Inhibition of energy production as a strategy for potentiation of anticancer chemotherapy was investigated using 1 glycolysis inhibitor and 1 fatty acid β-oxidation inhibitor—2-deoxyglucose and etomoxir, respectively, both known to be clinically well tolerated. Eighteen anticancer drugs were screened for potentiation by these inhibitors. 2-deoxyglucose potentiated acute apoptosis (24 hr) induced mainly by some, but not all, genotoxic drugs, whereas etomoxir had effect only on cisplatin. By contrast, etomoxir did potentiate the overall, 48 hr effects of some genotoxic drugs, and was in addition more efficient than deoxyglucose in potentiating the overall effects of several non-genotoxic drugs. Both types of potentiation were largely lost in the absence of p53. Because cisplatin was potentiated by both energy inhibitors in both types of assay, but not at additional concentrations and over longer time. Both energy inhibitors strongly potentiated non-apoptotic concentrations of cisplatin in p53-wildtype as well as in p53-deficient, cisplatin-resistant HCT116 colon carcinoma cells. Reduced ATP levels correlated with, but were not sole determinants, the antiproliferative effects. We conclude that the long-term effects of cisplatin potentiation are important and either p53-independent or improved by a lack of p53. We also conclude that although the potentiated drugs as yet have no obvious mechanistic factor in common, the strategy holds promise with genotoxic as well non-genotoxic anticancer drugs.

Key words: chemopotentiation; antiproliferation; apoptosis; glycolysis; β-oxidation

Interest in tumour cell energy metabolism as a therapeutic target is on the increase. It is well known that tumour cells are highly dependent on glycolysis for ATP production also under aerobic conditions (the Warburg effect) and that this is connected to decreased oxidative phosphorylation in mitochondria. Glycolysis produces pyruvate for the mitochondrial Krebs cycle which in turn provides reducing equivalents for the electron transport chain required for oxidative phosphorylation. In addition, glucose and hexokinase—the first enzyme in glycolysis—are required for subsequent NADPH and NADPH-dependent biosyntheses, e.g., purine synthesis. The glucose dependence of tumour cells is reflected in increased cellular uptake of glucose and increased expression of glycolysis-related genes such as glucose transporters, hexokinase and glyceraldehyde-3-phosphate dehydrogenase.1–5 The increased levels of glucose transporters and glucose uptake is indeed clinically exploited for imaging of tumours using positron emission tomography (PET) based on tumour uptake of [18]F-deoxyglucose (18-FDG).

Tumour biology and tumour cell phenotypic traits are greatly influenced by the changes in energy metabolism. It has thus been shown that tumour progression and drug resistance correlate with a set of metabolic shifts.5 In the first phase, tumour cells are still sensitive to therapy, show high mitochondrial inner membrane potential (Δψ), low use of fatty acids as energy source and, during cellular stress, production of cytotoxic levels of reactive oxygen species (ROS). In the second phase, cells become increasingly resistant to chemotherapy drugs, and this is at least in part due to efficient cellular scavenging of therapy-induced ROS. This capacity for scavenging may reflect increased endogenous protection against ROS formation that is due to electron leakage in the electron transport chain. Tumour cells in the second phase also show increased β-oxidation of fatty acids and increased glycolysis rates which likely compensate the reduction in cellular ATP production and support viability in the hypoxic tumour.5,6 Based on increased expression of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase and with repression of the catalytic subunit of the mitochondrial H⁺-ATP synthetase (β-F1-ATPase), a cellular bioenergetic index with prognostic value for various carcinomas has been created.4,5 Links between oncogenic and bioenergetic alterations have also been reported in a set of progressively transformed fibroblasts, i.e., transforming oncogenes were shown to lead to incrementally increased dependence on glycolysis and decreased dependence on mitochondrial ATP.5

Glycolysis can be targeted using the glucose analogue 2-deoxyglucose (DG) which is phosphorylated by hexokinase, the first enzyme in glycolysis, but which is not further metabolized in the glycolytic process. This titration of endogenous glucose therefore leads to decreased ATP and NADH levels. DG has been shown to sensitize gliomas and other cancer cells to radiation both in vitro and in vivo.7–11

The metabolic changes in tumours may also involve increased fatty acid turnover. Increased fatty acid synthesis in various types of carcinoma is indicated by high levels of fatty acid synthase expression and β-oxidation of long-chain fatty acids.5,12,13 Inhibitors of fatty acid synthase have been shown to have antiproliferative effects in tumor cells.14 As an alternative, we have here targeted tumour cell energy metabolism by inhibiting fatty acid β-oxidation using etomoxir. Etomoxir acts by inhibiting carnitine palmitoyl transferase-1, the enzyme that transports long-chain fatty acids into mitochondria. It was launched as a diabetes drug and has undergone clinical trials as treatment for cardiovascular disease.15 Both DG and etomoxir have thus been evaluated for clinical use.

