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Intrinsic and Acquired Forms of Resistance Against the Anticancer Ruthenium Compound KP1019 (FFC14A)

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d) Abbreviations used: AAS, atomic absorptions spectroscopy; ABC, ATP-binding cassette; ANOVA, analysis of variance; BCRP, breast cancer resistance protein; BSA, bovine serum albumin; ICP-MS, inductively-coupled plasma mass spectroscopy; KP1019, indazolium trans- [tetrachlorbisindazoleruthenat]; LRP, lung resistance protein; MDR, multidrug resistance; MOPS, 3-N-morpholinopropanesulfonic acid; MRP, multidrug resistance-related protein; PBS, phosphate-buffered saline; TfR, Transferrin receptor; TMAH, tetra methyl ammonium hydroxide;

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ABSTRACT

Indazolium *trans*-[tetrachlorobis(1H-indazole)ruthenate (III)] (KP1019; FFC14A) is a metal complex with promising anticancer activity. Since chemoresistance is a major obstacle in chemotherapy, this study investigated the influence of several drug resistance mechanisms on the anticancer activity of KP1019. Here, we demonstrate that the cytotoxic effects of KP1019 are neither substantially hampered by overexpression of the drug resistance proteins MRP1, BCRP, and LRP nor the transferrin receptor and only marginally by the cellular p53 status. In contrast, P-glycoprotein overexpression weakly, but significantly (up to 2-fold) reduced KP1019 activity. P-glycoprotein related resistance was based on reduced intracellular KP1019 accumulation and reversible by known P-glycoprotein modulators. KP1019 dose-dependently inhibited ATPase activity of P-glycoprotein with a K_i of ~31µM. Furthermore, it potently blocked P-glycoprotein-mediated rhodamine 123 efflux under serum-free conditions (EC_{50} ~8µM), however, with reduced activity at increased serum concentrations (at 10% serum EC_{50} ~35µM). Moreover, P-glycoprotein-mediated daunomycin resistance could only be marginally restored by KP1019 in serum-containing medium, also indicating an influence of serum proteins on the interaction between KP1019 and P-glycoprotein. Acquired KP1019 resistance was investigated by selecting KB-3-1 cells against KP1019 for more than one year. Only an ~2-fold KP1019 resistance could be induced which unexpectedly was not due to overexpression of P-glycoprotein or other efflux pumps. Accordingly, KP1019-resistant cells did not display reduced drug accumulation. Their unique cross-resistance pattern confirmed an ABC-transporter-independent resistance phenotype. Summarizing, the likeliness of acquiring insensitivity to KP1019 during therapy is expected to be low and resistance should not be based on overexpression of drug efflux transporters.

In 1969, Rosenberg discovered the pharmacological activity of cisplatin, which became the first purely inorganic antitumour drug introduced into the clinics. With this drug, testicular cancer, a disease which until the late 1970ies had been almost incurable, can be treated effectively today. Although cisplatin is now widely and successfully used against various types of malignancies, its benefits are limited due to intrinsic and acquired resistance mechanisms of tumour cells (Siddik, 2003). Nevertheless the success of cisplatin indicates that metal compounds are a valuable chance for new chemotherapeutical approaches. Besides platinum, a wide range of other metal compounds have been investigated for their anti-tumour activity. One of the most auspicious among these is indazolium *trans*-[tetrachlorobis (1*H*-indazole) ruthenate(III)] (KP1019; FFC14A). This ruthenium compound is supposed to be a prodrug, which is activated to a more reactive ruthenium (II) complex by the reducing environment of hypoxic regions which are abundant in solid tumour tissues (Brabec, 2002). KP1019 binds to serum proteins such as albumin (Kratz et al., 1992) (about 80%) and to the two iron(III)-binding sites of apotransferrin (Kratz et al., 1994) without significant loss of its heterocyclic ligand. It is assumed that bound KP1019 is transported (like iron) into tumour cells via the transferrin-dependent pathway (Kratz et al., 1992; Kratz et al., 1994; Pongratz et al., 2004) KP1019 already showed promising pharmacological properties in acetoxymethylmethylnitrosamine (AMMN)-induced colorectal carcinomas in rats, a model which has macro- and microscopically very similar characteristics as human colorectal tumours (Keppler et al., 1989). This led to the recent initiation of a clinical phase I study of KP1019 in patients with solid tumours.

One of the major impediments for successful chemotherapy is a phenomenon called multidrug resistance (MDR) (Gottesman et al., 2002). Prominent among possible mechanisms causing MDR are the broad specificity drug efflux pumps of the ATP binding cassette family (ABC-transporter family), (for a recent review see Gottesman, Fojo, and Bates, 2002). ABC proteins use ATP to drive the transport of various molecules including peptides, sugars, lipids, but also

chemotherapeutical drugs and hydrophobic compounds across biological membranes (Gottesman, Fojo, and Bates, 2002). A large number of compounds have been identified as substrates for diverse members of this transporter family, including most anticancer drugs as well as other cytotoxic agents (Ambudkar et al., 2003; Gottesman et al., 2002). Several drug-transporting ABC proteins have been characterised, which differ in their substrate specificity. However, often overlapping transport profiles are observed (Gottesman et al., 2002). Besides the activation of ABC transporters a number of other changes in cancer cells can lead to drug resistance ranging from very specific mechanisms active against a single agent to those inhibiting e.g. apoptosis which impacts on the outcome of practically each chemotherapy (Makin and Hickman, 2000). In this study we have investigated, whether several ABC family drug transporters, i.e. P-glycoprotein, multidrug-resistance protein1 (MRP1) and breast cancer resistance protein (BCRP), and other resistance-related mechanisms like transferrin receptor (TfR) (Chitambar et al., 1990), p53 status (Fojo and Bates, 2003), and the lung cancer resistance protein (LRP) (Mossink et al., 2003) lead to resistance against KP1019-induced cytotoxicity. Moreover, KB cells resistant against the cytotoxic activity of KP1019 have been generated and characterised with special attention to drug accumulation defects and overexpression of drug resistance proteins.

MATERIAL AND METHODS

Drugs. Indazolium *trans*-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019) was prepared at the Institute of Inorganic Chemistry as described previously (Keppler et al., 1989). The compound was dissolved in DMSO and diluted into the culture media at the concentrations indicated (DMSO concentrations were always below 1%). Verapamil was purchased from Abbott (Vienna, Austria), cyclosporin A from Sandoz (Basel, Switzerland), dipyridamole from Aldrich (Milwaukee, USA), BCNU from Bristol-Myers Squibb (Munich, Germany), paclitaxel from Rhone-Poulenc RORER (Essex, GB), TMAH and gallium nitrate octahydrate from Merck (Darmstadt, Germany). All other substances were purchased from Sigma-Aldrich (St. Louis, USA). All solutions were freshly prepared before usage.

Cell Culture. The following human cell lines and their chemoresistant sublines were used in this study: the epidermal carcinoma-derived cell line KB-3-1 and its P-glycoprotein-overexpressing subline KBC-1 (generously donated by Dr. D.W. Shen, Bethesda, USA) (Shen et al., 1986); the promyelocytic leukaemia cell line HL60 and its MRP1-overexpressing subline HL60/adr and P-glycoprotein-overexpressing subline HL60/vinc (by Dr. M. Center, Kansas State University, USA) (McGrath and Center, 1988), another HL60 parental cell line together with its TfR-overexpressing subline HL60/ga (by Dr. C. Chitambar, Medical College of Wisconsin, USA) (Chitambar et al., 1990); the small cell lung carcinoma cell line GLC-4 and its MRP1- and LRP-overexpressing subline GLC-4/adr (by Dr. E.G. deVries, Groningen, The Netherlands) (Zijlstra et al., 1987); the breast adenocarcinoma cell lines MCF-7 and MDA-MB-231 with their respective BCRP-transfected subclones MCF-7/bcrp and MDA-MB-231/bcrp (both by Prof. Ross, University of Maryland, Greenbaum Cancer Centre, USA) (Doyle et al., 1998). Additionally, the non-small cell lung cancer cell line A549 and the hepatocellular carcinoma cell line Hep3B (from American Type Culture Collection, Manassas, VA) were used. All cell lines were grown in RMPI 1640 supplemented with 10% fetal bovine serum with the exception of MCF-7 cells, which were grown in MEME with 10%

serum. Cultures were regularly checked for *Mycoplasma* contamination. A KP1019-resistant KB cell line was generated by continuous exposure of KB-3-1 cells to KP1019 at concentrations increasing stepwise from 20µM (concentration A) to 150µM (concentration N) over a period of one year. The ruthenium compound was administered to KB-3-1 cells twice a week at the day after passage, when cells had attached to the culture flasks. KP1019-resistant cells were termed KB-1019N.

