

# Reduced endocytosis and altered lysosome function in cisplatin-resistant cell lines

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We isolated human KB adenocarcinoma cisplatin-resistant (CP-r) cell lines with multidrug-resistance phenotypes because of reduced accumulation of cisplatin and other cytotoxic compounds such as methotrexate and heavy metals. The uptake of horseradish peroxidase (HRPO) and Texas Red dextran was decreased several-fold in KB-CP-r cells, indicating a general defect in fluid-phase endocytosis. In contrast, although EGF receptors were decreased in amount, the kinetics of EGF uptake, a marker of receptor-mediated endocytosis, was similar in sensitive and resistant cells. However, 40–60% of the <sup>125</sup>I-EGF released into the medium after uptake into lysosomes of KB-CP-r cells was TCA precipitable as compared to only 10% released by sensitive cells. These results indicate inefficient degradation of internalised <sup>125</sup>I-EGF in the lysosomes of KB-CP-r cells, consistent with slower processing of cathepsin L, a lysosomal cysteine protease. Treatment of KB cells by bafilomycin A<sub>1</sub>, a known inhibitor of the vacuolar proton pump, mimicked the phenotype seen in KB-CP-r cells with reduced uptake of HRPO, <sup>125</sup>I-EGF, <sup>14</sup>C-carboplatin, and release of TCA precipitable <sup>125</sup>I-EGF. KB-CP-r cells also had less acidic lysosomes. KB-CP-r cells were crossresistant to *Pseudomonas* exotoxin, and *Pseudomonas* exotoxin-resistant KB cells were crossresistant to cisplatin. Since cells with endosomal acidification defects are known to be resistant to *Pseudomonas* exotoxin and blocking of endosomal acidification mimics the CP-r phenotype, we conclude that defective endosomal acidification may contribute to acquired cisplatin resistance.

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Cisplatin (CP) is a component of standard treatment regimens for testicular, ovarian, bladder, cervical, head and neck and small-cell and nonsmall-cell lung cancers (Ozols and Williams, 1989; Perez, 1998). Adducts of DNA with CP induce apoptosis leading to cell death (Minn *et al*, 1995; Simonian *et al*, 1997). Intrinsic or acquired resistance of tumour cells to CP undermines its clinical effectiveness (Niedner *et al*, 2001). Mechanisms of resistance include decreased drug accumulation (Shen *et al*, 2000), changes in DNA repair proficiency (Fink *et al*, 1998; Zhen *et al*, 1992; Chu, 1994; Lai *et al*, 1995; Johnson *et al*, 1996), metallothionein (MT II) (Kelly *et al*, 1988; Kondo *et al*, 1995), glutathione-related enzymes (Moscow and Cowan, 1988; Godwin *et al*, 1992; Zaman *et al*, 1995), stress response proteins (Shen *et al*, 1995; Hettinga *et al*, 1997), proto-oncogenes or apoptosis-related genes, and cancer susceptibility genes (Fanidi *et al*, 1993; Lowe *et al*, 1993; DeFeudis *et al*, 1996; Husain *et al*, 1998; Moorehead and Singh, 2000). Protein kinases (PKs) like PKA and PKC have also been associated with CP-resistance (CP-r) and use of their specific inhibitors has been demonstrated to increase CP cytotoxicity in resistant tumour cells (Gosland *et al*, 1996). Recently, our laboratory (Shen *et al*, 2000)

reported decreased energy-dependent uptake of <sup>14</sup>C-carboplatin by CP-r cells. Copper transporters have recently been described to be involved in cisplatin uptake in yeast and mouse (Ishida *et al*, 2002), and in human cell lines (Katano *et al*, 2002). However, we have been unable to demonstrate any alternation in expression of the copper transporter CTR1 in our CP-r cell lines, and expression of CTR1 in our cells does not change cisplatin accumulation (data not shown). It was of interest to evaluate various uptake pathways in CP-r cells.

Results of the present study demonstrate the reduced uptake of fluid-phase endocytotic markers by CP-r cells. We also report here the incomplete degradation of internalised EGF and slow processing of lysosomal cysteine protease cathepsin L in CP-r cells. Treatment of normal cells with the vacuolar proton pump inhibitor bafilomycin A<sub>1</sub> mimics the CP-r phenotype in wild-type cells, suggesting that an endosomal/lysosomal acidification defect or another similar defect in the endocytic pathway may be plausible mechanisms contributing to CP-r.

## MATERIALS AND METHODS

### Cell lines and cell culture

The previously described human epidermoid carcinoma cell line KB-3-1 (IC<sub>50</sub> 0.1 µg ml<sup>-1</sup> cisplatin) and its CP-r derivative KB-CP20 (selected for resistance to 20 µg ml<sup>-1</sup> cisplatin in many steps) were

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used in the present study. These cells were grown as monolayer cultures in Dulbecco's modified eagle's medium (Quality Biological, Gaithersburg, MD, USA) containing  $4.5 \text{ g l}^{-1}$  glucose (Gibco-BRL, Grand Island, NY, USA), glutamine, penicillin, streptomycin and 10% foetal bovine serum at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator. The KB-CP20 cells were maintained in the above culture medium containing CP ( $20 \mu\text{g ml}^{-1}$ ) as described earlier (Shen *et al*, 1995). Cisplatin was removed from the culture medium 3 days prior to conducting experiments. KB-CP20-EGFR-cl-6 cells were cloned from transfectants of KB-CP20 cells with the pcDNA/EGFR expression vector, and used for studies on EGF binding and uptake. *Pseudomonas* exotoxin-resistant cell lines, ET-12, ET-22 and ET-28 (ET: EGF-toxin-resistant cells), were established and described in detail by Amano *et al* (1988). These ET cell lines were up to more than 10 000-fold resistant to PE-EGF conjugate in comparison with their parent wild-type KB-3-1 cells as determined by a colony-forming assay.

