Metabolic interaction between amino acid deprivation and cisplatin synergistically reduces phosphoribosyl-pyrophosphate and augments cisplatin cytotoxicity

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Cisplatin is a mainstay of cancer chemotherapy. It forms DNA adducts, thereby activating poly(ADP-ribose) polymerases (PARPs) to initiate DNA repair. The PARP substrate NAD⁺ is synthesized from 5-phosphoribose-1-pyrophosphate (PRPP), and we found that treating cells for 6 h with cisplatin reduced intracellular PRPP availability. The decrease in PRPP was likely from (1) increased PRPP consumption, because cisplatin increased protein PARylation and PARP1 shRNA knock-down returned PRPP towards normal, and (2) decreased intracellular phosphate, which down-regulated PRPP synthetase activity. Depriving cells of a single essential amino acid decreased PRPP synthetase activity with a half-life of ~8 h, and combining cisplatin and amino acid deprivation synergistically reduced intracellular PRPP. PRPP is a rate-limiting substrate for purine nucleotide synthesis, and cisplatin inhibited de novo purine synthesis and DNA synthesis, with amino acid deprivation augmenting cisplatin’s effects. Amino acid deprivation enhanced cisplatin’s cytotoxicity, increasing cellular apoptosis and DNA strand breaks in vitro, and intermittent deprivation of lysine combined with a sub-therapeutic dose of cisplatin inhibited growth of ectopic hepatomas in mice. Augmentation of cisplatin’s biochemical and cytotoxic effects by amino acid deprivation suggest that intermittent deprivation of an essential amino acid could allow dose reduction of cisplatin; this could reduce the drug’s side effects, and allow its use in cisplatin-resistant tumors.

Abbreviations
CDDP  Cisplatin
CDI  Coefficient of drug interaction
HPRT  Hypoxanthine phosphoribosyltransferase
NAD⁺  Nicotinamide adenine dinucleotide
PARP  Poly(ADP-ribose) polymerase
PRPP  5-Phosphoribose-1-pyrophosphate
PBS  Phosphate-buffered saline

Cisplatin is active against a wide variety of solid tumors, including bladder, head and neck, lung, ovarian, and testicular cancers, and is required for curative treatment of some of these cancers. Its use is limited by toxic side effects and tumor resistance, with both acquired and intrinsic resistance observed.

Cisplatin acts, in part, by covalently binding to the N7 position of adenine and guanine in DNA, generating intra- and inter-strand adducts. In response to such DNA damage, poly(ADP-ribose) polymerases (PARPs) are activated, catalyzing both auto-poly(ADP-ribosylation) (PARylation) of a central glutamate-rich domain, as well as PARylation of chromatin-associated proteins. This generates branching polymers of ADP-ribose, which serve as scaffolds for DNA repair factors, including those of the mismatch repair and nucleotide exchange repair pathways. PARPs use NAD⁺ as a substrate, and since NAD⁺ synthesis requires two molecules...
of 5-phosphoribose-1-pyrophosphate (PRPP), we hypothesized that cisplatin could deplete intracellular PRPP, a rate-limiting substrate for purine, pyrimidine, and pyridine nucleotide synthesis.

We have shown previously that depriving cells of a single essential amino acid decreases PRPP production, due in part to decreased activity of transketolase, a key enzyme in the non-oxidative pentose phosphate pathway. Both the oxidative and non-oxidative pentose phosphate pathways generate the PRPP precursor ribose 5-phosphate, with the relative contribution of each pathway varying depending on the cell type and culture conditions. The two main mammalian PRPP synthetases, which convert ribose 5-phosphate to PRPP, are multimers of a single polypeptide chain that aggregate into 4, 8, 16, and 32 subunit complexes, with highest activity in the most aggregated states. Aggregation is regulated, in part, by the intracellular phosphate concentration.

We found that cisplatin decreased intracellular PRPP, and that depriving cisplatin-treated cells of a single essential amino acid profoundly reduced PRPP availability and purine and DNA synthesis. The reduction in PRPP was due largely to decreased PRPP synthetase activity and increased PRPP consumption. The combination of cisplatin and amino acid deprivation reduced tumor cell growth both in vitro and in vivo, with the in vivo effect occurring under conditions where each modality alone had no significant effect.

**Results**

Cisplatin decreases PRPP availability, purine and DNA synthesis, and intracellular ATP and NAD: augmentation by amino acid deprivation.

**PRPP availability.** The intracellular concentration of PRPP is low and PRPP is relatively unstable, making it difficult to measure PRPP accurately. We, and others, therefore, have measured “PRPP availability” by following incorporation of radioactive hypoxanthine or adenine into purine nucleotides. This measures the amount of PRPP available to a cell over a set time interval, as opposed to measuring the PRPP concentration at one time point. We found that treating Dih10 mouse hepatoma cells for 6 h with 10 or 25 µM cisplatin minimally affected PRPP availability, whereas 50 and 100 µM cisplatin reduced PRPP availability by ~35% (Fig. 1A, light grey bars; in this figure, and subsequent figures, the control concentration is complete medium without cisplatin). The assay is dependent on the enzyme hypoxanthine–guanine phosphoribosyl-transferase (HPRT), and we showed that HPRT activity measured in cell extracts did not change after 6 h of 100 µM cisplatin treatment (Table 1). At the standard human cisplatin dose of 100 mg/m², the cisplatin plasma concentration reaches 30–50 µM; thus, the cisplatin concentrations used were within the range found in patients’ plasma.

Depriving Dih10 cells of lysine, arginine, or leucine for 6 h reduced PRPP availability by ~25% (Fig. 1A,B). Combining amino acid deprivation with cisplatin strikingly reduced PRPP availability. Augmentation by lysine deprivation occurred at 10, 25, 50, and 100 µM cisplatin, reducing PRPP availability at 50 and 100 µM cisplatin by ~85% (Fig. 1A). Arginine and leucine deprivation were studied at 100 µM cisplatin, the standard cisplatin concentration used in most of the remainder of studies in Dih10 cells, and we found that the combination of amino acid deprivation and cisplatin reduced PRPP availability by 79 and 76%, respectively (Fig. 1B). To determine if cisplatin and amino acid deprivation were synergistic in reducing PRPP availability, we calculated the coefficient of drug interaction (CDI), with amino acid deprivation considered as a drug (CDI calculation is described in Experimental Procedures). We found that 100 µM cisplatin combined with lysine, arginine, or leucine deprivation yielded CDI values of 0.3, 0.5, and 0.4, respectively, indicating strong synergy. We could not use the method of Chou and Talalay to calculate the combination index, because this method requires multiple concentrations of both drugs; at the low concentrations of amino acids where we would have to conduct the experiments, the dose response is steep and the amino acid concentration decreases substantially during the experiment, thus leading to major inaccuracy. Amino acid deprivation did not affect HPRT activity over the time course studied (Table 1).