Cytotoxicity Assays. Cells were plated ($2x10^4$cells/ml for KB, A549, MDA-MB-231, and Hep 3B cells; $5x10^4$cells/ml for HL60 and MCF-7 cells, and $4x10^4$cells/ml for GLC-4 cells) in 100µl per well in 96-well plates and allowed to attach for 24hrs. Drugs were added in another 100µl growth medium and cells exposed for 72hrs. The proportion of viable cells was determined by MTT assay following the manufacturer's recommendations (EZ4U, Biomedica, Vienna, Austria). Cytotoxicity was expressed as IC_{50} values calculated from full dose-response curves (drug concentrations including a 50% reduction of cell survival in comparison to the control cultured in parallel without drugs).

p53 Transfection. p53-positive cell clones were obtained from the p53(-/-) Hep3B cell line by transfection with the temperature-sensitive p53val143 vector (van Laar et al., 1996). To allow selection of transfected clones, cells were cotransfected with 1/10 amount of a pBabe puromycin resistance vector (donated by Dr. C. Cerni, Vienna). Hep3B/p53 and Hep3B/c (vector control) cells were plated on 96-well microstate plates at a density of $4x10^3$/100µl RPMI/well. After incubation for 24hrs cells were starved for another 24hrs to reduce cell proliferation. On the next day two test groups were defined: group 1 was transferred to 32°C (wt p53) for 12hrs, group 2 remained at 37°C (mutated p53). Subsequently, drugs were added in 100µl culture medium with 10% serum and after 1hr incubation the first group was transferred from 32 to 37°C. Exposure was continued for another 72hrs and cytotoxicity was measured by EZ4U kit.

Drug Accumulation Assay (Zeeman AAS and ICP-MS). Accumulation of KP1019 in

HL60, HL60/adr and HL60/vinc was monitored using Zeeman AAS. KP1019 with and without modulators was added to cell cultures in RPMI with 10% FCS and incubated for 1hr. Cells were washed twice with PBS, the pellet resuspended in 100µl tetramethylammoniumhydroxide (TMAH) and cell lysis proceeded at room temperature for 2 days. Cell lysates were vacuum dried, dissolved in 2M HCl and ruthenium concentrations were measured with a graphite furnace atomic absorption spectrometer (Perkrin Elmer Zeeman 5100) using the following parameters: pre-treatment temperature 1400°C; atomisation temperature 2500°C; wavelength 349.9nm.

Accumulation of KP1019 in KB-3-1, KBC-1 and KB-1019N was monitored using ICP-MS. Cells ($1x10^5$/well) were exposed to 50µM KP1019 for 60 minutes at 37°C. After 3 washes with PBS, cells were lysed by incubation at room temperature in 400µl TMAH. Lysates were diluted in 0.6N HNO_3 and ruthenium concentrations determined by inductively coupled plasma mass spectroscopy (ICP-MS) using an Elan 6100, Perkin Elmer/Sciex Corporation. Values represent means of at least three independent experiments. Statistical evaluation was performed using Two-Way-ANOVA test.

Measuring P-glycoprotein ATPase Activity. Preparation of plasma membrane vesicles from CCRF ADR5000 cells (gift of Dr. V. Gekeler) and measurement of P-glycoprotein ATPase activity were performed exactly as described (Schmid et al., 1999).

Rhodamine 123 Accumulation Studies. Rhodamine 123 accumulation assays were performed as previously described (Elbling et al., 1998). Briefly, $5x10^5$ or 1 $x10^6$ HL60 and HL60/vinc cells were incubated in RPMI/HEPES medium with and without serum for 1h at 37°C with rhodamine123 (0.25 mg/ml) both in the presence and in the absence of verapamil or KP1019 (5 to 50µM both) added 30 minutes before rhodamine 123. After 30, 60 and 120min exposure, fluorescence of rhodamine 123 was collected through a 530/30-nm bandpass filter on the FACS Calibur (Becton Dickinson, Palo Alto, CA).

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Western Blot Analysis. Cell fractionation, protein separation and western blotting were performed as described (Berger et al., 2000). The following antibodies were used: anti- P-glycoprotein monoclonal mouse C219 (Signet, Dedham, USA), dilution: 1:100; anti-LRP monoclonal mouse clone 42 (Transduction Lab., Lexington, USA), 1:1000; anti-BCRP monoclonal mouse MAB4146 (Chemicon, Temicola, USA), 1:500, anti-MRP1 monoclonal rat MRPr1 (Sanbio, Uden, Netherlands), 1:40; anti-MRP2 monoclonal mouse C250 (Alexis Corp., Lausen, Switzerland), 1:50; anti-MRP3 monoclonal mouse M_3II-9 (Alexis Corp., Lausen, Switzerland), 1:40. PARP rabbit polyclonal (Cell Signalling Technology, Beverly, MA), 1:1000. All secondary, peroxidase-labelled antibodies from Pierce were used at working dilutions of 1:10000.

Expression of Transferrin Receptor (TfR). Expression of TfR in HL60 and HL60/ga cells was analysed by flow cytometry (FACS Calibur, Becton Dickinson, Palo Alto, CA) using the monoclonal mouse antibody VIP-1 (generously donated by Dr. Majdic, Medical University of Vienna, Austria). Briefly, $5x10^5$ cells were washed with PBS/1%BSA and incubated for 30min with 20µg/ml primary antibody at 4°C. Bound antibody was stained for 30min at 4°C with an anti-mouse IgG (Fab specific) FITC conjugate (SIGMA, St. Louis, MO, USA) at a 1:150 working dilution.

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RESULTS

Cytotoxicity of KP1019 in Chemosensitive and Chemoresistant Cell Lines. Cytotoxic activity of KP1019 was tested using a panel of chemosensitive cell lines and their chemoresistant sublines expressing defined resistance mechanisms (Table 1). In general the sensitivity of parental, unselected tumour cell lines against KP1019 ranged from IC_{50} values of 56 μM (HL-60) to 179 μM (MCF7). With regard to the resistant sublines, MRP1 and LRP-overexpressing cells (GLC4/adr and HL60/adr) were comparably sensitive as the respective parental cell lines. In contrast, both P-glycoprotein-overexpressing cell lines (HL60/vinc and KBC-1) were moderately but significantly (~1.8-fold) chemoresistant against KP1019. Resistance against KP1019 was relatively low as compared to other known P-glycoprotein substrates including paclitaxel (>68-fold) and VP-16 (>51-fold) (compare Table 2). Interestingly, both cell lines transfected with BCRP (MCF7/bcrp, MDA-MB-231/bcrp) were slightly but significantly hypersensitive against KP1019, a phenomenon known as "collateral sensitivity" (Fattman et al., 1996).