### Uptake assays of horse radish peroxidase (HRPO) and Texas Red dextran-10

For the HRPO assay, KB-3-1 or KB-CP20 cells were seeded in each well of a six-well plate 18–24 h before the assay. The next day, the cells were washed three times with serum-free medium and incubated at  $37^\circ\text{C}$  with medium containing  $2 \text{ mg ml}^{-1}$  HRPO type VI. After various intervals of time, the uptake medium was removed and the cells were washed several times with ice-cold phosphate-buffered saline (PBS), and lysed in PBS containing 0.2% TritonX-100. The cell lysate was centrifuged ( $12\,000 \text{ g}$ ) in a microcentrifuge at  $4^\circ\text{C}$  and HRPO was assayed in the supernatant fraction by the method of West *et al* (1989). For uptake of Texas Red dextran-10 (Molecular Probes, Eugene, OR, USA), both KB-3-1 and KB-CP20 cells were incubated with this fluorescence-labelled marker at  $37^\circ\text{C}$  for 2 h, and then monitored under a laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA) at a  $\times 600$  magnification. A time course for influx of Texas Red dextran-10 was performed by incubation of cells with  $3 \text{ mg ml}^{-1}$  of this fluorescence marker at a desired period of time, and then analysed by a FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with Cell Quest software.

### $^{14}\text{C}$ -carboplatin uptake assay and metabolic labelling

The uptake of  $^{14}\text{C}$ -carboplatin was measured essentially by the method described earlier (Shen *et al*, 2000). Briefly,  $2 \times 10^6$  cells were plated in each well of a six-well Petri dish. The next day, the cells were washed with prewarmed DMEM and incubated with  $^{14}\text{C}$ -carboplatin in DMEM ( $2 \text{ mCi ml}^{-1}$ ). After 1 h, medium was removed, cells were washed  $3 \times$  with ice-cold PBS and harvested by trypsinisation.  $^{14}\text{C}$ -carboplatin taken up by cells was quantitated using a Beckman Liquid Scintillation Counter (LS 2800, Fullerton, CA, USA) after solubilising them in Formula 989 (Dupont, NEN, Boston, MA, USA). For metabolic labelling,  $2 \times 10^6$  cells were plated in each well of a six-well Petri dish. The next day, the cells were washed twice with methionine-free, cysteine-free medium containing 2 mM glutamine and preincubated in the same medium for 1 h before labelling in the presence of  $^{35}\text{S}$ -translabel ( $200 \mu\text{Ci ml}^{-1}$ ). After 30 min, the cells were washed three times with ice-cold PBS and either lysed in SDS buffer A (50 mM Tris-HCl pH 7.4, 150 mM KCl, 0.5% NP-40 and 0.05% SDS) immediately or fed with regular serum-free medium. After 30, 60, 120 and 240 min, the culture medium was collected. The cells were washed three times with ice-cold PBS and lysed. The cell lysate of the 30 min pulse period containing  $1 \times 10^6$  TCA precipitable counts, and an equal volume of lysate were used at the other chase periods. The lysates were immunoprecipitated with rabbit polyclonal antibody to the lysosomal cysteine protease cathepsin L as described previously (Chauhan *et al*, 1998). Similarly, secreted cathepsin L in

a volume of culture medium proportional to the amount of lysate from different chase periods was also immunoprecipitated. The immunoprecipitates were resolved on SDS – PAGE and subjected to autoradiography as described earlier (Chauhan *et al*, 1998). The 42 kDa unprocessed form of human cathepsin L or its 34 and 26 kDa processed forms were quantitated by a phosphorImager 425 (Molecular Dynamics, Sunnyvale, CA, USA).

### Surface binding of $^{125}\text{I}$ -EGF

KB-3-1 and KB-CP20 cells were plated in triplicate in 24-well tissue culture dishes at a cell density of  $2 \times 10^5$  cells  $\text{well}^{-1}$  in 0.5 ml containing 10% FBS. The following day, the cells were fed with fresh DMEM and incubated for 1 h at  $37^\circ\text{C}$ . Then the cells were chilled on ice for 30 min and  $^{125}\text{I}$ -EGF (8 nM, specific activity  $75 \text{ Ci mmol}^{-1}$ ) was added to each well. The incubation was continued on ice for an additional 2 h. Then the medium was aspirated off, cells were washed three times with ice-cold PBS and solubilised in 0.5 ml of 1 N NaOH, and  $^{125}\text{I}$ -EGF bound to the cell surface was determined by using a mini-gamma counter (LKB, Gaithersburg, MD, USA). To determine the nonspecific binding of radiolabelled EGF, cells were incubated with  $^{125}\text{I}$ -EGF in the presence of a 10 M excess of unlabelled EGF and processed similarly. This value was subtracted from the specific binding. The nonspecific binding was never observed to be more than 5% of the specific binding.

### Internalisation, degradation and release of $^{125}\text{I}$ -EGF

Measurement of internalisation and degradation of radiolabelled EGF by CP-r and cisplatin-sensitive (CP-s) cells was performed as described earlier (Das *et al*, 1989).  $^{125}\text{I}$ -EGF was allowed to bind to surface receptors at  $4^\circ\text{C}$  for 2 h as described above. While still on ice, the cells were washed with ice-cold PBS three times and fed with 1.0 ml complete media followed by incubation at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator for 0, 5, 15, 30, 60 and 120 min. At the end of each time period, the medium was saved and total and TCA precipitable radioactivity was determined. The cells were washed with ice-cold PBS and incubated on ice for 6 min with 1.0 ml of mild acid (0.2 M acetic acid – 0.5 M NaCl, pH 2.5). The acidic medium was carefully removed and counted (acid-dissociated radioactivity). The remaining cell-associated radioactivity was solubilised at  $60^\circ\text{C}$  with 1.0 ml of 1 N NaOH and counted in a gamma counter (non-dissociated radioactivity).