We have here examined the antiproliferative potential of DG and etomoxir not only as single drugs but in particular as low-dose chemopotentiators. To gain insight into which type(s) of known anticancer drugs that can be potentiated by these 2 inhibitors of energy metabolism, and whether they potentiate the same set of drugs, a collection of 18 chemotherapeutic agents—standard drugs as well as experimental, and representing various modes of action—was screened in the presence and absence of DG and etomoxir for potentiation of acute (stress-induced) apoptosis as well as of the total antiproliferative effect, as assessed by total protein levels.

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Material and methods

Cell culture and drug treatment

HCT116 colon carcinoma cell lines (p53 wt and a p53 −/− subclone) were maintained in McCoy’s 5A modified medium (16.7 mM glucose), and MDA-MB-231 human metastatic breast carcinoma cells in DMEM medium. The media were supplemented with 10% fetal calf serum, l-glutamine and penicillin/streptomycin, and cells were kept at 37°C in 5% CO2. Cells plated in plastic culture dishes were treated with drugs 1 day after plating, and the drugs were present throughout the indicated incubation periods. DG and etomoxir were obtained from Sigma-Aldrich (St. Louis, MO), and cisplatin (Platinol) was from Bristol-Myers-Squibb. The sources of drugs in the drug screen are indicated in Table I.

Assessment of ATP and reducing equivalents

ATP levels were determined at indicated time points using the luciferase-based Aposensor ATP depletion assay kit (Alexis Biochemicals, Lausen, Switzerland) according to the manufacturer’s instructions. The MTT assay (Promega Corp., Madison, WI) was used to indirectly assess short-term effects on reducing equivalents. The assay was performed at indicated timepoints and according to the manufacturers’ instructions.

Assessment of apoptosis in cell culture and in explant slices

Caspase-specific cleavage of cytokeratin-18 (CK18) results in a stable fragment with a neoptope (CK18-Asp396) that is recognized by the M30 antibody and quantified using the Apoptosense assay (PEVIVA AB, Stockholm, Sweden). The assay was performed according to the manufacturer’s instructions. Briefly, 8–10,000 cells/well were seeded in 96-well plates and after 1 day treated with drugs as indicated. At the end of the drug incubation period, NP-40 was added to the medium to 0.1% to lyse floating as well as attached cells. The method for tumour explant slices was adapted from Ref. 17. Briefly, SCID mice were intravenously injected with MDA-MB-231 human breast carcinoma cells. Tumors were excised and cut into approximately 200-μm thick slices using a vibratome, or precision cutting tissue slicer (Krumdieck, Alabama Research and Development Corp., Munsford, AL). The slices were kept in cell culture medium and were after 24 hr treated with the indicated drugs. Supernatant samples were collected every 24 hr for assessment of apoptosis using the Apoptosense assay.

LHD release and inhibition of cell growth

The lactate dehydrogenase release assay (Promega Corp., Madison, WI) was used to assess necrotic cell death and was performed according to the manufacturer’s instructions on both supernatants and on cell lysates to calculate the relative amounts of released LDH activity. Inhibition of cell growth was assessed by 2 methods. Total cellular protein remaining in microplate wells after treatment was quantitated after precipitation with trichloroacetic acid and staining with sulfonhideamine B, according to manufacturer’s instructions (Sigma-Aldrich kit), and results were calculated based on relative absorbance values in untreated and treated samples. In 24-well plates, absolute number of remaining attached cells were also determined by counting using a Burker chamber after incubation for 2 or 3 days as indicated. Data are presented as percent of cell numbers in untreated control samples at each timepoint.

Intracellular reducing power

The cell-permeable resazurin agent AlamarBlue is easily reduced intracellularly and can replace oxygen as electron acceptor. Both the non-reduced and reduced forms were quantitated spectrophotometrically in live cells to calculate the percentage of reduced AlamarBlue, all according to the manufacturer’s instructions (Biosource, Camarillo, CA).

Statistical methods

For data from 2 separate experiments (n = 2), statistical averages are shown, and for data involving more than 2 experiments, the standard deviation of the mean is also shown.