Influence of P-glycoprotein Modulation on KP1019 Cytotoxity. Several substances are known to inhibit P-gp-mediated efflux including verapamil, cyclosporin A, dipyridamole, and tamoxifen (Fojo and Bates, 2003). When co-administered with KP1019 to P-gp-overexpressing KBC-1 cells, these P-gp modulators were highly effective at restoring sensitivity against KP1019 (Fig. 1). In contrast, the MRP1 and MRP2 modulator probenecid (Versantvoort et al., 1995) showed no significant impact on KP1019 sensitivity in these cells.

Impact of Drug-Transporter Overexpression on Intracellular Accumulation of KP1019. To examine whether KP1019 resistance of P-glycoprotein-overexpressing cells is based on differences in the cellular drug accumulation, ruthenium levels in KP1019-treated HL60, HL60/vinc and HL60/adr cells were compared. As shown in Fig. 2A the amount of ruthenium

in P-glycoprotein expressing HL60/vinc cells was significantly lower than in parental HL60 or MRP1-positive HL60/adr cells. Coadministration with verapamil significantly increased (1.7-fold) KP1019 accumulation in the P-glycoprotein-overexpressing cell line HL60/vinc only. Comparable results could be observed in the KB model measured by ICP-MS, however with a lower efficacy of verapamil (1.4-fold at 10μM) in restoring intracellular ruthenium levels in KBC-1 cells (Fig. 2B).

Effect of KP1019 on P-glycoprotein ATPase activity. In order to analyse whether KP1019 directly interacts with P-glycoprotein, the impact on the P-glycoprotein ATPase activity was measured in the presence of ouabain, EGTA, and Na-azide to block the membrane-bound Na^+/K^+-, Ca^{2+}- and mitochondrial ATPases. Differences between the ATPase activity in P-glycoprotein-containing and wild-type vesicles were defined as basal P-glycoprotein ATPase activity (Schmid et al., 1999). Fig. 3 shows the concentration dependent effect of KP1019 on P-glycoprotein ATPase activity. While at lower KP1019 concentrations the ATPase activity was slightly but not significantly enhanced, it was potently inhibited at higher concentrations with an IC_{50} value of approximately 31μM. The effect of KP1019 on P-glycoprotein ATPase activity is thus comparable to that of the known P-glycoprotein substrate daunomycin, which is included in Fig. 3 for comparison.

Modulation of P-glycoprotein-mediated Resistance by KP1019. Many P-glycoprotein substrates also act as P-glycoprotein modulators and competitively inhibit the efflux of other substrate drugs (Fojo and Bates, 2003). To test whether KP1019 is able to modulate P-glycoprotein-mediated resistance, the compound was administered to P-glycoprotein-overexpressing cells together with the two well-characterised P-glycoprotein substrates daunomycin and etoposide (data not shown) as well as cisplatin which is not transported by P-glycoprotein (Fig. 4). Resistance of P-glycoprotein-overexpressing KBC-1 cells against

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daunomycin but not cisplatin was slightly but significantly reduced when KP1019 was added at low, non-toxic concentrations. Also in case of VP-16 a small but statistically significant chemosensitizing effect of KP1019 was detectable in KBC-1 cells (data not shown). In contrast, no modulation with regard to any of the tested drugs was detectable in chemosensitive KB-3-1 cells. These data argue in contrast to those from the ATPase assay (compare Fig.3) against a potent P-glycoprotein-inhibiting function of KP1019.

For further clarification, the P-glycoprotein-modulatory effect of KP1019 was studied in a drug retention assay using rhodamine 123 as the fluorescent substrate (Elbling et al., 1998). Drug-sensitive and -resistant HL60 cells were exposed to rhodamine 123 in the absence or presence of increasing concentrations of KP1019. The known P-glycoprotein modulator verapamil was used as a control. While KP1019 did not affect the accumulation of rhodamine 123 in HL60 cells, it significantly increased the intracellular fluorescence in a dose- and time-dependent manner in HL60/vinc cells (Fig. 5A). Complete revision occurred at 25μM KP1019. In this assay, rhodamine 123 efflux was inhibited by KP1019 to an extent which was comparably to that of verapamil.

As both P-glycoprotein ATPase measurements and rhodamine 123 accumulation assays indicated that KP1019 was a potent P-glycoprotein modulator, while cytotoxicity assays performed in the presence of 10% serum only indicated a moderate effect, the impact of serum was evaluated using the rhodamine 123 retention assay (Fig. 5B). Addition of 10% serum significantly reduced the potency of KP1019 at 5, 10 and 25 μM to restore rhodamine 123 accumulation in HL60/vinc cells. The respective EC_{50} values shifted from ~8μM under serum free conditions to ~35 μM KP1019. These data indicate a substantial influence of serum proteins on the interaction between KP1019 and P-glycoprotein.

Influence of Transferrin-Receptor Overexpression on the Cytotoxicity of KP1019. Overexpression of the TfR has been shown to be associated with acquired resistance against

gallium nitrate in HL60 cells (Chitambar et al., 1990). Since, similar to gallium, KP1019 binds to transferrin and is internalised by the cells via the TfR (Kratz et al., 1992), we were interested, whether changes in the TfR expression influence the anticancer activity of KP1019. In contrast to gallium nitrate, TfR-overexpressing HL60/ga cells (Fig. 6C) were not resistant but hypersensitive to KP1019 (Fig. 6A). Moreover, KP1019 accumulation was significantly increased in the gallium nitrate-resistant cell line when compared to its chemosensitive parental line (Fig. 6B). Thus overexpression of TfR, which is observed in many tumour tissues (Hogemann-Savellano et al., 2003), seems to induce hypersensitivity rather than resistance to KP1019.

Cellular p53 Status and its Relation to KP1019-induced Cytotoxicity. To determine a possible impact of the cellular p53 status on KP1019 cytotoxicity, we used p53-null Hep3B-cells transfected with a temperature-sensitive p53 variant (Hep3B/p53). The encoded p53 is in mutant conformation at 37°C and has wild-type conformation at 32°C. Independent of the p53 status, Hep3B cell proliferation was strongly reduced at 32°C leading to a distinctly decreased cytotoxic activity of KP1019 (data not shown). This indicated that cell proliferation is essential for the cytotoxic activity of KP1019. To avoid the influence of temperature on cell growth, we reduced proliferation in all experimental groups by serum starvation 24hrs prior to drug exposure. Two hrs after drug exposure cells were returned to 37°C to allow unimpeded proliferation. Using this experimental setting, a minor opposite influence of temperature was obvious because the vector control clone (Hep3B/c) was more sensitive to KP1019 when treated at 32°C as compared to 37°C (Fig. 7). This difference was slightly more prominent in p53-transfected Hep3B/p53 cells pointing towards a marginally enhanced activity of KP1019 under p53wt conditions. These data together with the potent cytotoxic effects of KP1019 against p53 null Hep3B and HL60 cells (Fig. 7, Table 1) argue against a major function of p53 in KP1019-induced cytotoxicity.

Generation of the KP1019-resistant Cell Line KB-1019N. A KP1019-resistant KB-3-1 subline was generated by stepwise selection against increasing concentrations of KP1019. During the selection process, drug doses had to be increased very slowly as compared to several other cytostatics used concurrently in the same experimental setting (data not shown). It took more than 12 months until cells displayed a significant (around 1.5-fold) resistance against KP1019 and the highest achievable concentration was 150µM (KB-1019N). Fig. 8, presents KP1019 dose-response curves at 72h drug exposure for KB-1019N as compared to KB-3-1 and KBC-1 cells. KB-1019N cells displayed a 1.8-fold resistance against the ruthenium drug, comparable to the highly P-glycoprotein-overexpressing KBC-1 cells (compare Table 2). The cross-resistance pattern of KB-1019N cells in comparison to KBC-1 cells against various chemotherapeutic drugs is shown in Table 2. As expected, KBC-1 cells were highly resistant to all P-glycoprotein substrates tested (paclitaxel, daunomycin, VP-16, vincristine, vinblastine). In contrast, KB-1019N cells displayed a distinctly different resistance phenotype. While low resistance against VP-16 became obvious, a tendency towards hypersensitivity against two other P-glycoprotein substrate drugs, paclitaxel and daunomycin, and significant collateral sensitivity against gallium nitrate were detectable.

