Datum: August 28th, 2013
Tijd: 9-12 pm
Zaal: 418
Docent: Dr. Sylvestre Bonnet

Voorzie het 1e blad van naam, adres, email, jaar van aankomst en nummer collegekaart.
Schrijf op de andere losse bladen alleen de naam. Bij het tentamen is het gebruik van de syllabus of mobiele telefoon niet toegestaan. Voor elke vraag is de waardering aangegeven.

On page 1, write your name, address, e-mail, year of enrolment and the number of the college card.
At the following pages not your name. It is not allowed to use the syllabus or a cell phone during the examination. For each question the rating is given.

When a justification is asked it counts at least as many points as the answer itself. A justification is preferably precise AND short.
The number of points per question is indicative and may be re-evaluated.

Part A. Resistances to cisplatin anticancer therapy (5 points)

The figure below shows three clinically approved anticancer drugs. Cisplatin is used in a large fraction of chemotherapeutic cocktails given to cancer patients throughout the world. However, certain cancer cells are naturally resistant to cisplatin therapy, or become resistant during the initial phases of the chemotherapeutic treatment.

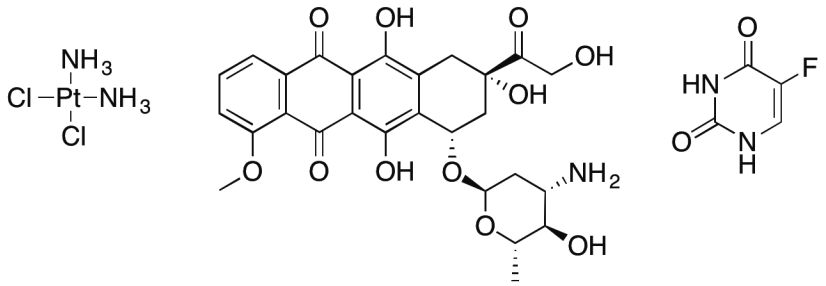

Figure 1. Chemical structure of cisplatin, doxorubicin, and fluorouracil (from left to right).

Answer the following questions:

1. Cite two major occupational risk factors for lung cancer. (0.25 point)
2. What is the oxidation state of platinum in cisplatin? (0.25 point)
3. What distinguishes the mode of action of cisplatin, compared to organic drugs such as doxorubicin or fluorouracil? (0.25 point)
4. How many isomers exist for cisplatin? Do they have the same biological activity? (0.25 point)
5. Cisplatin interacts with DNA. On the four base pair drawn below give two sites that preferentially bind to the platinum center? (0.25 point)

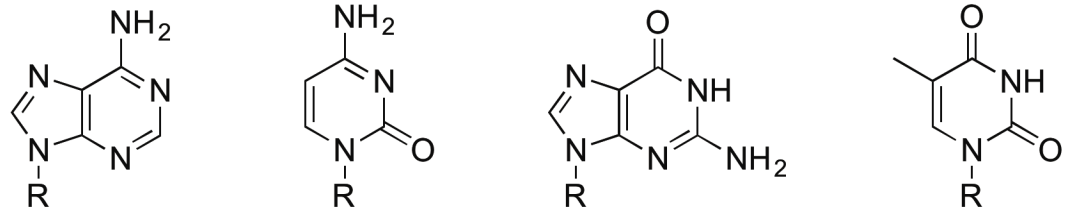

Table 1 gives the sensitivity of normal (H69) and cisplatin-resistant (H60/$CDDP_{0.2}$ and H60/CDDP) human lung cancer cells to several chemotoxic agents including cisplatin and cadmium dichloride. The two resistant cancer cell lines have different sensibilities to cisplatin: H69/$CDDP_{0.2}$ and H69/CDDP were more resistant to cisplatin than H69 with relative resistance of 6.2 and 10.9, respectively (the relative resistance is defined as IC_{50} of resistant cell line/IC_{50} of parental cell line).
By using radioactive platinum (^{195m}Pt) it is possible to measure platinum accumulation in the cells. Figure 2A and 2B give the accumulation and effluxes, respectively, of ^{195m}Pt-labelled cisplatin by the three cancer cell lines. To look at effluxes cells initially treated with cisplatin are washed with a buffer and incubated for an additional time in drug-free medium before determining the Pt remaining in the cells. Figure 2B is indirectly showing platinum effluxes as a function of incubation time in the drug-free medium.