To determine if cisplatin reduced PRPP availability in other cell types, we examined human ovarian cancer cells. We found that 6 h of cisplatin treatment significantly reduced PRPP availability in IGROV-1 cells as well (Fig. 1C; these studies were conducted at 35 µM cisplatin, because IGROV-1 cells are more sensitive to cisplatin than Dih10 cells). Combining lysine deprivation with cisplatin further reduced PRPP availability, yielding a CDI of 0.7, again indicating synergy (Fig. 1C).

**Purine and DNA synthesis.** PRPP is a key regulator of purine nucleotide synthesis, and we found that cisplatin treatment and lysine deprivation each reduced de novo purine synthesis by ~60%, and that combined together they reduced purine synthesis by ~80% (Fig. 1D). Reduced purine nucleotide synthesis might be expected to reduce DNA synthesis, and we found that cisplatin treatment and lysine deprivation individually reduced DNA synthesis by 43% and 59%, respectively, and that the combination of the two modalities reduced DNA synthesis by 83% (Fig. 1E; data are for a 3 h incubation; at 6 h DNA synthesis was completely halted under the condition of combined cisplatin treatment and lysine deprivation).

**ATP concentration.** Decreased PRPP availability and purine nucleotide synthesis should decrease cellular ATP, and we found that 14 h of cisplatin treatment decreased the intracellular ATP concentration, with lysine deprivation of cisplatin-treated cells causing little additional decrease in ATP (Fig. 1F). The decrease in cellular ATP is noticeably less than the decrease in PRPP availability and purine synthesis, likely because the cellular ATP pool is large and decreased purine synthesis is reflected slowly in the total ATP concentration. Consistent with this notion, we found no change in the intracellular ATP concentration at treatment times of 8 h or less.

**NAD concentration.** PRPP is also required for synthesis of pyridine nucleotides, i.e., NAD and NADP; and 14 h of cisplatin treatment decreased intracellular NAD (Fig. 1G). Depriving cisplatin-treated cells of lysine had no
Figure 1. Cisplatin decreases PRPP availability and purine and DNA synthesis, and reduces intracellular ATP and NAD: augmentation by amino acid deprivation. Dih10 cells (A, B, D–G) or IGROV-1 cells (C) were incubated for 6 h (A–D), 3 h (E), or 14 h (E,G) in complete medium or lysine-, arginine-, or leucine-deficient medium in the absence or presence of the indicated concentrations of cisplatin (A), 100 µM cisplatin (B,D–G), or 35 µM cisplatin (C). (A–C) [8-14C]-hypoxanthine incorporation into purine nucleotides was measured during the last 20 min of the incubation to assess PRPP availability. (D) [14C]-formate incorporation into purines was measured during the last 90 min of the incubation as a measure of de novo purine synthesis. (E) [3H]-thymidine incorporation into DNA was measured during the last 60 min of the incubation. (F,G) The intracellular content of ATP and NAD were measured by a luciferase-based and a cycling colorimetric assay, respectively. In all panels, each symbol in the bar graphs is the mean of duplicate samples from an independent experiment, with the bar height representing the mean value of all experiments and the error bars indicating the standard deviation. *, **, ***, and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively, for the indicated comparisons. # in Panel A indicates $p < 0.001$ for comparison between complete and lysine-deficient medium for each of the four cisplatin concentrations. CDDP, cisplatin; PRPP, phosphoribosyl-pyrophosphate.
additional effect (Fig. 1G). Again, these changes are noticeably less than the decrease in PRPP, likely because the NAD pool is also relatively large.

Cisplatin decreases glucose incorporation into DNA and RNA via the non-oxidative pentose phosphate pathway, and reduces PRPP synthetase activity by reducing intracellular phosphate; augmentation by amino acid deprivation. Pentose phosphate pathway. PRPP can be produced via the oxidative and non-oxidative pentose phosphate pathways. Assessing carbon flow through the oxidative pentose phosphate pathway is generally done by following [1-14C]glucose oxidation to [14C]carbon dioxide (Fig. 2A), realizing that some oxidation of the C-1 carbon of glucose can occur in the tricarboxylic acid cycle17. Using this method, we found no change in oxidative pentose phosphate pathway activity after 6 h of cisplatin treatment, lysine deprivation, or the combination of the two (Fig. 2B). Assessing carbon flow through the non-oxidative pentose phosphate pathway is more difficult, because the pathway is not linear and several reactions are at equilibrium. We have shown previously that following [1-14C]glucose incorporation into nucleic acids can be used as a measure of non-oxidative pentose phosphate pathway activity, recognizing this method also assesses PRPP synthetase activity as well as multiple downstream steps (Fig. 2A)6, 7, 18. Using this method, we found a marked reduction in glucose incorporation into DNA and RNA after 6 h of either cisplatin treat-

### Table 1. HPRT activity. Dih10 mouse hepatoma cells were incubated for 6 h in full medium (Control), lysine-free medium (Lys-), full medium containing 100 µM cisplatin (CDDP), or lysine-free medium containing 100 µM cisplatin (Lys-/CDDP). The cells were extracted, and HPRT activity in the extracts was measured following [8-14C] hypoxanthine incorporation into nucleotides in the presence of 1 mM PRPP. Data are the mean ± S.D. of three independent experiments.

| Condition       | Enzyme activity (nmol/min/mg protein) |
|-----------------|---------------------------------------|
| Control         | 1.10 ± 0.19                           |
| Lys-            | 1.21 ± 0.17                           |
| CDDP            | 1.14 ± 0.16                           |
| Lys-/CDDP       | 1.03 ± 0.18                           |

### Table 2. Mouse serum amino acid concentrations. Mice were injected in the flank with Dih10 hepatoma cells, and received either normal rodent chow (Control) or three cycles of lysine-deficient chow (Lys-) for 5 days followed by 3 days of normal chow as depicted in Fig. 4A. Some animals on either normal chow or lysine-deficient chow received one injection of cisplatin (CDDP). At the end of the third cycle of the lysine-deficient diet, the animals were euthanized, and blood was obtained by cardiac puncture. Amino acids in the serum were measured by high performance liquid chromatography coupled to post-column derivitization with ninhydrin. Data are mean values from five animals in each group; *** = p < 0.001 compared to control animals.