MDR Protein Expression in KB-1019N Cells. Expression levels of several drug resistance proteins (P-glycoprotein, MRP1, MRP2, MRP3, MRP5, BCRP and LRP) in KB-1019N cells as opposed to the one of parental KB-3-1 and several other MDR cell models (KBC-1, HL60/adr, HL60/ar, HL60/vinc and the NSCLC cell line A549) are shown in Fig. 9. Generally, selection against P-glycoprotein substrate drugs is known to readily induce overexpression of P-glycoprotein (Gottesman et al., 2002). However, unlike in KBC-1 and HL60/vinc cells, P-glycoprotein expression was not increased in KB-1019N cells. In comparison to KB-3-1, KB-1019N cells showed a reduced MRP1 expression, while MRP2

and MRP5 levels were unchanged. With the exception of A549 cells, which also intrinsically overexpressed MRP1 and MRP2, LRP and BCRP expression were absent in all tested cell lines (Kohno et al., 1988). Similarly, no MRP3 expression was detectable (data not shown).

KP1019 Accumulation in KB-1019N Cells.

In order to test whether the KP1019 resistance of KB-1019N cells was based on a drug accumulation deficiency, the intracellular amount of ruthenium was measured by ICP-MS as shown in Fig. 10. Unlike KBC-1 cells, KB-1019N cells did not display an accumulation defect. Moreover, preincubation with verapamil failed to increase intracellular ruthenium levels in KB-1019N cells (data not shown).

DISCUSSION

As multidrug resistance (MDR) is a major impediment for successful chemotherapy in cancer patients (Gottesman, et al. 2002), the influence of MDR mechanisms on the cytotoxic activity of new drugs entering clinical development is a matter of particular interest. In this study we investigated the anticancer activity of KP1019 in regard to the influence of intrinsic and acquired resistance mechanisms. P-glycoprotein-mediated resistance to KP1019 was based on reduced intracellular drug accumulation and reversible by P-glycoprotein inhibitors. However, the KP1019 resistance levels in highly P-glycoprotein-overexpressing cells were up to 100-fold lower than those which would be expected against known P-glycoprotein substrates in the identical cell models. Since P-glycoprotein mediated resistance to KP1019 is marginal as compared to other widely used anticancer drugs (Gottesman et al., 2002), this mechanism might be of limited relevance for the development of resistance in a clinical setting.

In addition, our data show that KP1019 acts as a substrate and as an inhibitor of P-glycoprotein. It restored rhodamine 123 accumulation in P-glycoprotein-overexpressing cells with a potency comparable to that of the known P-glycoprotein modulator verapamil. The effects of KP1019 on P-glycoprotein ATPase activity resembled those of daunomycin (Schmid et al., 1999). In contrast, many other P-glycoprotein substrates and/or modulators including taxanes, vinblastine and verapamil stimulate P-glycoprotein ATPase activity with higher potency (Litman et al., 1997). In the rhodamine 123 accumulation assay the P-glycoprotein-modulating activity of KP1019 was strongly reduced by addition of 10% serum. This suggests, that binding to transferrin (and other serum proteins) influences the affinity of KP1019 towards P-glycoprotein like it has already been shown for doxorubicin-transferrin conjugates (Fritzer et al., 1996). Correspondingly, in cytotoxicity assays (performed at 10% serum) KP1019 (20μM) only marginally reversed resistance against the P-glycoprotein substrates daunomycin and VP-16. With regard to clinical trials it is conceivable that in

combination regimens KP1019 might sensitize P-glycoprotein-expressing tumours to other chemotherapeutics. The balance between P-glycoprotein substrate and modulator activities of KP1019 in combination chemotherapy settings has to be further evaluated.

In order to investigate acquired resistance, we grew KB-3-1 cells under increasing concentrations of KP1019. It took more than one year to establish cells with a ~1.7-fold resistance as compared to the parental KB-3-1 cells. This low resistance value is in sharp contrast to published data about selection against diverse ABC-transporter substrate drugs (McGrath and Center, 1988; Shen et al., 1986; Zijlstra et al., 1987). Accordingly, we succeeded in attaining resistance values up to 200-fold by selection against other cytotoxic metal compounds within the same time period (data not shown). Moreover, selection against P-glycoprotein substrate drugs frequently leads to overexpression of P-glycoprotein (Lee et al., 1997). However, we were unable to detect any P-glycoprotein expression in KB-1019N cells. Furthermore, KB-1019N cells showed a unique cross-resistance pattern, with no general resistance against P-glycoprotein substrate drugs. Protein expression and cross-resistance data are in line with the observation that ruthenium accumulation in KB-1019N cells was not reduced in comparison with the chemosensitive parental KB-3-1. These data indicate that the KP1019 resistance of KB-1019N cells is not based on active drug export through the plasma membrane.

The reason why KP1019 selection does not induce overexpression of P-glycoprotein, as do several other P-glycoprotein substrate drugs in the identical KB-3-1 cell line, remains unclear. Until now most metal compounds have been identified as substrates for MRP1 (Sb-, Co-, Al-, Co- complexes (Vernhet et al., 2000)) or MRP2 (Cisplatin-glutathione conjugates (Liedert et al., 2003)). Moreover, MRP-related proteins were shown to be involved in metal resistance in other eukaryotic organisms (Broeks et al., 1996; Szczypka et al., 1994). Especially, MRP2 has been shown to be induced by selection against cytotoxic metal compounds (Gerk and Vore, 2002). However, KB-1019N cells neither overexpressed MRP2

nor did they exhibit cross-resistance against the known MRP2 substrate cisplatin (Liedert et al., 2003). Only a few other ruthenium compounds have been investigated in regard to the impact of resistance mechanisms. Interestingly, a number of Ru (II) organometallic arene complexes have been identified as P-glycoprotein but not as MRP2 substrates in ovarian carcinoma cell models (Aird et al., 2002). This might indicate, that in contrast to other metal compounds, ruthenium compounds are generally more likely P-glycoprotein than MRP substrates. Moreover, the ruthenium complex group is very heterogeneous with respect to the chemical ligands thus differing in chemical properties and biological activity (Brabec, 2002). Based on this heterogeneity it has to be suggested that each ruthenium compound might elicit an individual resistance profile.

KP1019 is known to bind to iron transport proteins like apotransferrin and lactotransferrin (Kratz et al., 1994; Kratz et al., 1992) and to be transported into tumour cells via the transferrin-dependent pathway (Pongratz et al., 2003). Based on these observations we tested the influence of TfR overexpression in HL60 cells. The cell line was selected against another metal compound, gallium nitrate (Chitambar and Wereley, 1997). HL60/ga cells accumulated a higher amount of the ruthenium drug and were consequently hypersensitive to the anticancer activity of KP1019. These results correspond with published experiments which demonstrated that binding to iron-loaded transferrin enhances the cellular accumulation of KP1019 (Pongratz et al., 2003). Cancer cells generally express elevated levels of TfR to serve their higher need for iron (Chen et al., 1982). This leads to preferential accumulation of KP1019 in tumour tissue and might explain the limited toxicity of KP1019 in animals (Keppler et al., 1989) and cancer patients (personal communication Dr. Scheulen).