Results

ATP depletion by etomoxir and 2-deoxyglucose

Incubation of HCT116 colon carcinoma cells with the β-oxidation inhibitor etomoxir at 100 and 200 μM for 5 hr led to ATP levels at 80 and 75%, respectively, of the level in untreated cells (Fig. 1a). Glycolysis was blocked using the unmetabolizable glucose analogue DG. Treatment of HCT116 cells with 10 and 20 mM DG for 5 hr led to almost 50% loss of cellular ATP (Fig. 1a). In accordance with these inhibitors affecting 2 different pathways to ATP production, their combined effect was nearly additive (Fig. 1a). The “energy sensor” AMP-activated kinase (AMPK) is activated by an increase in the AMP:ATP ratio, and activation was here assessed as levels of phosphorylated AMPK. DG (20 mM) had induced AMPK activation by 16 hr, but not 8 hr, whereas etomoxir did not activate it at all (Fig. 1b), suggesting that overall

<table>
<thead>
<tr>
<th>Name</th>
<th>Conc. (μM)</th>
<th>Mechanism</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cisplatin</td>
<td>12</td>
<td>DNA &amp; protein adducts</td>
<td>Bristol Myers Squibb</td>
</tr>
<tr>
<td>2. Oxaliplatin</td>
<td>10</td>
<td>DNA adducts</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>3. Etoposide</td>
<td>5</td>
<td>Topo II inhibitor</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>4. Ellipticine</td>
<td>2.5</td>
<td>DNA adducts; topo II inhibitor</td>
<td>NC1 mechanistic set</td>
</tr>
<tr>
<td>5. U0126</td>
<td>10</td>
<td>MEK1 inhibitor</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>6. MG132</td>
<td>0.04</td>
<td>Proteasome inhibitor</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>7. Curcumin</td>
<td>10</td>
<td>Antioxidant, gene regulation</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>8. Chlorotriethylphosphine;gold(I) (TEP Au)</td>
<td>0.2</td>
<td>Alkalylator; oxidative stress</td>
<td>NC1 mechanistic set</td>
</tr>
<tr>
<td>9. Betulinic acid</td>
<td>5</td>
<td>Apoptosis via mitochondria</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>10. Arsenic trioxide</td>
<td>3</td>
<td>Oxidative stress?</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>11. Lonidamine</td>
<td>5</td>
<td>Energy metab. inhibitor</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>12. 5-Fluorouracil</td>
<td>0.75</td>
<td>Nucleoside analogue</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>13. Methotrexate</td>
<td>25</td>
<td>Antimetabolite</td>
<td>Schir Laboratories</td>
</tr>
<tr>
<td>14. Quercetin</td>
<td>150</td>
<td>Redox effects; gene regulation</td>
<td>Sigma Aldrich</td>
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<tr>
<td>15. Piroxicam</td>
<td>0.1</td>
<td>Topo I inhibitor</td>
<td>NCI mechanistic set</td>
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<tr>
<td>16. Camptothecin</td>
<td>1250</td>
<td>GSK3β inhibitor</td>
<td>Sigma Aldrich</td>
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<tr>
<td>17. LICI</td>
<td>10</td>
<td>DNA intercalation; binds to HIF1alpha promoter</td>
<td>Sigma Aldrich</td>
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</table>
depletion is greater with DG. To our knowledge, the use of higher concentrations of etomoxir has not been reported in the literature.

Screening of 18 anticancer agents for apoptosis potentiation by 2-deoxyglucose and etomoxir

Eighteen anticancer agents—standard as well as experimental—were selected to represent a variety of mechanisms of action (Table I). Primary or stress-induced apoptosis occurring within 24 hr, was quantified using the Apoptosense \( ^{1} \) ELISA-type assay based on caspase-specific cleavage of cytokeratin-18 (CK18) to a stable fragment. \(^{16} \) For each drug, 1 low-apoptotic concentration (<2-fold apoptosis induction over 24 hr) was first identified in HCT116 cells. The cells were then treated for 24 hr with this concentration of drug in the presence or absence of 10 mM DG or 200 \( \mu \)M etomoxir. These concentrations induced no or little apoptosis in HCT116 parental and p53-deficient cells, nor in MDA-MB-231 or MCF-7 breast carcinoma cells (not shown). After 24 hr, accumulated apoptosis was quantitated and results are summarized as box plots in Figure 2a. With potentiation defined as a >2.4-fold increase compared to drug alone, DG potentiated primary apoptosis induced by the genotoxic drugs cisplatin and ellipticine, the MEK1 inhibitor U0126 and the proteasome inhibitor MG132 (Table IIA). Reducing DG to 5 mM still allowed potentiation of cisplatin- and ellipticine-induced apoptosis only (not shown). With etomoxir, cisplatin and lithium chloride were the only drugs to be potentiated (Table IIA). Using DG, the apoptosis screen was performed at the same concentrations also on HCT116 p53-/- cells. The absence of p53 was protective (Fig. 2a), except for potentiation of cisplatin- and piroxicam-induced apoptosis (Table IIB).