| Amino acid | Control µmol/L | Lys− µmol/L | CDDP µmol/L | Lys−/CDDP µmol/L |
|------------|----------------|-------------|-------------|------------------|
| Ala        | 382            | 355         | 382         | 522              |
| Arg        | 115            | 108         | 117         | 117              |
| Asn        | 36             | 33          | 32          | 39               |
| Asp        | 22             | 21          | 19          | 17               |
| Cys        | 5.5            | 3.9         | 4.8         | 4.0              |
| Gln        | 505            | 464         | 442         | 447              |
| Glu        | 72             | 56          | 61          | 51               |
| Gly        | 391            | 415         | 393         | 636              |
| His        | 81             | 95          | 91          | 92               |
| Ile        | 80             | 92          | 98          | 86               |
| Leu        | 129            | 152         | 147         | 130              |
| Lys        | 296            | 141***      | 304         | 129***           |
| Met        | 57             | 54          | 83          | 96               |
| Phe        | 64             | 71          | 74          | 70               |
| Pro        | 40             | 44          | 41          | 51               |
| Ser        | 162            | 192         | 173         | 220              |
| Thr        | 216            | 209         | 217         | 278              |
| Trp        | 40             | 34          | 34          | 34               |
| Tyr        | 68             | 47          | 64          | 56               |
| Val        | 153            | 185         | 189         | 192              |
ment or lysine deprivation, with the combination of the two modalities further reducing glucose incorporation (Fig. 2C). The lack of a change in glucose oxidation by cisplatin treatment, lysine deprivation, or the combination of the two indicates that the cause of their reduction in glucose incorporation into nucleic acids occurred distal to glucose uptake and phosphorylation.

**PRPP synthetase activity and intracellular phosphate.** PRPP synthetases are highly regulated and are rate-limiting enzymes for purine nucleotide synthesis\(^4\). To determine if the enzymes were affected by cisplatin treatment and/or amino acid deprivation, we measured enzyme activity in cell extracts, and found no change in activity in extracts derived from cisplatin-treated cells in the presence of 40 mM phosphate in the assay buffer, a phosphate concentration where the enzymes should be activated maximally (Fig. 2D)\(^9,10\). Under these conditions, lysine deprivation significantly reduced enzyme activity, and the combination of cisplatin and lysine-deprivation yielded similar activity as lysine deprivation alone (Fig. 2D). Since lysine deprivation quickly reduces protein synthesis\(^4\), these data suggest that PRPP synthetase(s) may have a relatively short half-life. Repeated measures of PRPP synthetase activity over 8 h of lysine deprivation yielded a linear decrease in activity, with a 50% loss of activity by ~8 h (Fig. 2E).

We next measured PRPP synthetase activity in the presence of 1 mM phosphate in the assay buffer, a phosphate concentration that is limiting to enzyme activity so that phosphate in the cell extracts will contribute to enzyme activity. We now found decreased enzyme activity in cisplatin-treated cells (Fig. 2F). Lysine deprivation decreased enzyme activity to a similar extent as in high phosphate buffer, and the combination of cisplatin and lysine deprivation further decreased enzyme activity (Fig. 2F). These data suggested that cisplatin decreased the intracellular phosphate concentration, which could occur through increased protein PARylation, since each ADP-ribose unit contains two phosphate groups; previous workers have observed increased protein PARylation in cisplatin-treated cells\(^20,21\). We, therefore, measured inorganic phosphate in cell extracts and found that cisplatin reduced the phosphate concentration by 16% compared to control cells (Fig. 2G). Although this is a relatively small decrease in phosphate concentration, it could have a major impact on PRPP synthetase activity, due to the enzymes’ high phosphate sensitivity at phosphate concentrations <10 mM\(^2\). Lysine deprivation had no effect on intracellular phosphate, and the combination of cisplatin and lysine deprivation yielded a similar decrease in intracellular phosphate as cisplatin alone (Fig. 2G).

**Cisplatin reduces PRPP availability through increased protein PARylation and PRPP consumption; amino acid deprivation prevents the increased protein PARylation.** The amount of PRPP available to a cell is determined by the amount of PRPP synthetase activity and the amount of PRPP consumed in PRPP-dependent reactions. NAD synthesis consumes two PRPP molecules, and since NAD is the substrate of PARP, increased protein PARylation could reduce PRPP availability. To assess this possibility, we first confirmed increased protein PARylation in cisplatin-treated Dih10 cells (Fig. 2H; note increased PARylation of proteins at molecular weights of ~160, 38, and 33 kDa; the 38 and 33 kDa bands are quantified). The increased protein PARylation occurred in the absence of a significant change in PARP1 expression (Fig. 2I). We then used an shRNA approach to reduce PARP1, which accounts for most cellular PARylation activity\(^3\), and derived independent clones of cells infected with control virus or the PARP1 shRNA virus. PARP1 protein was reduced an average of 58% in cells infected with the PARP1 shRNA vector, compared to cells infected with control vector (Fig. 2I). In cisplatin-treated cells, PARP1 knock-down increased PRPP availability almost two-fold, to within 80% of that found in cells infected with control vector (Fig. 2J). PARP1 knock-down also increased PRPP availability in cells in control medium, lysine-free medium, and lysine-free medium in the presence of cisplatin, although the increase was less than in cells treated with cisplatin alone (Fig. 2J; PARP1 availability increased by 20, 44, and 38% in control, lysine-deprived, and lysine-deprived cells treated with cisplatin, respectively). Of note, protein PARylation was unchanged in lysine-deprived cells, but lysine deprivation prevented the increase in protein PARylation found in cisplatin-treated cells (Fig. 2H). The inhibition of protein PARylation in lysine-deprived cells occurred in the absence of a significant change in PARP1 protein (Fig. 2I). We will consider the implications of these findings in the Discussion.

**Cisplatin cytotoxicity and induction of apoptosis and DNA strand breaks are augmented by amino acid deprivation.** Since cisplatin's biochemical effects were augmented by amino acid deprivation, we assessed whether cisplatin's cytotoxic effects were also amplified by amino acid deprivation. Culturing Dih10 cells for 72 h in medium containing limiting concentrations of lysine, arginine, or leucine increased the cells’ sensitivity to the growth inhibitory effects of 0.1–10 µM cisplatin, reducing the IC50 in each case by ~50% (Fig. 3A–C). The concentrations of lysine, arginine, and leucine were 75, 125, and 150 µM, respectively, compared to 798, 398, and 801 µM in full medium, respectively. Consistent with these data, cisplatin’s inhibition of clonal cell growth was augmented by limiting the lysine concentration, both in Dih10 cells and the ovarian cancer cell lines (Fig. 3D,E).