### Drug-sensitivity assay

Dose – response curves were determined by seeding  $5 \times 10^4$  cells in 1 ml of medium in each well of a 24-well plate. Drugs at desired concentrations were introduced into the wells at the time of cell seeding. After  $37^\circ\text{C}$  incubation for 3 days, cells were counted by a Coulter Counter. An  $\text{IC}_{50}$  was measured as the concentration of drug reducing the growth of cells to 50% of that in control (drug-free) medium. A relative resistance factor value for each drug was calculated by dividing the  $\text{IC}_{50}$  value of each cell line by that of the wild-type KB-3-1 cells. The values are means of triplicate determinations.

### Measurement of endosomal and lysosomal pH

Cells ( $200 \text{ ml}$ ) at a density of  $1 \times 10^6$  cells  $\text{ml}^{-1}$  were placed onto an  $18 \times 18 \text{ mm}$  coverslip and allowed to grow for 3 days. KB-3-1 and KB-CP20 cells were incubated with 1.5 mM LysoSensor DND-189 (Molecular Probes, Eugene, OR, USA) in DMEM medium for 45 min, and then replaced with fresh medium. Acidified compartments were visualised by laser confocal microscopy. pH determination by LysoSensor-binding fluorescence studies was performed according to the manufacturer's instructions. For analysis of

lysosomal distribution, cells were incubated with 100 nM LysoTracker Red DND-99 (Molecular Probes, Eugene, OR, USA) for 30 min, then monitored under a laser scanning confocal microscope as described above. For direct measurement of endosomal pH, cellubrevin pHluorins were applied as described by Miesenbock *et al* (1998).

## RESULTS

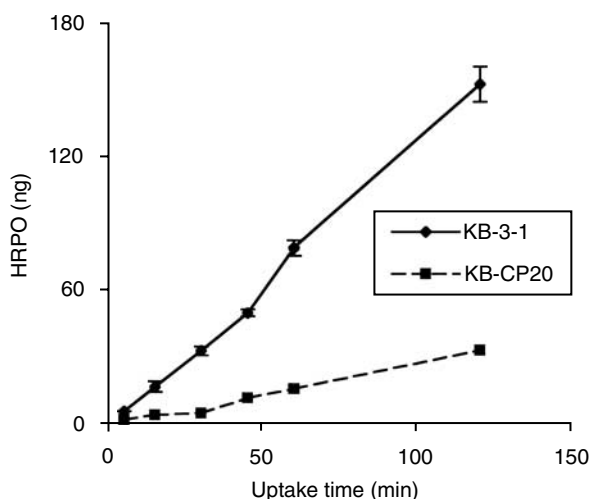
### Measurement of fluid-phase endocytosis

To determine nonreceptor-mediated fluid-phase endocytosis, we measured uptake of HRPO at different intervals of time ranging from 5 to 120 min to assess fluid-phase endocytosis in CS-s (KB-3-1) and CS-r (KB-CP20) cells, and the results are given in Figure 1. We observed a five-fold decrease in the uptake of HRPO by KB-CP20 cells as compared to their sensitive counterparts. This difference in HRPO uptake was consistently seen at all intervals of time ranging from 5 to 120 min.

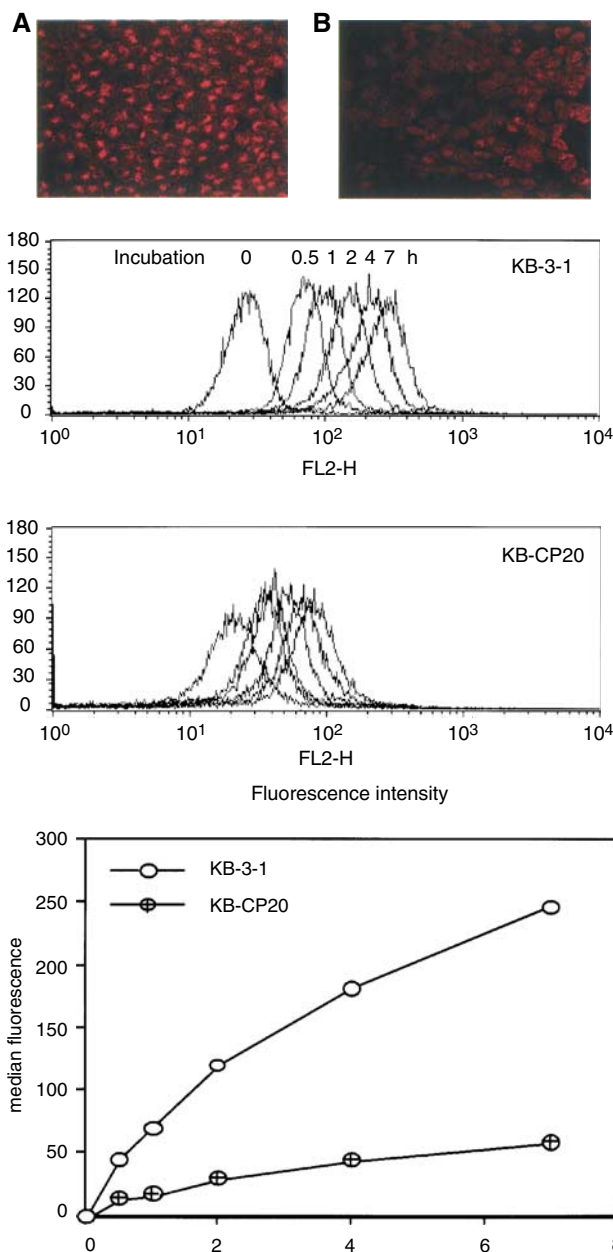
Accumulation of Texas Red dextran-10, another fluid-phase endocytosis marker, was also significantly reduced in the KB-CP20 cells as compared to the KB-3-1 cells during a period of 2 h incubation as seen in Figure 2A and B. We observed that accumulation of Texas Red dextran-10 in the KB-3-1 cells was located mostly at the TGN (Trans-Golgi Network) region, while in KB-CP20 cells the Texas Red dextran-10 was reduced in amount and deposited at the cytoplasm near the peripheral membrane, indicating a dysfunctional uptake pathway for the marker in CP-r cells. FACS analysis on a time course of up to 7 h incubation of cells with Texas Red dextran-10 indicated a four-fold more uptake in the KB-3-1 cells (Figure 2C) than in the KB-CP20 cells (Figure 2D) during the incubation period. A relative semiquantitative measurement based on the data from the FACS analysis is shown in Figure 2E.