Overall, the cell biological and molecular mechanisms underlying the cytotoxic effects of KP1019 on tumour cells have not been extensively investigated yet. We found that KP1019 induced DNA damage in malignant cells and consequently leads to apoptotic cell death (Kapitza et al. 2004). The tumour suppressor gene p53, mutated in at least 50% of human

cancers, is well known to be involved in the induction of apoptosis following DNA damage by cytotoxic drugs (El-Deiry, 2003). However, in our hands p53-dependent signalling had only a very minor influence on the cytotoxic activity of KP1019. This is supported by the fact that HL60 and Hep3B cells, which both are p53 (-/-) (Banerjee et al., 1995), are sensitive to KP1019-induced apoptosis. Consequently, p53 mutation should not be a limiting factor in successful application of KP1019 in cancer chemotherapy.

In summary, our *in vitro* data suggest that a rapid acquisition of KP1019 resistance during chemotherapy is supposed to be unlikely, a feature which distinguishes this ruthenium compound from many other anticancer drugs. Only, a weak intrinsic resistance against KP1019 in highly P-glycoprotein-overexpressing tumours will have to be taken into consideration. However, such high P-glycoprotein levels as present in the drug-selected cell models used in this study are very rarely observed in clinical samples at least in chemo-naive patients. Moreover, due to the low KP1019 resistance levels even at high P-glycoprotein expression, dose escalation protocols might be successfully applied.

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LEGENDS FOR FIGURES

Figure 1. Modulation of P-glycoprotein-mediated resistance against KP1019. KB-3-1 cells and the P-glycoprotein-overexpressing subline KBC-1 were incubated for 72hrs with increasing concentrations of KP1019 in combination with the P-glycoprotein modulators tamoxifen (10μM) and dipyridamole (10μM), the P-glycoprotein and MRP modulators verapamil (10μM) and cyclosporin A (1μM), as well as the MRP modulator probenecid (1mM). IC_{50} were calculated from whole dose response curves. Values given are means ± SD from at least three independent experiments performed in triplicate.

Figure 2. Ruthenium accumulation in KP1019-treated MDR cell models. (A) Ruthenium accumulation in HL60 cells and their MRP1- and P-glycoprotein-overexpressing sublines HL60/adr and HL60/vinc were measured by AAS after one hr incubation with KP1019 (50μM) with and without the MDR modulator verapamil (10μM). Ruthenium accumulation in HL60/vinc was significantly ($p<0.01$) lower than in HL60 and HL60/adr and could be significantly ($p<0.01$) enhanced by co administration of the P-glycoprotein inhibitor verapamil. (B) Ruthenium accumulation in KB-3-1 and P-glycoprotein-overexpressing KB-C1 cells treated like in (A) were measured by ICP-MS. Accumulation in KBC-1 was significantly ($p<0.001$) lower than in KB-3-1 and could be significantly ($p<0.01$) enhanced by coadministration of verapamil. Mean ± SD of at least five experiments are given.

Figure 3. Effects of KP1019 on P-glycoprotein ATPase activity. The rate of ATP hydrolysis in P-glycoprotein containing plasma membrane vesicles was measured as described (Schmid et al., 1999) under increasing concentrations of KP1019 (♦) as indicated. For comparison P-glycoprotein ATPase activity under the influence of daunomycin (*) is shown. The dose-response curves were fitted to the data points by non-linear regression analysis.

Figure 4. Modulation of P-glycoprotein-mediated resistance in KBC-1 by KP1019. Dose-response curves for daunomycin and cisplatin with and without KP1019 (20μM) and the known P-glycoprotein modulator verapamil (10μM) in KB-3-1 and KBC-1 cells as indicated are shown. Data given are means±SD derived from three independent 72hrs drug incubation experiments.

Figure 5. Modulation of rhodamine 123 accumulation by KP1019 and influence of serum. (A) Rhodamine 123 accumulation in HL60 and HL60/vinc cells with and without coadministration of KP1019 and verapamil at the indicated concentrations was measured at the given time points by FACS analysis. (B) Influence of 10% serum on the P-glycoprotein-modulating activity of KP1019 as compared to verapamil was measured by rhodamine 123 accumulation at the indicated concentrations in HL60 and HL60/vinc cells. Expose time was 1h.

Figure 6. Cytotoxic activity of KP1019 against a TfR-overexpressing, gallium nitrate-resistant HL60 cell line. (A) Dose-response curves of KP1019 for the promyelocytic leukaemia cell line HL60 and its TfR-overexpressing subline HL60/ga were generated by 72hrs continuous drug exposure assays. One experiment performed in triplicate is given representative for three experiments with comparable results. (B) Amount of intracellular ruthenium in HL60 and HL60/ga after 1hr incubation with 50μM KP1019 determined by ICP-MS as described under Material and Methods. (C) Expression of TfR on HL60 and HL60/ga cells. TfR was detected via FACS analysis as described under Material and Methods. Full line: HL60; scattered line: Hl60/ga.

Figure 7. Impact of p53 on KP1019-induced cytotoxicity. Hep3B cells (p53 -/-) have been transfected with a vector containing a temperature-sensitive p53 gene (wild-type at 32°C, mutated at 37°C) (Hep3B/p53) or the control vector (Hep3B/c). Growth arrested cells were treated with KP1019 as described in Material and Methods. Dose-response curves derived from 2 independent experiments in triplicates are shown.

Figure 8. Generation of the KP1019-resistant cell line KB-1019N. Dose-response curves of KP1019 for the parental, chemosensitive KB-3-1 cells line in comparison with its drug-selected sublines KB-1019N and KBC-1 are shown.

Figure 9. Expression of P-glycoprotein, MRP1, MRP2, MRP5, BCRP or LRP was visualized via Western blotting (representative experiments are shown in the left panel) and quantified by scanning densitometry (right panel): KB-3-1 (1), KBC-1 (2), KB-1019N (**3**), HL60 (4), HL60/ar (5), HL60/adr (6), A549 (7), and HL60/vinc cells (8). Antibodies used are described under Material and Methods.

Figure 10. The amount of intracellular ruthenium in KB-3-1, KB-1019N and KBC-1 cells after one hr treatment with 50μM KP1019 was measured via ICP-MS as described under Materials and Methods. One experiment performed in triplicate is given representative for three experiments with comparable results.

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Table 1. KP1019-induced cytotoxicity against various chemosensitive cell lines and their drug-resistant sublines at 72hrs continuous drug exposure.

	IC_{50} (μM)		Relative resistance	Resistance
Cell line	Mean[a]	±SD	(-fold)	mechanisms
KB-3-1	82.6	0.6		
KBC-1	[b]138.4***	12.5	1.8	P-glycoprotein
HL60	56.0	19.0		
HL60/vinc	95.2**	17.7	1.7	P-glycoprotein
HL60/adr	51.8	21.3	0.9	MRP1
GLC-4	65.0	24.0		
GLC-4/adr	64.6	24.5	1.0	MRP1, LRP
MCF7	179.0	18.0		
MCF7/bcrp	152.0*	19.0	0.8	BCRP
MDA-231	126.7	26.9		
MDA-231/bcrp	97.6*	3.7	0.9	BCRP

[a] Means and SD were calculated from at least three independent experiments in triplicates.

[b] Significantly different from the parental cell line at * $p< 0.05$, ** $p<0.01$, and *** $p>0.0001$ by two-sided, unpaired Student´s t test.

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Table 2. Sensitivity of KB-3-1, KBC-1 and KB-1019N cells against the indicated chemotherapeutic agents was determined by MTT assay after 72hrs continuous drug exposure. The relative resistance was obtained by dividing the IC_{50} of the chemoresistant KBC-1 and KB-1019N by the respective IC_{50} values for the parental KB-3-1 cell line.