Cisplatin cytotoxicity is mediated primarily via apoptosis\(^3\), but the combination of cisplatin and amino acid deprivation could potentially cause cellular necrosis. Over the 6 h time frame of the biochemical experiments presented in Figs. 1 and 2, Dih10 cells showed no significant increase in apoptosis at 10, 25, 50, or 100 µM cisplatin, as measured by caspase 3 activation (Fig. 3F; the blot in Fig. 3G shows 100 µM cisplatin). Similarly, 6 h of lysine deprivation did not increase apoptosis of Dih10 cells (Fig. 3F,G). However, the combination of 50 and 100 µM cisplatin with lysine deprivation significantly increased apoptosis (Fig. 3F,G). The increased apoptosis under the condition of 50 and 100 µM cisplatin combined with lysine starvation correlates with the profound decrease in PRPP that occurred under these conditions (Fig. 1A). We found no increase in cell necrosis as measured by lactate dehydrogenase loss into the medium at 10, 25, 50, or 100 µM cisplatin alone or when combined with...
Cisplatin and intermittent amino acid deprivation decrease tumor growth in mice. The increased cytotoxicity observed by combining amino acid deprivation with cisplatin suggested that the anti-tumor effects of cisplatin could be augmented by amino acid deprivation in vivo. We chose to use a nude (Nu/Nu) mouse allograft tumor model with intermittent deprivation of lysine. Seven days after injecting Dih10 cells into the flanks of the mice, we randomized them into four different groups: Group 1, fully supplemented diet; Group 2, fully supplemented diet with one injection of 7.5 mg/kg cisplatin; Group 3, three 5 d periods of a lysine-free diet separated by two 3 d periods of a fully-supplemented diet; and Group 4, the combination of cisplatin and lysine deprivation (Fig. 3H). We conclude that amino acid deprivation augments cisplatin-induced apoptosis, but does not induce cellular necrosis.

DNA strand breaks are characteristic of cisplatin treatment, and 100 μM cisplatin increased the number of double-stranded DNA breaks in Dih10 cells more than 16-fold [Fig. 3I; DNA breaks were measured by counting γ-H2AX-positive foci]. Lysine deprivation increased DNA breaks 3.8-fold, which was not significant compared to Group 1; 1.3% decrease in Group 2; 10.3% decrease in Group 3, and 11.6% decrease in Group 4. In animals from pilot studies, a lysine deprivation protocol that reduced the serum lysine concentration by about 50%, decreased DNA strand breaks by 28%, of the full range of the three independently-derived clones, each measured in duplicate, and the horizontal line in the box is the mean value. *, **, ***, and **** indicate p < 0.05, < 0.01, < 0.001, and < 0.0001, respectively, for the indicated comparisons, or in Panel H to the control condition. CDDP, cisplatin; PARP1, poly(ADP-ribose) polymerase-1; PPP, pentose phosphate pathway; PRPP, phosphoribosyl-pyrophosphate. lysine deprivation (Fig. 3H). We conclude that amino acid deprivation augments cisplatin-induced apoptosis, but does not induce cellular necrosis.

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A. Full medium and 75 μM lysine
B. Full medium and 125 μM arginine
C. Full medium and 150 μM leucine

D. Dih10 Control, Low Lys, CDDP

E. IGROV-1 Control, Low Lys, CDDP

F. Cleaved Caspase 3 (absorbance units normalized to actin)

G. Western blot

H. LDH in the medium (% of total)

I. Vehicle

0.28 ± 0.23
1.07 ± 0.35

CDDP

4.73 ± 1.27**
9.82 ± 2.33****
We found that cisplatin and deprivation for a single essential amino acid individually reduced cellular availability of PRPP. When combined together, the two modalities profoundly and synergistically decreased PRPP, decreased tumor cell growth in vitro and in vivo, and decreased DNA synthesis, while increasing cellular apoptosis and DNA strand breaks (Fig. 4F).

The decrease in PRPP in cisplatin-treated cells was in part from increased PRPP consumption due to increased protein PARylation (Fig. 4F), because PARP1 knock-down using an shRNA returned PRPP availability in cisplatin-treated cells to values near those found in control cells. We also tried a pharmacological approach to knock down PARP activity, but three different PARP inhibitors—olaparib, rucaparib, and E7449—unexpectedly decreased PRPP availability at concentrations that inhibit PARP possibly through off-target effects. The PARP1 shRNA did not fully restore PRPP to control values in cisplatin-treated cells, likely because: (1) PARP1 knock-down was incomplete as shown in Fig. 2J; (2) other PARPs could be present in Dih10 cells; and (3) cisplatin shRNA did not fully restore PRPP to control values in cisplatin-treated cells. We also tried a pharmacological approach to dissolve the stain in acid, and are the mean ± SD of three independent experiments derived from duplicate wells. (F–I) Dih10 cells were cultured for 6 h in complete DMEM (control) in the presence of 100 µM cisplatin (G,I) or the indicated cisplatin concentration (F,H). As noted, some cells were cultured in lysine-deficient medium (Lys-). (F,G) Cells were extracted, and proteins were analyzed by SDS-PAGE/immunoblotting using antibodies against actin and cleaved caspase-3. (F) Protein bands were scanned on a Li-Cor Odyssey instrument, and the sum of the density of the 17 and 19 kDa cleaved caspase-3 bands was normalized to the respective actin bands. Each symbol is from an independent experiment, with the bar height representing the mean value of all experiments and the error bars indicating the standard deviation. (G) A full blot is shown. (H) The medium and cells were harvested separately, and lactate dehydrogenase (LDH) activity in the medium and cell extracts was measured in a coupled spectrophotometric assay. Data are presented as the percent of LDH activity in the medium. (I) Cells were stained using an anti-γ-H2A.X antibody and a Texas Red-conjugated secondary antibody, and nuclei were counterstained with DAPI. The scale bar is 50 µm and magnification was 20 ×. The number of γ-H2A.X-positive foci per cell were counted in 50 cells per condition in four independent experiments, yielding a total of 200 cells counted per condition. The numbers shown are the mean ± SD of the number of foci per cell. *, **, ***, and **** indicate p < 0.05, <0.01, <0.001, and <0.0001, respectively, for the indicated comparisons or, in Panels D and E, for comparison to the control. # and ## in Panels D and E indicate p < 0.05 and <0.01, respectively, for comparison to the individual conditions of cisplatin treatment or lysine deprivation. CDDP, cisplatin.

Discussion

We found that cisplatin and deprivation for a single essential amino acid individually reduced cellular availability of PRPP. When combined together, the two modalities profoundly and synergistically decreased PRPP, decreased tumor cell growth in vitro and in vivo, and decreased DNA synthesis, while increasing cellular apoptosis and DNA strand breaks (Fig. 4F).