### Measurement of EGF binding, uptake, release and degradation

We used  $^{125}\text{I}$ -EGF uptake to measure receptor-mediated endocytosis in KB-3-1 and KB-CP20 cells. The binding of radiolabelled EGF on the cell surface was at least three-fold less in KB-CP20 cells

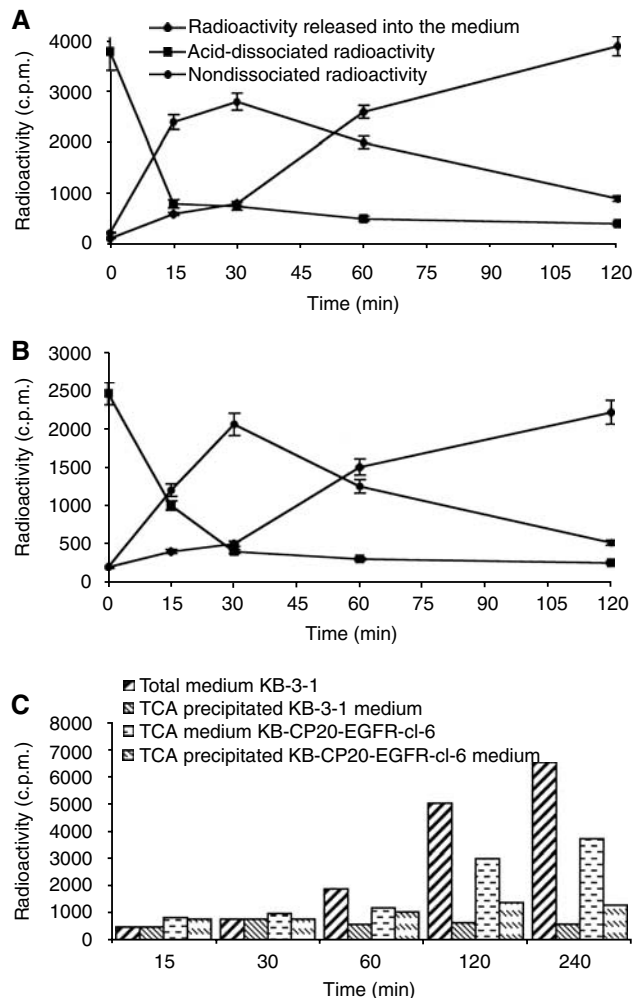


**Figure 1** Kinetics of HRPO uptake by KB-3-1 and KB-CP20 cells. Cells were incubated with  $2\text{ mg ml}^{-1}$  HRP at  $37^\circ\text{C}$ . After various time intervals, the cells were washed several times, lysed in PBS containing 0.2% Triton X-100, and HRP in the cell lysate was assayed. Values expressed are mean  $\pm$  s.d. from three independent experiments.



**Figure 2** Reduced accumulation of Texas Red dextran-10, a fluid-phase marker, in CP-r cells. (A, B) Cells were incubated with  $10\text{ mg ml}^{-1}$  of Texas Red dextran-10 at  $37^\circ\text{C}$  for 2 h, then monitored under a laser scanning confocal microscope (Bio-Rad, Molecular Probes, Eugene, OR, USA) at a  $\times 600$  magnification. (C, D) A time course of Texas Red dextran-10 accumulation was performed by FACS analysis. Cells were incubated with  $3\text{ mg ml}^{-1}$  of Texas Red dextran-10 at  $37^\circ\text{C}$  for up to 7 h. (E) A semiquantitative analysis based on the data of Figure 2C and D.

as compared to the KB-3-1 cells, indicating fewer functional EGF receptors on their surface. This result has been confirmed by quantitation of EGF receptors in the plasma membrane as determined by Western blot (data not shown). In order to compensate for the reduced number of EGF receptors on the surface of KB-CP20 cells, these cells were transfected with EGF receptor cDNA. A clone (KB-CP20-EGFR-cl-6) with receptor number more comparable to KB-3-1 cells was used for the following experiments. As shown in Figure 3A and B, both KB-3-1 and KB-CP20-EGFR-cl-6 cells internalised the majority of the