Using high doses, genotoxic treatment may lead to increased necrosis rather than apoptosis, due to depletion of NAD\(^{+}\) and/or excessively decreased ATP levels. \(^{18,19} \) Genotoxic treatment at lower doses may furthermore take more than 24 hr to lead to apoptosis. Drugs that were not potentiated by DG in parental HCT116 cells were therefore combined with DG for 48 hr. Because etomoxir was less efficient than DG both in decreasing ATP and in potentiating apoptosis, it was not examined for necrosis. Release of phosphorylated (activated) AMPK and total AMPK were determined by western blotting. Antibodies were from Cell Signaling and were used at 1:1,000. The experiment was repeated with identical results.

![FIGURE 1](image1.png)  
**FIGURE 1** – Effects of 2-deoxyglucose and etomoxir on cellular ATP levels. (a) ATP levels in HCT116 cells were assessed after 5 hr treatment with indicated doses of etomoxir (ETO) and 2-deoxyglucose (DG). Data are from 3 separate experiments. (b) HCT116 cells were incubated with 10 mM DG or 200 \( \mu \)M etomoxir for 8 or 16 hr. Levels of phosphorylated (activated) AMPK and total AMPK were determined by western blotting. Antibodies were from Cell Signaling and were used at 1:1,000. The experiment was repeated with identical results.

![FIGURE 2](image2.png)  
**FIGURE 2** – Screening of anticancer drugs for apoptosis potentiation by DG and etomoxir. (a) Eighteen anticancer agents, experimental as well as standard therapeutic drugs, chosen to represent different mechanisms of action (Table I) were first tested for dose-dependent apoptosis induction over 24 hr in HCT116 cells (not shown). Using concentrations which induced up to 2-fold apoptosis, the drugs were screened for apoptosis potentiation by DG at 10 mM or etomoxir at 200 \( \mu \)M. Cells were treated in triplicate samples for 24 hr, and accumulated apoptosis in total lysates was then assessed using the Apoptosense \( ^{1} \) assay, and the average fold potentiation of drug-induced apoptosis was calculated. Drug combinations with DG were repeated in HCT116 p53-/- cells. The box plots each show the median, and lower and upper quartiles, respectively, of the resulting values from 2 experiments. T-bars indicate highest and lowest values. (b) Drugs that were not potentiated in the screen were assessed over 48 hr using accumulated apoptosis and LDH release as endpoint read-outs. For each sample, apoptosis was thus specifically quantitated as fold increase in levels of DEVDase-cleaved CK-18 released into the supernatant (top), and cell death as fold increase in released LDH (bottom). In these experiments, etoposide, curcumin, TEPAu, 5-fluorouracil and piroxicam were not potentiated by DG (not shown). The data shown for oxaliplatin (OXP), lonidamine (LA) and lithium chloride represent the average of 2 separate experiments with quadruplicate samples. (Top) MDA-MB-231 breast carcinoma xenograft explants were kept in culture and treated with DG (10 mM), camptothecin (CPT; 5 \( \mu \)M), etoposide (VP16; 5 \( \mu \)M) or echinomycin (ECH; 5 \( \mu \)M) per se, and in the indicated combinations. At 24, 48 and 72 hr, supernatant samples were taken for apoptosis analysis using Apoptosense \( ^{1} \). Shown are the averages of 2 samples. (Bottom) MDA-MB-231 breast carcinoma xenograft explants were kept in culture and treated for DG (10 mM), cisplatin (cDDP; 12 \( \mu \)M) and etomoxir (ETO; 200 \( \mu \)M), alone and in combinations. At 24, 48 and 72 hr, supernatant samples were taken for apoptosis analysis using Apoptosense \( ^{1} \). Shown are the averages of 2 samples.
DG and etomoxir on p53 induction were examined; DG (10 mM; effects were all but lost (Table IIB and not shown). The effects of were similar to those in the parental cells but potentiation per se cisplatin. Using the Apoptosense side and echinomycin, or with combinations of DG, etomoxir and potentiation of selected drugs in this tumour model, explant slices such samples also contain infiltrating stromal cells. To determine to being thick enough to retain a certain level of hypoxia, of LDH through rupturing membranes was used to determine necrosis, and the Apoptosense\textsuperscript{a} assay to detect any concomitant apoptosis. By either assay, 5 of the drugs—etoposide, curcumin, betulinic acid, piroxicam and echinomycin—were again not potentiated by DG (not shown). By contrast, DG combined with oxaliplatin, lonidamine or lithium chloride induced release of LDH in the total absence of caspase-dependent apoptosis, showing that with these drugs, the effect of DG involves necrosis rather than apoptosis (Fig. 2b).