The decrease in PRPP in cisplatin-treated cells was in part from increased PRPP consumption due to increased protein PARylation (Fig. 4F), because PARP1 knock-down using an shRNA returned PRPP availability in cisplatin-treated cells to values near those found in control cells. We also tried a pharmacological approach to knock down PARP activity, but three different PARP inhibitors—olaparib, rucaparib, and E7449—unexpectedly decreased PRPP availability at concentrations that inhibit PARP possibly through off-target effects. The PARP1 shRNA did not fully restore PRPP to control values in cisplatin-treated cells, likely because: (1) PARP1 knock-down was incomplete as shown in Fig. 2J; (2) other PARPs could be present in Dih10 cells; and (3) cisplatin decreased intracellular phosphate, thereby decreasing PRPP synthetase activity and PRPP production (discussed in the next paragraph). The PARP1 shRNA increased PRPP availability under all conditions tested, including in control cells, suggesting that a substantial amount of PRPP is consumed by protein PARylation, even under normal, non-stressed conditions. In lysine-starved, cisplatin-treated cells, the PARP1 shRNA had only a modest effect on PRPP availability, and this is likely due to low PRPP synthetase activity. Overall, these data demonstrate a dynamic balance between the activities of PRPP synthetase and PARP1 in determining PRPP availability.

We did not expect cisplatin would reduce intracellular phosphate, but each ADP-ribose unit in poly(ADP-ribose) contains two phosphate moieties, and multiple proteins undergo PARylation after cisplatin generation of DNA adducts and strand breaks23. Since each poly(ADP-ribose) chain can contain up to 250 ADP-ribose units and each protein can have multiple chains attached, it seems possible that cisplatin could transiently decrease the intracellular phosphate concentration via increased PARP activity. At the normal intracellular concentration of 1–2 mM inorganic phosphate, even a modest decrease in phosphate will profoundly reduce PRPP synthetase activity, due to a steep slope in the phosphate response curve of the enzyme between 0 and 10 mM phosphate24.

The half-lives of the two main PRPP synthetases have not been reported previously, due in part to a lack of specific antibodies. Although depriving cells of lysine does not immediately nor fully stop protein synthesis4, we found that PRPP synthetase activity was decreased by 50% after ~ 8 h of lysine deprivation. This measurement almost certainly overestimates the enzymes’ true half-lives, but it indicates that PRPP synthetase activity would be reduced by about 40% during the 6 h time frame of most of our in vitro experiments. The 40% reduction in enzyme activity by amino acid deprivation combined with a 16% reduction in inorganic phosphate by cisplatin would be expected to reduce cellular PRPP synthetase activity, and thus PRPP production. Reduced PRPP synthetase activity during amino acid deprivation is yet another mechanism, in addition to the reduction in transketolase activity that we found previously4, of reduced PRPP production in cells deprived of an essential amino acid.

Reduced PRPP availability likely led to the decrease in purine and DNA synthesis and increase in DNA strand breaks in cells treated with cisplatin and deprived of an amino acid. PRPP is a rate-limiting substrate for...
Scientific Reports | (2020) 10:19907 | https://doi.org/10.1038/s41598-020-76958-7

A

Day 0 7 12 15 20 23 28
Cell Injection
Lysine-deficient diet
Full diet
Lysine-deficient diet
Full diet
Lysine-deficient diet
Euthanasia

B

Tumor Volume (mm³)

Control diet
CDDP, 7.5 mg/kg
Lysine-deficient diet
Lysine-deficient diet, CDDP, 7.5 mg/kg

7 14 21 28

C

Tumor Weight (g)

Control CDDP Lys Lys/CDDP

D

Control Diet Lys-deficient Diet

CDDP Lys-deficient Diet/CDDP

E

Control CDDP Lys Lys/CDDP

4X

20X

F

Cisplatin Treatment

DNA Breaks

↓ Protein PARylation

PRPP Consumption

↓ PRPP

PO₄

PRPP Synthetase

Protein PARylation

↓ Nucleotide Synthesis

Purine, Pyrimidine, & Pyridine

DNA Synthesis

Cell Growth

↓ NAD

Repair of DNA Breaks

Deprivation of Single EAA
Figure 4. Cisplatin and intermittent amino acid deprivation decrease tumor growth in mice. (A) Nude (Nu/Nu) mice were injected on day zero with 1.7 × 10^6 Dih10 cells subcutaneously into both flanks. On day 7, the mice were randomly assigned to four groups: Group 1, fully-supplemented diet throughout the study; Group 2, fully-supplemented diet until day 36, with one injection of 7.5 mg/kg cisplatin; Group 3, three 5-d periods of lysine-free diet followed by two 3-d periods of fully-supplemented diet; Group 4, cisplatin injection followed by the cyclical lysine-free/fully supplemented diet. (B) Tumor volumes were measured weekly using a digital caliper. Group 1 mice, solid line, n = 5; Group 2 mice, dotted line, n = 5; Group 3 mice, dashed line, n = 6; Group 4 mice, line with alternating dots and dashes, n = 6. All mice had bilateral tumors, except for one mouse each in Groups 2 and 3, which had only one tumor. Thus, each symbol represents the mean of 10 tumors in each of the four conditions. In Panels B and C, ** indicates p < 0.01. (E) Hematoxylin and eosin stains of representative tumors from each of the four groups of mice. The samples are shown under 4 and 20 × magnification. All four samples showed some fibrosis with prominent spindle cells. (F) Cisplatin induces DNA breaks, which increase protein PARylation and PRPP consumption, leading to a decrease in intracellular phosphate. Depriving cells of an essential amino acid decreases PRPP synthetase activity, and when combined with a decrease in intracellular phosphate, further decreases enzyme activity. The decrease in PRPP synthetase activity and increase in PRPP consumption markedly reduces PRPP availability, thereby reducing purine nucleotide synthesis and retarding protein PARylation and repair of DNA breaks; although not studied here, the decrease in PRPP likely also decreases pyrimidine and pyridine nucleotide synthesis. Ultimately, the decrease in PRPP likely also decreases pyrimidine and pyridine nucleotide synthesis. Ultimately, the decrease in PRPP availability likely explains the marked increase in DNA strand breaks in cisplatin-treated, lysine-deprived cells, and suggests the breaks were from decreased DNA repair, rather than from an increase in the number of breaks (Fig. 4F). The block of protein PARylation by lysine deprivation occurred in the absence of a significant change in total cellular NAD^+ or PARP1 protein. This suggests, as for DNA synthesis, that protein PARylation may draw from a pool of newly-synthesized NAD^+ that was contracted due to the striking reduction in PRPP in cisplatin-treated, lysine-deprived cells.