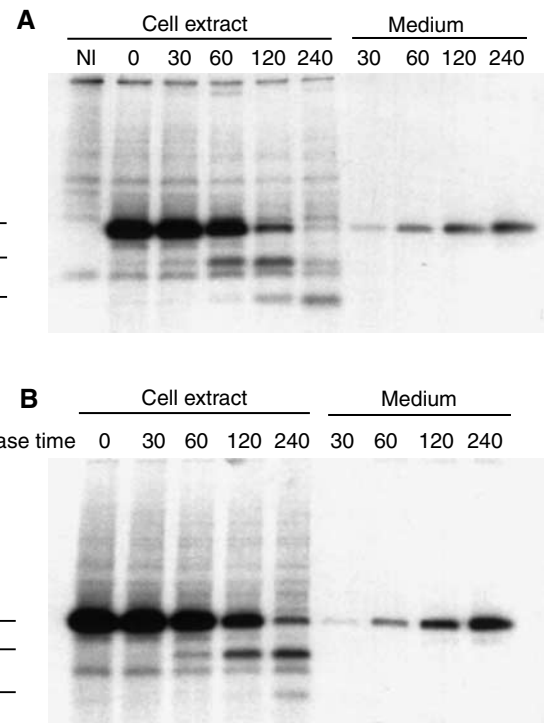


**Figure 3** Kinetics of <sup>125</sup>I-EGF uptake, degradation and release into the medium by KB-3-1 (**A**) and KB-CP20 cells (**B**). Details of the experimental procedure are given in Materials and Methods. Values are mean  $\pm$  s.d. from three independent experiments. (**C**) Kinetics of TCA precipitable <sup>125</sup>I-EGF release by KB-3-1 and KB-CP20 cells. Total medium at different time points of <sup>125</sup>I-EGF uptake was divided into two equal parts. Total radioactivity was determined in one half and the radioactivity in the TCA-precipitated fraction of the remaining half was also determined. Values expressed here are the mean of two independent experiments.

surface bound ligand within 30 min (nondissociated radioactivity). Similarly, the release of radioactive ligand into the culture medium after internalisation followed similar kinetics. However, after 120 min, for KB-CP20-EGFR-cl-6 cells 45% of the radioactive ligand released into the medium, after internalisation, could be precipitated by TCA compared to only 12% in the case of KB-3-1 cells (Figure 3C). These results indicate that degradation of internalised radiolabelled EGF is more efficient in CP-s cells than in the resistant cells, suggesting that CP-r cells may have a defect in lysosomal acidification.

### Secretion and processing of procathepsin L

Malignantly transformed cells are known to secrete a large amount of the 42 kDa procathepsin L into the culture medium (Miesenbock *et al* 1998). To understand if cellular secretion of this protease is affected by the development of the CP-r phenotype in KB-3-1 cells, we studied the kinetics of procathepsin L secretion into the culture medium by KB-3-1 and KB-CP20 cells. Our results demonstrate that both KB-3-1 and KB-CP20 cells secrete procathepsin L into the

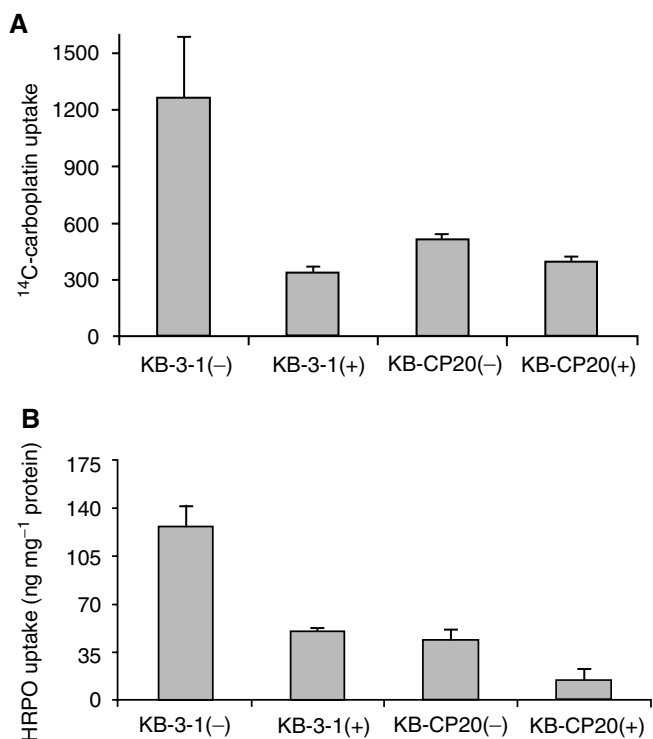


**Figure 4** Kinetics of human procathepsin L secretion and processing in KB-3-1 (**A**) and KB-CP20 (**B**) cells. Cells were radiolabelled briefly for 30 min with Trans-<sup>35</sup>S-label and chased for varying time periods (0 – 240 min, as shown on the top of the gel panels A and B) in serum-free medium. At the end of each time period, medium was saved and cells were lysed after washing with ice-cold PBS. The radiolabelled human procathepsin L was immunoprecipitated using a polyclonal antibody. The immunoprecipitates were resolved on SDS – PAGE and subjected to fluorography. The values given on the left side of the panels indicate the molecular weight of different forms of cathepsin L in kDa.

culture medium in comparable amounts (Figure 4A, B). However, the kinetics of processing of procathepsin L is different in the two cell lines. In KB-3-1 cells, the unprocessed 34 kDa cathepsin L is detectable in the first 30 min of the chase period and the 26 kDa mature cathepsin L appears at 60 min. In these cells, the majority of the 42 kDa procathepsin L form is processed to the 26 kDa mature form of cathepsin L by 240 min, and the 34 kDa form is barely detectable at this period. In contrast, in KB-CP20 cells the 34 kDa band appears after a 60 min chase, and the 26 kDa mature cathepsin L is barely detectable even after 240 min. At this time in KB-CP20 cells, all three forms of cathepsin L (42, 34 and 26 kDa bands) are detectable, the 34 kDa form being predominant. However, at 240 min, the 26 kDa form is predominant in KB-3-1 cells and the other two forms of cathepsin L are barely detectable. These results indicate that the rate of processing of 42 kDa procathepsin L into its enzymatically active 26 kDa mature form is dramatically slowed in KB-CP20 as compared to the sensitive parental cells. However, the secretory pathway for procathepsin L appears to be intact.

