_2 phases of the cell cycle were derived from three independent experiments

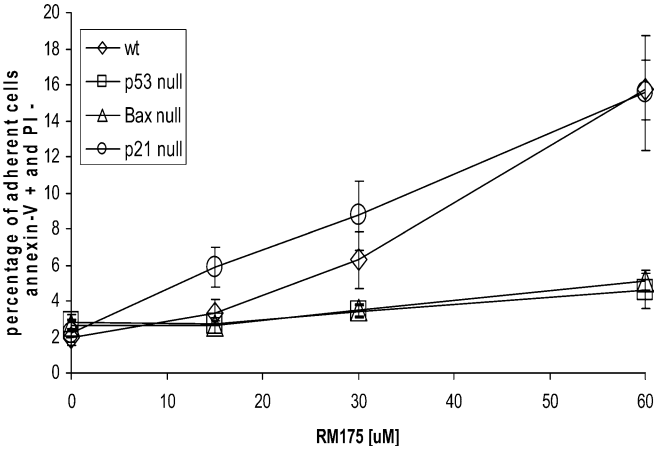

Fig. 7 RM175 induced apoptosis in HCT116-wt, HCT116-ch3 and HCT116 p21-null cells, but not in HCT116 p53-null or HCT116 Bax-null cells. Cells were treated with RM175 as indicated. After 24 h 1 million adherent cells were harvested and incubated in Annexin-V binding buffer containing PI and Annexin-V-FITC. Fluorescence was measured on a flow cytometer within 1 h. The mean and standard errors for the proportion of cells in the early stages of apoptosis (Annexin-V but no PI staining) were derived from at least three independent experiments

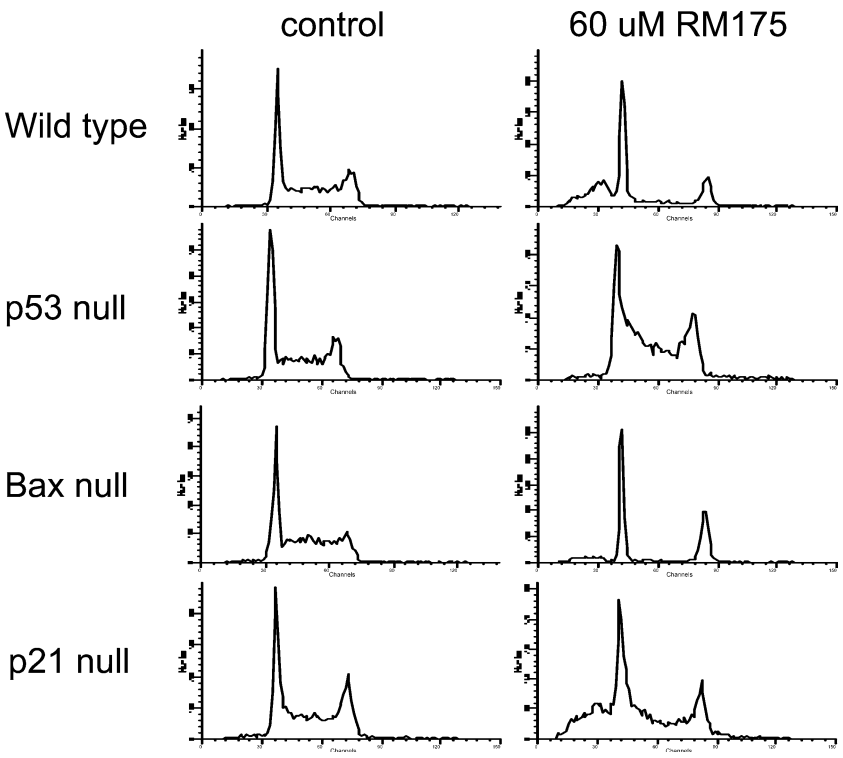

Fig. 8 RM175 induced a sub-G_1 peak in HCT116-wt and p21-null cells. The sub-G_1 component was p53- and Bax-dependent. Cells were treated in triplicate with RM175 as indicated. Wells were replenished with fresh drug-free medium at 24 h. Adherent and floating cells were harvested 48 h after the start of treatment and the cell cycle distribution determined by flow cytometry following ethanol fixation, ribonuclease A treatment and PI staining. Representative histograms are shown

status. Thus the currently unidentified primary cytotoxic lesion, whilst generating p53-dependent early growth arrest and apoptotic responses via p21/WAF1 and Bax, respectively, induces a longer-term loss of clonogenicity in a manner independent of these genetic determinants.

The nature of the primary lesion and the mechanism of its effect on clonogenicity remains to be determined. It may be that short-term in vitro assays of drug response and longer-term in vitro clonogenic assays of cytotoxicity will yield complementary information relevant to different aspects of antitumour chemotherapeutic activity in vivo [31, 32]. It remains to be seen whether the determinants of cellular response identified here will be reflected in the antitumour activity of RM175 in vivo.

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