Consistent with our results, previous workers have found that PRPP synthetase is highly expressed in cisplatin-resistant human breast cancer cells and that silencing the enzyme reverses cisplatin resistance. Moreover, knockdown of transketolase-like 1, which functions similarly to transketolase, increases cisplatin cytotoxicity in nasopharyngeal carcinoma cells by reducing ribose 5-phosphate, and therefore PRPP. We specifically chose lysine as the main essential amino acid to be restricted both in the cell and animal studies, because of its limited intracellular role, other than being required for protein synthesis. Lysine can be a precursor to carnitine, via trimethyllysine; however, carnitine is present in fetal bovine serum and most animals receive sufficient carnitine in their diets and do not synthesize it from lysine. Thus, the effects of lysine starvation we observed, both in vitro and in vivo, are likely from reduced lysyl t-RNA charging and protein synthesis. Arginine and leucine, the two other amino acids we restricted, are involved in other biochemical reactions—including sensing of amino acid availability by mTORC1. Since they had similar effects as lysine restriction, it appears that at least in regulating PRPP availability, they likely acted through reduced protein synthesis.

Mice on the lysine-deficient diet lost about 11% of their body weight. Although an 11% weight loss would be hazardous for a non-obese human, the metabolic rate of a 30 g mouse is seven times greater than that of a 70 kg human. Thus, a nutritionally-deficient diet will have a much smaller effect on a human than a mouse, and multiple studies in cancer patients have shown that depriving patients of the essential amino acid methionine for recurring cycles of 3 to 7 days does not lead to weight loss or other adverse effects. Similarly, recurrent cycles of arginine deiminase or arginase, both of which markedly lower serum arginine concentrations, are well tolerated by cancer patients. Combining amino acid deprivation with cisplatin could allow the drug to be used in tumors that show resistance to the drug. Thus, we found a reduction in tumor size when lysine deprivation was added to cisplatin at the highest cisplatin dose tolerated by nude mice, which by itself had no effect. This is consistent with hepatomas being largely unresponsive to most standard chemotherapeutic agents, including cisplatin.

Our study has several limitations. First, most of the work was done in mouse hepatoma cells. Since the two main PRPP synthetases are ubiquitously expressed, the results should be applicable to other cell types, but confirmatory experimentation is needed. Second, in several assays we followed the incorporation of radioactively-labeled compounds into labeled products. Such assays are highly sensitive, but they rely on equal cellular uptake of the labeled compounds and no change in their intracellular pools under the conditions tested. Third, we
focused on changes in PRPP and PRPP synthetase activity. Other metabolites or enzymes could have changed during the 6 h of lysine deprivation in the cultured cell experiments, but identifying such changes and whether they augmented cisplatin efficacy was beyond the scope of this work. And fourth, we used only male mice in the in vivo studies; while seemingly unlikely, it is possible that female mice could have responded differently.

In conclusion, combining cisplatin with brief cycles of deprivation for an essential amino acid could be beneficial in cancer treatment, particularly in cisplatin-resistant tumors. The potential of nutrient limitation in cancer therapy is exemplified by combining chemotherapeutic agents with L-asparaginase—which lowers serum asparagine concentrations—as a standard approach to treat childhood acute lymphoblastic leukemia, but asparagine auxotrophy is limited to a subset of leukemias.

Methods

Materials. Reagents were of the highest grade commercially available. Cisplatin was made fresh just before use by dissolving it in an aqueous solution of 0.9% sodium chloride.

Table 3. Key resources. Reagents were of the highest grade commercially available. Cisplatin was made fresh just before use by dissolving it in an aqueous solution of 0.9% sodium chloride.

| Product name | Company | Cat # |
|--------------|---------|------|
| Antibodies   |         |      |
| β-actin      | Santa Cruz | SC-47778 |
| P-Histone H2AX | Cell Signaling | 2577 |
| Cleaved caspase-3 | Cell Signaling | 9661 |
| PARP1        | Cell Signaling | 9532 |
| PAR polymers | Trevigen | 4335-MC-100-AC |
| Amino acid-deficient culture medium |         |      |
| DME high glucose minus arg, leu, lys, met | Athenaes | 0420 |
| DME low glucose minus arg, gin, leu, lys | Sigma | D9443 |
| RPMI minus arg, lys | Thermo-Fisher | 89984 |
| Radioactive chemicals |         |      |
| [8-14C]Hypoxanthine | Moravek, Inc | MC-147 |
| [1-14C]D-Glucose | Moravek, Inc | MC228W |
| [14C]Formic acid sodium salt | Moravek, Inc | MC173 |
| [Methyl-3H]Thymidine | Moravek, Inc | MT6039 |
| Reagents |         |      |
| Cell Titer-Glo 2.0 Assay | Promega | G9248 |
| Cis-Diammineplatinum(II) dichloride | Millipore-Sigma | P-4394 |
| Human epidermal growth factor | Millipore-Sigma | E8644 |
| Lipofectamine 2000 | Thermo-Fisher | 11668019 |
| 5-Phospho-D-ribose 1-diphosphate, pentasodium salt | Millipore-Sigma | P-8296 |
| D-Ribose 5-phosphate, disodium salt | Millipore-Sigma | 83875 |
| Thiazoyl blue tetrazolium bromide | Millipore-Sigma | M2128 |
| Rodent chow |         |      |
| Amino acid-defined diet | Teklad | 86529 |
| Amino acid-defined diet minus lysine | Teklad | 99386 |

focused on changes in PRPP and PRPP synthetase activity. Other metabolites or enzymes could have changed during the 6 h of lysine deprivation in the cultured cell experiments, but identifying such changes and whether they augmented cisplatin efficacy was beyond the scope of this work. And fourth, we used only male mice in the in vivo studies; while seemingly unlikely, it is possible that female mice could have responded differently.

In conclusion, combining cisplatin with brief cycles of deprivation for an essential amino acid could be beneficial in cancer treatment, particularly in cisplatin-resistant tumors. The potential of nutrient limitation in cancer therapy is exemplified by combining chemotherapeutic agents with L-asparaginase—which lowers serum asparagine concentrations—as a standard approach to treat childhood acute lymphoblastic leukemia, but asparagine auxotrophy is limited to a subset of leukemias.

Methods

Materials. Reagents were of the highest grade commercially available; the source of antibodies and key reagents is shown in Table 3. Cisplatin was made fresh just before use by dissolving solid cisplatin in aqueous 0.9% sodium chloride.