	KB-3-1		**KBC-1**			**KB-1019N**		
Drugs	**IC_{50} Mean**	**±SD**	**IC_{50} Mean**	**±SD**	**Relative resistance**	**IC_{50} Mean**	**±SD**	**Relative resistance**
KP1019 (µM)	82.75	±0.6	138.40	±12.5	1.7-fold	143.38	±2.3	1.7-fold
Daunomycin (µM)	23.94	±19.2	>200	-	>8.3-fold	19.47	±16.1	0.8-fold
Etoposide (µM)	0.49	±0.1	>25	-	>51.0-fold	4.33	±0.2	6.1-fold
Paclitaxel (nM)	7.40	±3.7	>500	-	>67.6-fold	5.81	±0.3	0.8-fold
Vinblastine (nM)	17.94	±23.3	>200	-	>11.1-fold	21.60	±26.6	1.2-fold
Vincristine (nM)	1.18	±0.5	>200	-	>170-fold	1.91	±0.2	1.6-fold
BCNU (µM)	15.03	±3.8	9.59	±4.2	0.6-fold	18.28	±11.5	1.2-fold
$GaNO_3$ (µM)	774.91	±139.3	409.65	±60.6	0.5-fold	473.85	±80.3	0.6-fold
Cisplatin (µM)	0.90	±0.0	0.93	±0.0	1.0 fold	0.88	±0.0	1.0 fold

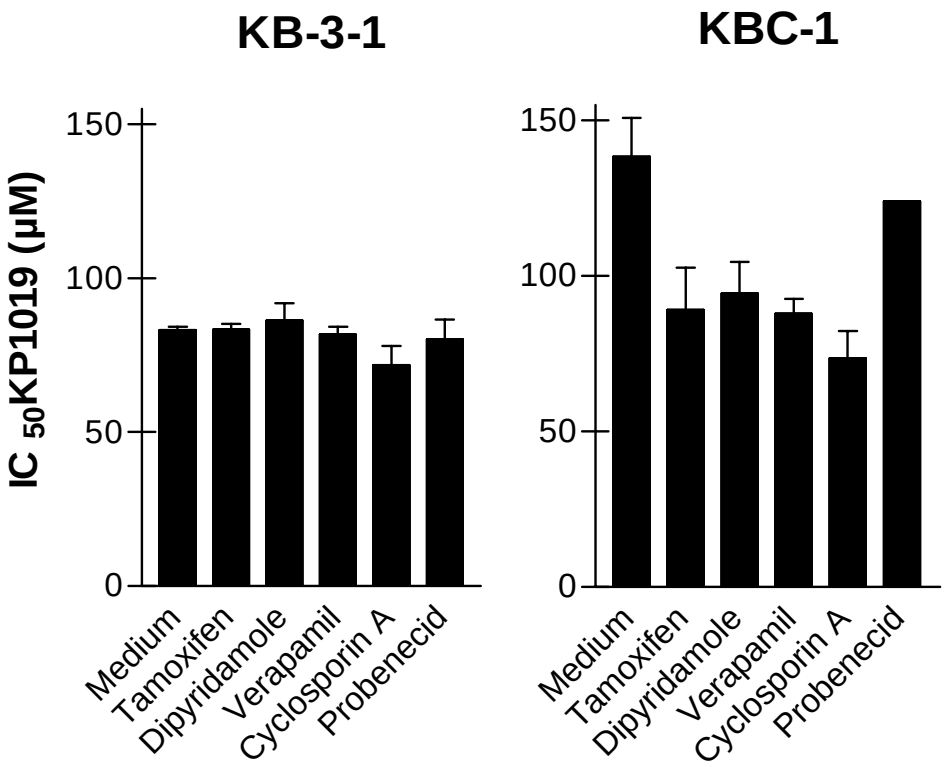


Figure 1

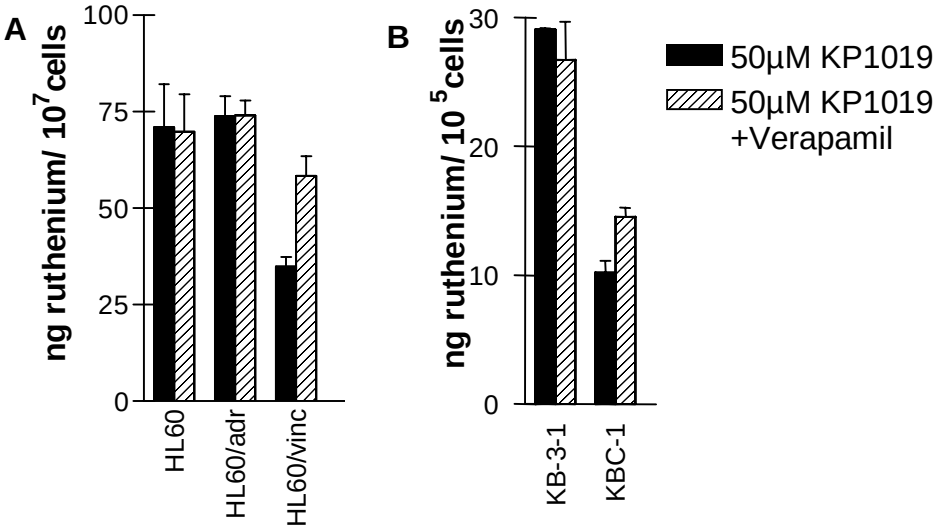
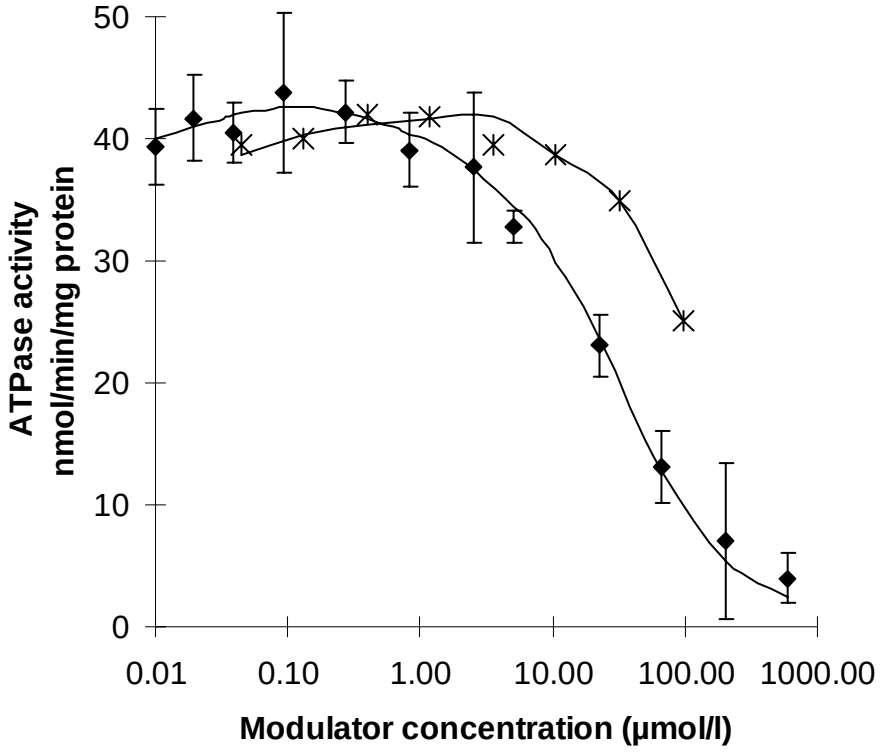