### Bafilomycin A<sub>1</sub> treatment and uptake of <sup>14</sup>C-carboplatin and HRPO

Ligands internalised by receptor-mediated endocytosis may be degraded by proteases in the lysosomal compartment. The above results suggested a possible lysosomal defect in the CP-r cells. Since acidic lysosomal pH plays a very important role in the processing of proteases and degradation of proteins in this

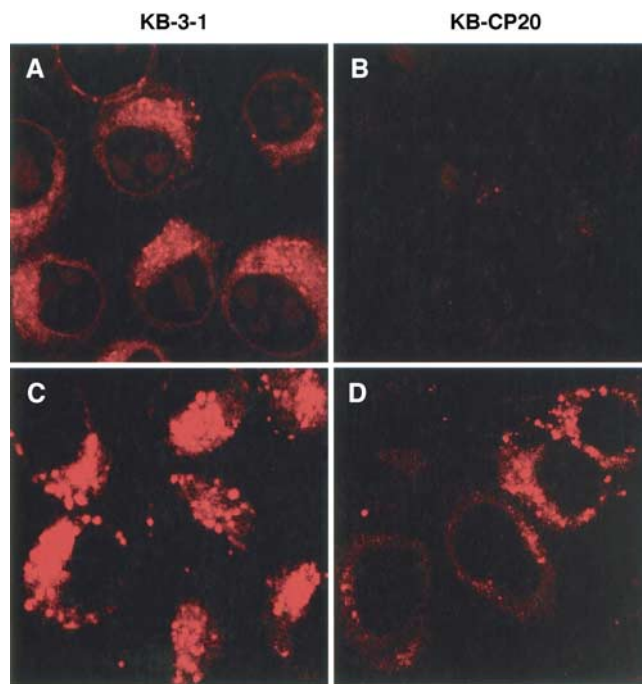


**Figure 5** Effect of bafilomycin A<sub>1</sub> treatment on the uptake of <sup>14</sup>C-carboplatin (A) and HRPO (B) by CP-r and CP-s cells. Cells were washed twice with prewarmed serum-free DMEM and incubated at 37°C with the same medium in the presence (+) or absence (-) of 1 μM bafilomycin A<sub>1</sub>. After 30 min medium was removed and fresh DMEM containing <sup>14</sup>C-carboplatin (2 μCi ml<sup>-1</sup>) or HRPO (2 mg ml<sup>-1</sup>) with (+) or without (-) bafilomycin A<sub>1</sub> was added. Then the incubation was continued at 37°C in a CO<sub>2</sub> incubator. After 1 h for <sup>14</sup>C-carboplatin uptake and 2 h for HRPO uptake, cells were processed as described in Materials and Methods. Values are mean ± s.d. from three independent experiments.

compartment, we altered lysosomal/endosomal pH by treatment with bafilomycin A<sub>1</sub> and studied its effect on the uptake of <sup>14</sup>C-carboplatin by CP-r and CP-s cells. The results are given in Figure 5A. Treatment with bafilomycin A<sub>1</sub> (1 μM) resulted in a four-fold decrease in the accumulation of <sup>14</sup>C-carboplatin by KB-3-1 cells. This treatment resulted in little or no decrease in the accumulation of <sup>14</sup>C-carboplatin in KB-CP20 cells. We observed a 2 – 2.5-fold decrease in the uptake of HRPO (Figure 5B) by bafilomycin A<sub>1</sub> treatment in both CP-r and CP-s cells. These results demonstrate that blocking of lysosomal/endosomal acidification in cells has effects similar to the phenotype of CP-r on uptake of HRPO and <sup>14</sup>C-carboplatin in KB-3-1 cells. Additional studies showed that bafilomycin treatment of KB-3-1 cells also blocked the degradation of <sup>125</sup>I-EGF as was seen in untreated KB-CP20 cells (data not shown).

### Changes of lysosome pH in CP-r cells

A pH-sensitive fluorescent dye LysoSensor DND 198 (Molecular Probes, Inc., Eugene, OR, USA) was used to determine the intracellular lysosomal pH in living cells. The intracellular fluorescence intensity was greatly decreased in the CP-r cells (Figure 6A, B), consistent with an increase in lysosomal pH in the resistant cells. Use of LysoTracker Red DND-99, which is a lysosomal dye not sensitive to pH, showed the presence of lysosomes in the CP-r cells (Figure 6D), although perhaps somewhat decreased in number in comparison to the CP-s cells



**Figure 6** Confocal microscopic fluorescence images of lysosomal pH (A, B) by LysoSensor DND 198 and lysosomes (C, D) by LysoTracker Red DND-99 in KB-3-1 and KB-CP20 cells. (A, C): KB-3-1 cells; (B, D): KB-CP20 cells.