Cell origin and culture. Dih10 cells were derived previously from a diethylnitrosamine-induced mouse hepatoma, and were confirmed as hepatic in origin, since they stained positive for alpha fetoprotein and produced albumin. They were grown in DMEM containing 4.5 g/L glucose, supplemented with 20% fetal bovine serum, 10 mg/L insulin, 10 mg/L hydrocortisone, 20 µg/L epidermal growth factor, and 1 mM phenobarbital. The IGROV-I ovarian cancer cell line was established in France in 1984 from a patient with Stage III ovarian cancer, and was received as a gift; it stained positive for PAX8, a marker of Mullerian differentiation. The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. Both cell lines were used between passages three to ten, and were negative for Mycoplasma infection.

General experimental conditions. Cells were seeded the evening before an experiment in their normal growth medium at a density corresponding to 350,000 cells per well of a 12 well plate, unless stated otherwise. On the day of the experiment, the cells were washed once with phosphate-buffered saline (PBS), and incubated for the indicated times in 0.5 ml experimental medium per well of a 12 well plate (correspondingly lower and higher volumes for smaller and larger wells, respectively). This medium was made fresh the day of the experiment, and consisted of either DMEM containing 4.5 g/L glucose (Dih10 cells) or RPMI-1640 medium (IGROV-I...
cells). The DME medium lacked arginine, leucine, lysine, and methionine; the RPMI medium lacked arginine and lysine. To generate full medium, the missing amino acids were added to their normal concentrations of 398 μM arginine, 801 μM leucine, 798 μM lysine, and 200 μM methionine for DME, and 948 μM arginine and 219 μM lysine for RPMI. To generate medium deficient in one amino acid, the other amino acids were added to their normal concentration, and the deficient one was either not added to make medium completely deficient in that amino acid, or added to the indicated concentration. In some experiments, lysine-deficient DMEM contained glucose at 1 g/L, and either lacked sodium bicarbonate or contained 15 mM NaHCO₃; in the latter cases, 20 mM Hepes, pH 7.4 was added to the medium. All experimental media were supplemented with 10% fetal bovine serum that had been dialyzed against 0.9% sodium chloride.

**Measurement of PRPP availability, and purine and DNA synthesis.** Cells were incubated in 12 well (PRPP availability and DNA synthesis) or 6 well plates (purine synthesis) for the indicated times.

For PRPP availability, [8,14C]-hypoxanthine (1 μCi, 58 mCi/mmol) was added during the last 20 min of incubation, and the cells were washed twice with PBS, extracted in 1 mM NaOH, and the extracts spotted onto squares of diethylaminoethyl cellulose paper. The paper squares were washed three times with 1 mM ammonium formate, dried, and radioactivity on the squares was measured.

For purine synthesis, [14C]-formate (10 μCi, 56 mCi/mmol) was added during the last 90 min of incubation, and the cells were extracted in 0.4 N perchloric acid. The extracts were boiled for 70 min to reduce all purine nucleotides and nucleosides to purine bases, and supernatants were applied to Dowex 50 columns eluted with 6 N HCl.

For DNA synthesis, [3H]-thymidine (1 μCi, 70 Ci/mmol) was added during the last 60 min of incubation, and the cells were washed once with PBS and extracted with 10% trichloroacetic acid. The resulting precipitates were collected on glass fiber discs, which were washed with trichloroacetic acid, dried, and transferred to scintillation vials.

In all cases, radioactivity was measured by liquid scintillation counting using 2,5-diphenyloxazole and p-bis-[2-(5-phenyloxazolyl)]-benzene as primary and secondary fluorophores, respectively.

**Measurement of ATP.** Cells were incubated in 96 well plates, and at the end of the incubation period, ATP was measured against a standard curve using the Cell Titer- Glo 2.0 kit (Promega) following the manufacturer's instructions.

**Measurement of NAD.** Cells in 12 well plates were extracted in perchloric acid, and NAD+ in the neutralized extracts was measured in an enzyme-cycling colorimetric assay following thiazolyl blue tetrazolium bromide reduction.

**Measurement of glucose oxidation via the oxidative pentose phosphate pathway and incorporation into DNA and RNA via the non-oxidative pentose phosphate pathway.** To measure glucose oxidation via the oxidative pentose phosphate pathway, cells were incubated in 725 flasks in bicarbonate-deficient DMEM containing 1 g/L glucose and 20 mM Hepes. [1-14C]-glucose (1 μCi, 58 mCi/mmol) was added during the last 60 min of incubation, and the flasks were immediately capped with rubber stoppers that had an attached center well containing fluted filter paper. At the end of the incubation period, perchloric acid was injected into the medium to a final concentration of 0.4 N, and 10 mM NaOH was injected into the center well. The perchloric acid simultaneously extracts the cells and converts HCO₃⁻ to CO₂; the CO₂ was collected in the wells by conversion back to HCO₃⁻ by incubating the flasks overnight. The collected radioactive CO₂ was quantified by transferring the wells to scintillation vials and measuring radioactivity by liquid scintillation counting.

To measure glucose incorporation into DNA and RNA via the non-oxidative pentose phosphate pathway, cells were incubated in 12 well plates in DMEM containing 1 g/L glucose, with [1-14C]-glucose (1 μCi, 58 mCi/mmol) added during the last 60 min of incubation. The cells were washed once at the end of the incubation period with PBS and extracted in 10% trichloroacetic acid. The extracts were washed with trichloroacetic acid and heated at 80 °C for 30 min to solubilize precipitated DNA and RNA; the resulting supernatants were transferred to scintillation vials, and radioactivity was measured.

**Measurement of HPRT and PRPP synthetase activity.** Cells in six well plates were washed with ice-cold Tris-buffered saline, and extracted either by repeated passage through a 27 g needle (HPRT) or by three freeze–thaw cycles (PRPP synthetase). The extracts were clarified by centrifugation, and incubated with [8,14C]-hypoxanthine (58 mCi/mmol, final concentration 20 μM) in the presence or absence of 0.4 mM ATP (HPRT), or with 0.7 mM ATP and [8,14C]-adenine (54 mCi/mmol, final concentration 30 μM) in the presence or absence of 0.5 mM ribose 5-phosphate (PRPP synthetase). Sufficient adenine phosphoribosylpyrophosphate transferase activity was present in the extracts that exogenous enzyme was not necessary in the PRPP synthetase assay. The samples were then processed as for measuring PRPP availability. In the PRPP synthetase assay, sodium phosphate, pH 7.4 was added to the final concentration indicated. Enzyme activity was calculated as the difference between the presence and absence of PRPP or ribose 5-phosphate for HPRT or PRPP synthetase, respectively, and was linear with time and protein concentration.
Measurement of inorganic phosphate. Cells in six well plates were washed twice with Tris-buffered saline and extracted in 6% sodium dodecyl sulfate. The extracts were sonicated, and phosphate was measured in an improved ammonium molybdate-malachite green assay.

shRNA knockdown of PARP1. A PARP1 shRNA plasmid containing the sequence CCTTGAAACAT GATGIAGCTC (SHCLNG-NM_007415) and a non-target shRNA control plasmid (SHC016) were purchased from Millipore-Sigma. They were co-transfected into 293 T cells with pCMV-Δ8.2R and pCMV-VSV-G expression vectors using Lipofectamine 2000. Medium supernatant containing assembled lentivirus was harvested 48 h later, filtered, and added twice, 24 h apart, to Dih10 cells. Twenty-four hours after the second addition, the Dih10 cells were placed in 1 µg/ml puromycin, and stable clones were selected by limiting dilution. PARP protein was assessed by Western blotting and normalized to actin. Experiments were done with cells obtained from three independent infections.