Figure 2



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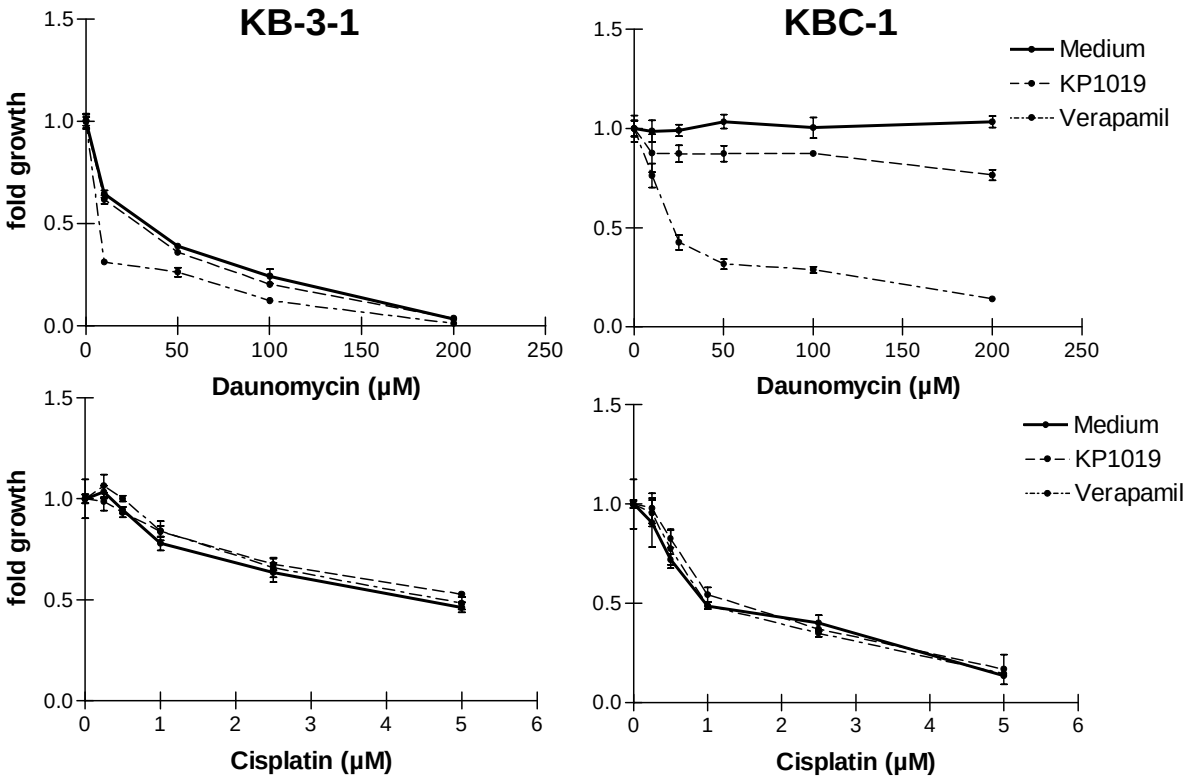

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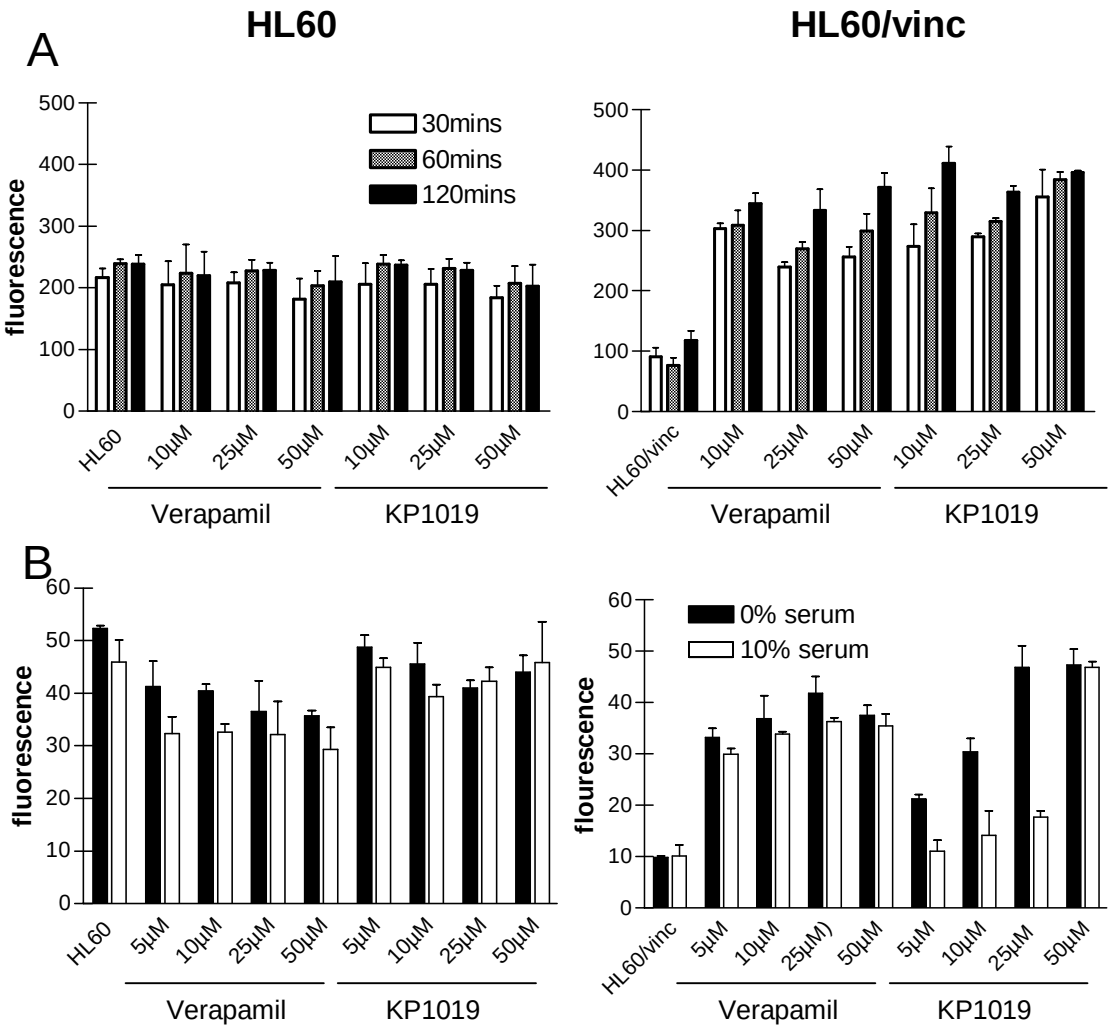

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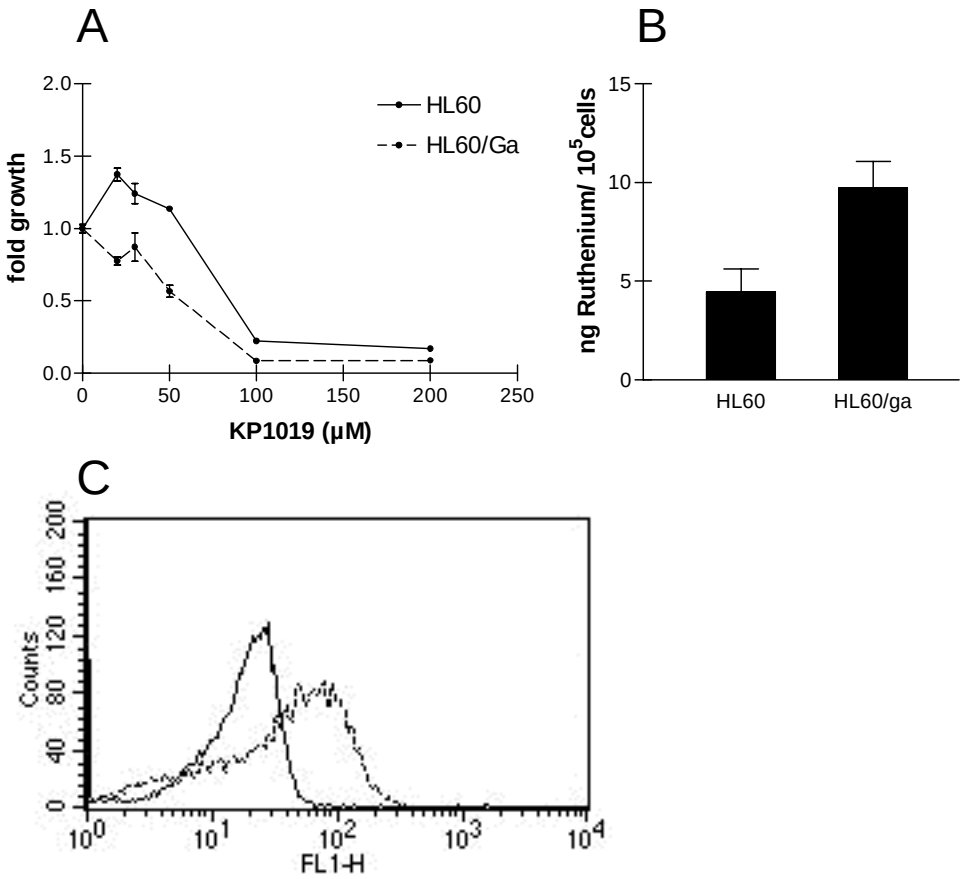