DG and etomoxir potentiate apoptosis in tumour tissue explants

It is well known that compared to monolayer culture 3D culture is more physiological and leads to a different gene expression pattern.\textsuperscript{20,21} To validate the use of DG and etomoxir in a 3D model and in another cell line (MDA-MB-231 breast carcinoma harboring P-gp protein and mutant p53), MDA-MB-231 xenograft tumours were excised from mice and cut into 200 μm slices that can be kept in culture for treatment over several days.\textsuperscript{17} In addition to being thick enough to retain a certain level of hypoxia, such samples also contain infiltrating stromal cells. To determine potentiation of selected drugs in this tumour model, explant slices were treated with DG in combinations with camptothecin, etoposide and echinomycin, or with combinations of DG, etomoxir and cisplatin. Using the Apoptosense\textsuperscript{a} assay, accumulated apoptosis was quantitated in supernatant samples collected at 24, 48 and 72 hr. It should be noted that being based on cleavage of CK18, which is not expressed in stromal cells, this assay is specific for tumour cell apoptosis. The results (Fig. 2c) show that DG and etomoxir potentiated drug-induced apoptosis also in this system. Similar results have been obtained also using human ovarian cancer biopsies (unpublished).

Screening for potentiation of total antiproliferative effects

Primary apoptosis and/or necrosis are not the only possible antiproliferative effects. Many anticancer agents, in particular at lower doses, require days to induce an antiproliferative sequence of events involving, e.g., cell cycle arrest, autophagy or mitotic catastrophe. We therefore examined DG- and etomoxir-mediated potentiation of the total antiproliferative effect of each drug after 48 hr and at the same low-apoptotic drug concentration as in the apoptosis screen. DG was reduced to 5 mM to prevent a too large effect per se. Potentiation was assessed using protein precipitation and sulforhodamine B staining for spectrophotometric quantitation. A box-plot representation based on the percentual increase in total effect compared to the effect of DG or etomoxir alone (Fig. 3a) shows that although DG potentiated more drugs than did etomoxir, the effect of etomoxir was greater when it did potentiate. In the HCT116 p53 \textsuperscript{+/−} cells, effects of DG and etomoxir per se were similar to those in the parental cells but potentiation effects were all but lost (Table IIB and not shown). The effects of DG and etomoxir on p53 induction were examined; DG (10 mM;
24 hr) led to 1.4-fold induction of p53, compared to almost 2-fold by 12 μM cisplatin (Fig. 3b). Etomoxir at 100 μM had no effect, while 200 μM led to 1.3-fold induction (Fig. 3b). Although the effects are small, it is thus conceivable that such induction may play a role in potentiation (Table IIb). Because cisplatin was potentiated by both drugs this potentiation was further investigated separately (see below).

The overall potentiation was greatest for drugs which per se had no or little antiproliferative effect (Fig. 3c). The total effects of oxaliplatin, lonidamine, MG132, curcumin and methotrexate were not at all potentiated by either drug (not shown). Together with Figure 2b, this indicates that with oxaliplatin and lonidamine the main effect of DG is to switch the response from apoptosis to necrosis. Altogether, the data (Fig. 3c; Table IIc) show that while DG and etomoxir have similar effects on etoposide, camptothecin and U0126, they differ greatly in their effects on quercetin, betulinic acid, arsenic trioxide and TEPAu. Furthermore, the effect of combining DG and etomoxir per se was large (Fig. 3c; Table IIc), in accordance with their having different modes of action.

### Table IIc – Summary of Potentiation Effects—Potentiation (>20%) of Total Antiproliferative Effect Over 48 HR in HCT116 Cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Effect of 5 mM DG (% increase; corrected for effect of DG only)</th>
<th>Effect of 200 μM ETO (% increase; corrected for effect of ETO only)</th>
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<td>Etomoxir</td>
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<tr>
<td>Etoposide</td>
<td>34 29</td>
<td></td>
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<tr>
<td>U0126</td>
<td>27 23</td>
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</tr>
<tr>
<td>Quercetin</td>
<td>22 57</td>
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</tr>
<tr>
<td>LiCl</td>
<td>20 Not potentiated</td>
<td></td>
</tr>
<tr>
<td>Betulinic acid</td>
<td>Not potentiated</td>
<td>43</td>
</tr>
<tr>
<td>As2O3</td>
<td>20 Not potentiated</td>
<td>43</td>
</tr>
<tr>
<td>TEPAu</td>
<td>Not potentiated</td>
<td>38</td>
</tr>
</tbody>
</table>