Immunoblotting. Cells were incubated in 12 well plates for the indicated times, and extracted in situ in sodium dodecyl sulfate sample buffer. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and the membrane was incubated with the indicated primary antibody, followed by a hors eradish peroxidase-conjugated secondary antibody. Proteins were quantified by chemiluminescence using a Li-COR Odyssey Scanner and Image Studio Version 5.2 software.

Assessment of cell growth. Cells were seeded at 30,000 cells per well in 24 well plates, and cultured under the indicated conditions. After 72 h, cells from triplicate wells were counted twice using a hemocytometer.

Clonogenic assay. Cells were seeded at 300 cells per well in six-well plates and cultured under the indicated conditions. After 2 weeks of culture, they were fixed in methanol, stained with crystal violet, and photographed. Stained cells were quantified by dissolving the crystal violet in acetic acid, and measuring optical density at 590 nm.

Measurement of lactate dehydrogenase (LDH) activity. Cells in a 96 well plate were placed in 0.2 ml of experimental DMEM medium containing 10% dialyzed heat-inactivated (60 °C for 30 min) fetal bovine serum. After 6 h of incubation, the media was harvested and the cells were extracted in situ in 0.9% Triton X-100. LDH activity in the media and cell extracts was measured separately in a coupled reaction following (1) lactate oxidation to pyruvate, with consequent NAD reduction to NADH, and (2) NADH reduction of iodonitrotetrazolium chloride to a colored formazan product measured at 490 nm. The assay was linear with time and cellular protein concentration, and results are presented as the percent of total LDH activity in the medium.

Immunofluorescence staining. Cells plated on glass slide cover slips (10,000 cells per slip) were incubated for the indicated times, fixed with 3.7% formaldehyde in PBS, permeabilized with 0.5% Triton X-100, and blocked with 2% bovine serum albumin. They were stained with phosphohistone H2A.X antibody followed by a Texas red-conjugated secondary antibody. Nuclei were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI).

Mouse xenograft model. Nude (Nu/Nu) male mice 8–12 weeks old and weighing an average of 28.2 g were housed four animals per cage in a pathogen-free environment with a 12 h light–dark cycle. At the start of the experiment, they were anesthetized briefly with 1.5% isoflurane, and injected subcutaneously in both flanks with 1.7 × 10⁶ Dih10 cells mixed 1:1 with Matrigel. For 7 d they received fully supplemented rodent chow (Teklad Amino Acid-Defined Diet), at which time they were randomly divided into four groups, assuring that the average tumor volume was similar in each group. Group 1 animals received the fully supplemented chow for the remainder of the study. Group 2 animals received fully supplemented chow and a single intraperitoneal injection on day seven of 7.5 mg/kg cisplatin in 0.9% NaCl, ~280 nl per mouse. Group 3 animals received the following diet: three 5 d periods of lysine-free chow (same formulation as fully supplemented chow but lacking lysine, Teklad Custom Diet Minus Lysine) separated by two 3 d periods of fully supplemented chow (Fig. 4A). Group 4 animals received the same 7.5 mg/kg cisplatin injection as Group 2 mice followed by the cyclical lysine-free/full-supplemented chow diet as Group 3 mice.

Animal Research (ILAR) Guide to the Care and Use of Laboratory Animals. The studies were conducted according to the National Academies of Sciences, Engineering, and Medicine Institute for Laboratory Animal Research (ILAR) Guide to the Care and Use of Laboratory Animals.
Measurement of serum amino acids. Blood obtained by cardiac puncture at the time of euthanasia was allowed to clot, and the resulting serum was extracted using 2% 5-sulfosalicylic acid. Amino acids in the serum were analyzed on a Biochrom 30 amino acid analyzer using a PEEK (polyether-ketone-ketone) lithium ion exchange column, with ninhydrin post-column derivatization. They were quantified by comparison to authentic standards using calibration curves with R^2 > 0.99.

Data analysis. Symbols in bar graphs represent the mean of duplicate samples, with each symbol in a given condition derived from an independent experiment. Comparisons among conditions were made by a one-way ANOVA, except in Fig. 2K where a two-way ANOVA was used. In all cases, Sidak’s multiple comparisons post-test analysis was used in GraphPad Prism 7.0. A p value of < 0.05 was considered significant; the absence of a comparison between two conditions means the difference was not significant. Further statistical details are in the figure legends.

To determine if combining cisplatin with amino acid deprivation yielded an additive, synergistic, or antagonistic effect, we considered cisplatin as one drug and amino acid deprivation as a second drug. We then calculated the coefficient of drug interaction (CDI) using the formula CDI = AB/A × B, where A, B, and AB are the ratios of the results with cisplatin alone, amino acid deprivation alone, and the combination of cisplatin and amino acid deprivation, respectively, over the result in control untreated cells in full medium. CDI = 1 indicates no effect, CDI > 1 indicates synergism, and CDI < 1 indicates antagonism.

Data availability
The data of this work are all within the manuscript.

Received: 9 July 2020; Accepted: 4 November 2020
Published online: 16 November 2020

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Acknowledgements

We thank M Karin and SB Howell for providing Dih10 cells and IGROV-1 cells, respectively, and N-W Chi and SB Howell for technical advice.

Author contributions

N.W., D.D., R.B.P., and G.R.B. conceived and designed the study, N.W., D.D., S.Z., A.C., D.E.C., and H.K. conducted the experiments, and N.W., R.B.P., and G.R.B. wrote the manuscript. All authors have read and approved the manuscript.

Funding

The work was supported in part by NIH Grants R01-AR068601, R01-HL132141, and P30-NS047101, and by funds from the Department of Medicine, University of California, San Diego. DD was supported by an ALF Liver Scholar award and NIH grant KL2TR001444. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Competing interests

The authors declare to have no competing interests.

Additional information

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