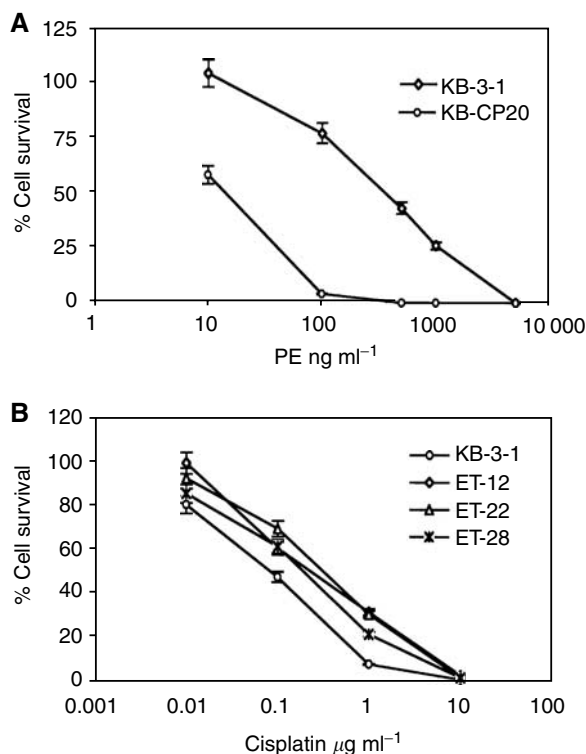
(Figure 6C). Using the cellubrevin pHluorins, it was found that the sensitive cells have an average ratio of 1.1 – 1.4 (corresponding to an endosomal pH of 5.5 – 6.0). The resistant cells have an average ratio of 2.0 – 2.6, corresponding to an endosomal pH of 7.2 – 7.5 (data not shown), suggesting an alkalinisation of the endosomes in the CP-r cells.

### Crossresistance of CP-r cells and *Pseudomonas* exotoxin (PE)-resistant KB cells and reduced accumulation of <sup>14</sup>C-carboplatin

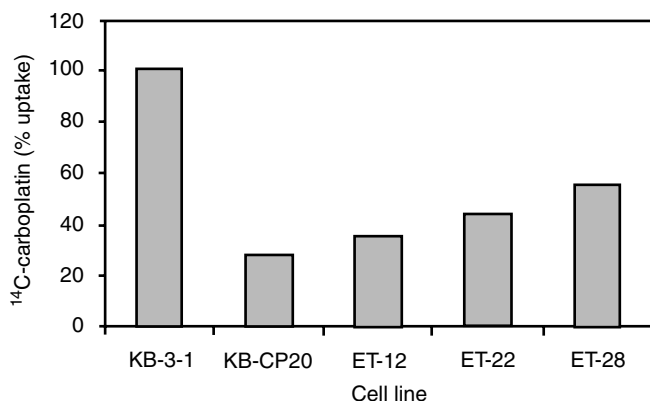
The killing curves shown in Figure 7A indicate that the KB-CP20 cells were about 12-fold more resistant than the KB-3-1 cells to PE. Furthermore, cell lines previously selected for resistance to *Pseudomonas* toxin (ET cells) (Amano *et al*, 1998) were also more crossresistant to cisplatin by four-fold compared to the parental wild-type KB-3-1 cells (Figure 7B). Uptake assays of <sup>14</sup>C-carboplatin in these ET cells demonstrate a significant reduction in accumulation in these ET cell lines (Figure 8).

### DISCUSSION

Cisplatin is a potent anticancer drug, but its clinical effectiveness is undermined by the inherent and acquired resistance of tumour cells to this drug. Although the mechanism of CP-r has been postulated to be multifactorial, decreased accumulation of this drug has been consistently observed in resistant tumour cells (Naredi *et al*, 1994). Previously, we and others observed reduced uptake of cisplatin or its analogue carboplatin in CP-r cells, with no difference in the efflux of this drug between sensitive and resistant cells (Loh *et al*, 1992; Mistry *et al*, 1992; Shen *et al*, 2000). These studies indicate that defective uptake of CP was one of the mechanisms responsible for the resistance. In the present study, receptor-mediated and fluid-phase endocytosis were assessed using the well-characterised ligand EGF to understand the



**Figure 7** Killing curves of KB-3-1 and KB-CP20 cells with unconjugated PE (A), and human *Pseudomonas* exotoxin-resistant cell lines ET-12, ET-22 and ET-28 to cisplatin in comparison to their wild-type KB-3-1 cells (B) were determined as described in Materials and Methods.



**Figure 8** Uptake assay of <sup>14</sup>C-carboplatin. Cells were incubated with <sup>14</sup>C-carboplatin at 37°C for 1 h. Radioactivity was measured as described in Materials and Methods.

molecular basis of reduced CP uptake leading to CP-r. Fluid-phase endocytosis, as measured by uptake of HRP and Texas Red dextran-10, was found to be reduced in CP-r cells. While receptor-mediated endocytosis for EGF was intact, trafficking of EGF to lysosomes and/or lysosomal degradation of EGF appeared to be defective in CP-r cells.

We have previously reported a pleiotropic defect in the expression of many cell surface proteins in CP-r cells (Shen *et al*, 1998). The decreased uptake and cell surface binding of radiolabelled EGF by CP-r cells observed in the present study suggests the downregulation of this receptor on the cell surface or its decreased synthesis in these cells. Western blot analysis

revealed decreased levels of the EGF receptor in CP-r cells (unpublished data), an observation consistent with our previous results (Mistry *et al*, 1992). However, expression of the EGF receptor in KB-CP20 cells at a level comparable to the KB-3-1 cells also did not result in a proportionate increase in cell surface binding of radiolabelled EGF (data not shown). These results suggest that in addition to the decreased synthesis of EGF receptor, CP-r cells may also harbour a defect in targeting it to the cell surface or in membrane protein recycling. In recent studies (Liang XJ, Shen DW, Garfield S, and Gottesman MM, submitted), we have clearly demonstrated a defect in cell surface localisation for two additional cell surface proteins.