**Figure 4** – Further potentiation of cisplatin by DG and etomoxir. (a) HCT116 wildtype-p53 and HCT116 p53−/− cell lines were treated in quadruplicates for 24 hr with cisplatin (cDDP) and DG as indicated. Accumulated apoptosis was assessed in total cell lysates using the Apoptosense<sup>®</sup> assay. Data are from 2 separate experiments, and similar results were obtained when experiments were repeated. (b) HCT116 cells were treated with indicated concentrations of cisplatin, DG and additional pyruvate. Apoptosis was quantitated after 24 hr using supernatant samples and the Apoptosense<sup>®</sup> assay. (c) Potentiation of 1 μM cisplatin by 5 mM DG was assessed in HCT116 and HCT116 p53−/− cells by determining the number of attached cells remaining after 48 and 72 hr treatment. Data represent averages of 3 separate experiments and are presented as percent survival in relation to cell counts in controls at the same timepoints. Bars: standard deviation of the mean. (d) Potentiation of 3 μM cisplatin by 200 μM etomoxir was assessed in HCT116 and HCT116 p53−/− cells by determining the number of attached cells remaining after 48 hr treatment. Data represent averages of 3 separate experiments and are presented as percent survival in relation to cell counts in controls at the same timepoints. Bars: standard deviation of the mean. (e) HCT116 parental cells (500 cells/10-cm dish) were treated with indicated doses of etomoxir alone (empty squares) or etomoxir combined with 1 μM cisplatin (black squares) or with 5 mM DG (empty circles). The medium was changed after 16 hr and cultures were postincubated for 10 days. After fixation and staining, the colonies in each dish were counted. Results are the averages of 2 separate experiments shown as percent of untreated control. Note the log scale of the y-axis. (f) ATP levels in HCT116 cells were assessed after 3 hr treatment with indicated combinations of etomoxir (ETO; 200 μM), cisplatin (cDDP; 12 μM) and DG (10 nM). Data are from 3 experiments and bars represent standard deviation of the mean.
combinations with arsenic trioxide (Fig. 3e), nor with cisplatin or etoposide (not shown), suggesting that the particular signaling induced by quercetin or betulinic acid is such that the additional and potentiating effect of etomoxir involves intracellular redox regulation. An increased electron flow as indicated by these experiments may lead to induction of ROS. To examine involvement of ROS, cells were treated with selected combinations in the presence or absence of the ROS scavenger N-acetylcysteine (NAC; 5 mM) and effects on apoptosis and total protein were assessed after 24 hr. NAC was found to completely block DG-mediated potentiation of cisplatin-induced apoptosis and had a small inhibitory effect on potentiation of ellipticine (not shown). Drug combinations with etomoxir were examined using total protein as read-out. NAC did not inhibit etomoxir potentiation of any of the tested drugs (quercetin, betulinic acid, etoposide or camptothecin) (not shown), indicating that ROS are not involved in potentiation.

Potentiation of the antiproliferative effects of cisplatin

In the above experiments, the only drug to be well potentiated by both DG and etomoxir was cisplatin, a cornerstone chemotherapeutic drug which induces p53-dependent apoptosis. For reference, in HCT116, 20 μM cisplatin induces caspase activation and nuclear fragmentation in approximately 50% of the cells within 24 hr, while in HCT116 p53−/− cells, this dose induces <10% apoptosis [22 and not shown]. Here, DG combined with cisplatin at 12 μM led to apoptosis potentiation in wildtype HCT116 whereas the effect was far lesser in HCT116 p53−/− cells (Fig. 4a, Table IIB), suggesting that a certain minimal level of apoptotic signaling must be present for this type of potentiation. Potentiation was furthermore prevented by pyruvate, the end-product of glycolysis (Fig. 4b). Similar results were obtained in MDA-MB-231 cells (not shown).

In the screening experiments, a single dose of each drug was used. To further validate the observed potentiation of cisplatin, effects were examined at different time points. We have earlier shown that cisplatin at <10 μM induces senescence rather than apoptosis in HCT116 cells.22 To examine whether DG can affect non-apoptotic cisplatin doses, HCT116 parental and HCT116 p53−/− cells were treated with 1 and 3 μM cisplatin in the presence or absence of 5 mM DG. After 48 and 72 hr, survival was assessed by counting cells. DG was found to potentiate the effect of as little as 1 μM cisplatin (Fig. 4c). Taking into consideration that survival is here expressed as percent of cells in untreated controls at a certain time point, and that the controls are proliferating, the effect was particularly remarkable in the p53−/− cells (Fig. 4c). Thus, cisplatin-only treatment did not lead to a net loss of cells, and they in fact resumed growth. By contrast, DG co-treatment prevented such restoration.