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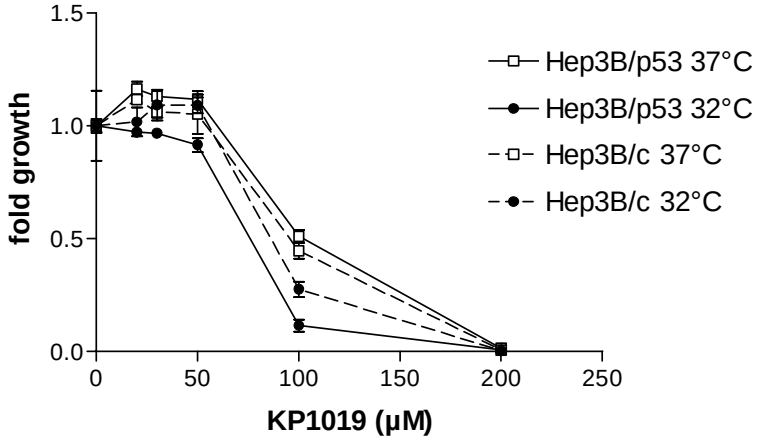

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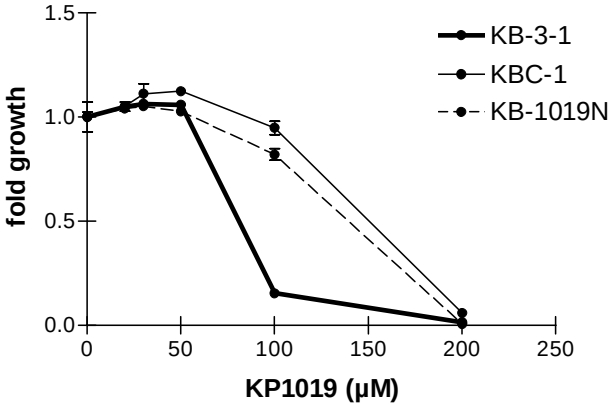

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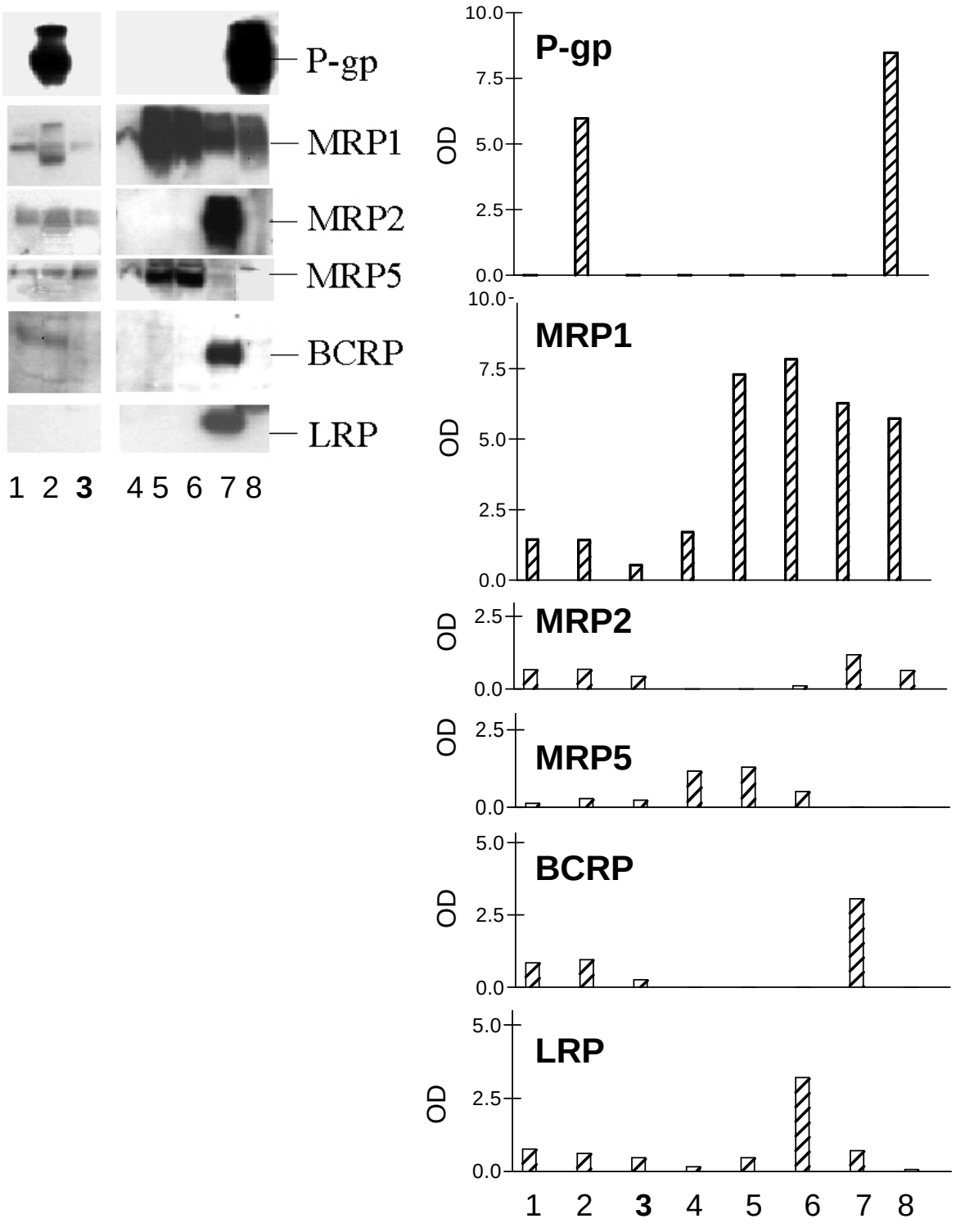

Figure 9

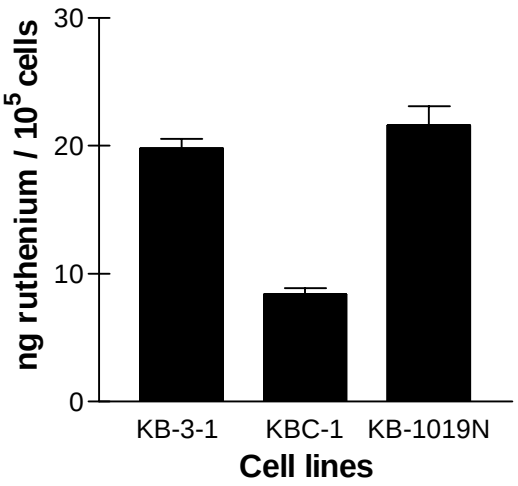

Figure 10