In receptor-mediated endocytosis, ligand-receptor complexes are internalised and transported via clathrin-coated vesicles to the endosomes. Many endocytosed ligands including EGF dissociate from their receptor in the acidic environment of endosomes and are finally degraded in the lysosomal compartment (Maxfield and Yamashiro, 1991). Approximately 90% of the endocytosed <sup>125</sup>I-EGF is degraded into monoiodotyrosine (which cannot be precipitated by TCA) by human fibroblasts in 0.5–2.0 h (Carpenter and Cohen, 1976). Our results (Figure 3) suggest that the degradation of endocytosed EGF is not as efficient in KB-CP20 cells as in KB-3-1 cells. Since internalised EGF is degraded in the lysosomal compartment, decreased degradation in the resistant cells could be because (i) internalised EGF is not reaching the lysosomal compartment, (ii) the lysosomal compartment in KB-CP20 cells does not contain proteases and (iii) functionally inactive proteases are present in the lysosomes.

We have previously demonstrated that KB-3-1 cells express large quantities of cathepsin L, a lysosomal cysteine protease, the majority of which is secreted into the culture medium (Chauhan *et al*, 1998). In the present study, we used cathepsin L to compare the expression of lysosomal proteases in KB-3-1 and KB-CP20 cells, and our results demonstrate that both CP-r and CP-s cells express and secrete this protease at comparable levels (unpublished data). Thus, these results rule out the possibility of proteases being absent in CP-r cells and demonstrate that there is no defect in the secretory mechanism of these cells. Human cathepsin L is synthesised as a 42 kDa preproenzyme and autoprocessed into a 34 kDa proenzyme and enzymatically active 26 kDa forms in the lysosomes (Salminen and Gottesman, 1990; Chauhan *et al*, 1998). Lysomotrophic agents like NH<sub>4</sub>Cl and chloroquine inhibit the processing of this enzyme to smaller forms, indicating the requirement of acidic pH for this purpose (Chauhan S and Gottesman MM, unpublished results). In the present study, we observed delayed processing of cathepsin L in the lysosomes of CP-r cells (Figure 5), consistent with the reduced degradation of <sup>125</sup>I-EGF.

Bafilomycin A<sub>1</sub>, a macrolide antibiotic that has been demonstrated to inhibit vacuolar type H<sup>+</sup>-ATPase, inhibits endosomal and lysosomal acidification and blocks lysosomal degradation of EGF in A431 cells (human epidermoid carcinoma cells) (Yoshimori *et al*, 1991; Melikova *et al*, 2001), blocked degradation of <sup>125</sup>I-EGF in KB-3-1 cells. Therefore, since acid lysosomal pH is essential for proper modification, processing and trafficking of proteins, we wished to determine whether the inefficient acidification of endosomal/lysosomal compartment(s) was responsible for the inefficient degradation of <sup>125</sup>I-EGF in KB-CP20 cells. Using a pH-sensitive fluorescence-labelled probe (LysoSensor DND-189, Molecular Probes, Eugene, OR, USA), a reduction in lysosomal pH and intensity of lysosomal staining was detected in KB-CP-r cells as compared to the KB-3-1 cells. Thus, a defect in lysosomal acidification may play a role in the improper processing of cathepsin L as well as inefficient degradation of internalised <sup>125</sup>I-EGF in CP-r cells. Additional data also show that the early endosome compartment in CP-r cells is more basic than in the CP-s cells, while the Golgi complex appears to have a pH similar to the CP-s cells. In a recent publication, the intracellular pH of CP-r cells



was also found to be significantly higher than that of the sensitive parental cells (Murakami *et al*, 2001).

Low endosomal pH has been demonstrated to be essential for *Pseudomonas* exotoxin cytotoxicity in mammalian cells (Pastan *et al*, 1992). Inhibitors of endosomal acidification protect cells from diphtheria toxin and *Pseudomonas* exotoxin cytotoxicity (Olsnes and Sandvig, 1988; Pastan *et al*, 1992). Lyall *et al* (1987) isolated human clones of KB-3-1 cells resistant to EGF-*Pseudomonas* exotoxin conjugates. In the present study, these cells were found to be crossresistant to CP (Figure 7A). Similarly, CP-r cells were found to be crossresistant to *Pseudomonas* exotoxin (Figure 7B) and to show reduced accumulation of <sup>14</sup>C-carboplatin (Figure 8). These results suggest that the endosomal/lysosomal acidification defect may be responsible for some of the observed CP-r in KB-CP20 cells.

Conclusions of this study are in agreement with a previous report, which demonstrated increased sensitivity of tumour cells to cisplatin at lower cellular pH (Laurencott *et al*, 1995). The involvement of defective vacuolar acidification in CP-r was further confirmed by the four-fold reduction in carboplatin uptake by

KB-3-1 cells after bafilomycin A<sub>1</sub> treatment. In addition to reduced carboplatin uptake, treatment of KB-3-1 cells with bafilomycin A<sub>1</sub> blocked <sup>125</sup>I-EGF degradation and HRPO uptake (Figure 5). Our results clearly demonstrate that CP-r cells, which exhibit reduced uptake of <sup>14</sup>C carboplatin and HRPO, harbour an endosomal acidification defect (Figures 1–6). Blocking endosomal/lysosomal acidification by bafilomycin A<sub>1</sub> in KB-3-1 cells mimics the CP-r phenotype in terms of <sup>14</sup>C-carboplatin and HRPO uptake. Therefore, we conclude that defective endosomal/lysosomal acidification may, at least, partly be responsible for CP-r due to its reduced uptake, resulting in less drug reaching the cytotoxic targets.

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