As shown above, etomoxir potentiated cisplatin-induced apoptosis over 24 hr both in HCT116 wt-p53 and p53−/− cells (Table IIB). Similar to DG, when combined with non-apoptotic doses of cisplatin, etomoxir potentiated the total antiproliferative effect, as assessed by cell counting (Fig. 4d). The effect was similar in the p53-wt and the p53−/− cells (Fig. 4d). In colony formation assays, etomoxir dose-dependently potentiated the effect of 1 μM cisplatin, while the effect of DG plus etomoxir remained similar to that observed in the total-protein assay (Figs. 4e and 3b).

The effects on ATP levels of cisplatin combined with DG or etomoxir were examined. The 2 cisplatin combinations had similar and, compared to each drug per se, additive effects (Fig. 4f), suggesting that this increased loss correlates with subsequent potentiation. The combination of DG plus etomoxir had a similar additive effect on ATP levels (Fig. 4f). This combination, however, was less efficient in the clonogenic assay (Fig. 4e), indicating that a separate cisplatin-induced factor is indeed required for potentiation.

Discussion

Compared to normal cells, tumour cell ATP production depends greatly on glycolysis and often also on fatty acid β-oxidation. Targeting these bioenergetic processes has therefore recently attracted interest as a strategy for tumour-specific therapy.5–11 Here, we have screened for potentiation effects of 2 small-molecule inhibitors of glycolysis DG and of β-oxidation (etomoxir), respectively, on the apoptotic as well as total antiproliferative effects induced by 18 anticancer drugs. The choice of DG and etomoxir was in part based on their documented lack of adverse effects in human subjects. Tumour-specific uptake of the glucose analogue DG is reflected in its use as fluorodeoxyglucose (FDG) for PET imaging. It is also well tolerated by humans, with up to 2 mg/kg body weight.23 In clinical trials for DG-mediated potentiation of irradiation of gliomas, 4 weekly fractions of oral DG (200 mg/kg) were administered, resulting in blood levels of 5–6 mM DG after 1 hr of treatment and in excellent tolerance in all 20 patients.24 This concentration in blood is particularly encouraging in view of our present finding that single-dose administration of 5 mM DG potentiated the effect of cisplatin also in resistant p53-null cells. Eto- mocixir has undergone clinical trials for treatment of cardiovascular disease, and healthy human subjects have also been treated with 3 × 120 mg etomoxir over 36 hr without reported adverse effects.24 In accordance with effects on energy metabolism, both DG and etomoxir rapidly decreased cellular ATP levels. However, in the combinations with genotoxic drugs, DG and etomoxir differed with respect to their ability to potentiate induction of acute apoptosis (<24 hr). Some, but not all, of the potentiated drugs were genotoxic, suggesting that the effect of DG does not directly involve DNA damage response and repair as such. Differential activation of, e.g., PARP, may contribute to the total effect of the combination treatment. Thus, DNA alkylation, PARP activation, concomitant depletion of NAD+ and thereby loss of glycolytic ATP23 might explain the observed loss of ATP by cisplatin alone. ATP levels were then further reduced by co-treatment with DG or etomoxir (Fig. 4f). Since the combination of DG plus etomoxir led to a similar loss of ATP as well as to increased apoptosis, the ATP levels at 5 hr correlated with apoptosis responses of these combinations. They did not, however, correlate with loss of clonogenicity, since combining DG with etomoxir did not greatly reduce viability in this assay.

Loss of ATP beyond a certain threshold leads to necrosis. This likely explains necrosis in DG combinations with oxaliplatin and the mitochondrial inhibitor lonidamine. DG did not potentiate these drugs, as seen in the total-protein assay, but rather switched the response from apoptosis to necrosis. This necrotic response is also in accordance with more toxic DNA lesions by oxaliplatin than by cisplatin.26 By contrast, apoptosis induced by the genotoxic drug ellipticine was potentiated to a similar degree as cisplatin. Because it shares several features with cisplatin, e.g., nucleus-independent apoptosis induction and induction of an ER-stress-like effect,27–29 it is possible that such pathways contribute to apoptosis potentiality by DG. Among the potentiated non-genotoxic drugs, U0126 is used as a MEK1 inhibitor but it has also been shown to have a weak indirect inhibitory effect on mitochondrial FIFO-ATPase activity29 which may contribute to apoptosis through non-necrotic energy depletion. A similar case is seen with lithium chloride which has been reported to inhibit citric acid cycle activity.30 Lithium was potentiated by etomoxir and in combination with DG led to massive necrosis.

Acute, stress-induced apoptosis often involves formation of ROS. When used with the 2 most potentiated drugs, the ROS scavenger NAC blocked potentiation of only cisplatin, while ellipticine potentiation was only partially inhibited, suggesting that ROS are not an absolute requirement for potentiation. Etomoxir data showed a striking potentiation especially of non-genotoxic drugs (Table IIC); the non-apoptotic potentiation over 24 hr was not prevented by co-treatment with NAC (not shown).

Acute apoptosis was not, however, a key determinant of drug potentiation by either DG or etomoxir. The data indicate that longer-term effects on viability are important, and such long-term cell death may include, e.g., secondary apoptosis, mitotic catastro-
phe, premature senescence, autophagic processes and combina-
tions thereof. These outcomes are drug- as well as dose-dependent.
In addition to loss of ATP, glycolysis inhibition leads to reduced
levels of NADH and acetyl-CoA which has consequences for
redox regulation and macromolecule synthesis, and inhibition of
β-oxidation leads to accumulation of long-chain fatty acids, some
of which may be cytotoxic.31 These effects in combination with
signaling induced by certain drugs are candidates for long-term
effects on viability.

The outcome also depends on the particular mechanisms of
each drug in the screen. Etoposide exemplifies a slower, low-apo-
ptotic development of potentiation effects, as observed in the
HCT116 total-protein assay and in the MDA-MB-231 xenograft
cultures, but despite a wide range of concentrations in both cell
lines over 24 and 48 hr etoposide did not show any apoptosis or
necrosis potentiation (not shown). A similar case is presented also
carbohydrate culture and 3D growth.20,21 This phenomenon may explain the
apoptotic sensitivity of the MDA-MB-231 xenograft explants to
DG potentiation of etoposide.

Additional drug-specific mechanisms with long-term effects
include the downregulation of glycolytic enzymes reported for
some genotoxic drugs including cisplatin and etoposide.32 With
genotoxic and non-genotoxic drugs alike, autophagy presents an
interesting and complex possibility, in that it can to some extent
protect cells from temporary energy depletion but will in the long
term lead to cell death. Autophagy has furthermore been reported to
be p53-dependent,18 wherefore 1 may speculate that the lack of
its protective function contributes to potentiation of cisplatin in
HCT116 p53+/− cells both by DG and etomoxir. Several non-
genotoxic drugs, in particular quercetin, were strikingly potenti-
ated by etomoxir (Table IIC). Quercetin is a bioflavonoid which
induces, e.g., autophagy in Ras-transformed cells,33 anti- as well as
pro-oxidant effects,34 inactivation of EGF receptor and FAK ty-
rosine kinases,35 inactivation of AKT1 phosphorylation.36 Similar to
the rapid effect on redox regulation indicated by the quercetin
experiments here, these effects are compatible with a role in the
mechanism(s) of potentiation. Arsenic trioxide was also potenti-
ated by etomoxir and, like quercetin, has multiple effects, e.g.,
autophagic cell death,37 induction of ceramide synthesis,38 lypo-
soomal destabilization,39 all of which may be important for potenti-
ability. However, arsenic trioxide differed from quercetin in that
we did not see any rapid increase in intracellular reducing
power.

In summary, we have investigated inhibition of energy produc-
tion as a strategy for chemopotentiation. Using 2 inhibitors known
to be clinically well tolerated, we show that the strategy holds
promise with genotoxic as well non-genotoxic anticancer drugs. It
may furthermore be advantageous especially in tumour cells with
mutant or deficient p53, since such cells are often more dependent
on aerobic glycolysis. The antiproliferative effect of cisplatin was
potentiated by both DG and etomoxir, and the clinical importance,
but also the clinical oto- and nephrotoxicity, of cisplatin make
chemopotentiation an attractive possibility for extending its clini-
cal use. The explant experiments demonstrate that in this more
physiological system, multiresistant MDA-MB-231 cells can be
sensitized to chemotherapy-induced apoptosis using DG as well as
etomoxir. Finally, potentiation was observed with single-
dose administration, wherefore protocols involving even lower
doses and preincubation and/or repeated boosting with DG or
etomoxir are likely to further underscore their potential as chemo-
potentiators.

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