Table 1 ***Sensitivities for various agents of H69 and its cisplatin-resistant cell lines***

Agents	H69	IC_{50} (μg/ml) H69/CDDP$_{0.2}$	H69/CDDP
Cisplatin	0.076 ± 0.003[a]	0.47 ± 0.07 (6.2)[b]	0.83 ± 0.13 (10.9)
254-S	0.21 ± 0.006	ND[c]	0.86 ± 0.02 (4.1)
hCPA	0.26 ± 0.017	0.56 ± 0.06 (2.2)	0.73 ± 0.11 (2.8)
ACNU	0.52 ± 0.12	1.8 ± 0.2 (3.5)	2.2 ± 0.1 (4.2)
ADM	0.058 ± 0.007	ND	0.063 ± 0.005
VCR	0.017 ± 0.008	ND	0.017 ± 0.008
CPT-11	0.85 ± 0.16	ND	0.79 ± 0.12
VP-16	0.82 ± 0.11	ND	0.55 ± 0.19
$CdCl_2$	9.6 ± 0.7	14.7 ± 1.5 (1.5)	19.8 ± 1.0 (2.1)

[a] Each value is the mean ± SD of the three independent experiments.
[b] Relative resistance value: IC_{50} value of resistant cells/IC_{50} value of parental cells.
[c] ND, not determined.

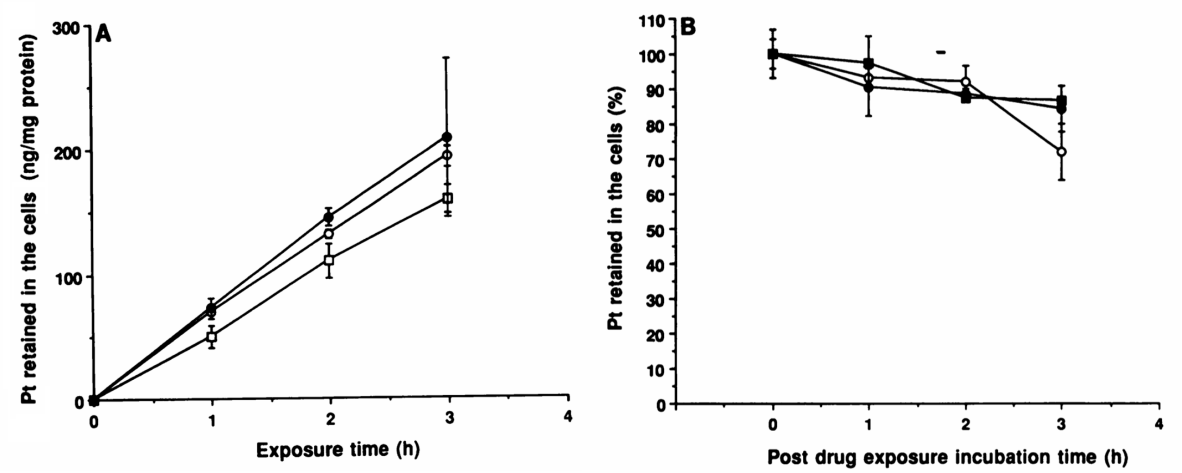

Figure 2. Accumulation (A) and efflux (B) of ^{195m}Pt-cisplatin in H69 and its cisplatin-resistant cell lines. A, incorporation of radiolabeled cisplatin into H69 parental and drug-resistant cells. All cells were treated with 5 μg/mL of cisplatin for 1, 2, or 3 h. B, efflux of ^{195m}Pt-cisplatin from H69 cells. Cells treated with 5 μg of cisplatin per ml were washed with cold PBS and incubated for 1, 2, or 3 h in drug-free medium before determination of Pt remaining in the cells. This graph is thus related to platinum effluxes out of the cell. All data were normalized to that of H69 cells with no drug-free incubation period (control). O, H69; □ H69/CDDP$_{0.2}$; • H.69/CDDP. Bars: Standard Deviations.

6. Is cisplatin taken up by the cell? Justify. (0.25 point)
7. Is the resistance mechanism based on reduced uptake of the drug? Justify. (0.5 point)
8. Is the resistance mechanism based on increased efflux of the drug? Justify. (0.5 point)

In order to elucidate the role of the potential drug detoxification systems in the acquisition of cisplatin resistance the content of glutathione (GSH) and metallothionein (MT) were measured, as well as the activity of glutathione S-transferase (GST). Glutathione is a hydrophilic tripeptide shown in Figure 3. It is present in high (mM) concentrations inside most cells. GST is an enzyme catalyzing the nucleophillic attack of GSH onto exogenic organic substrates; GSH-tagged toxic compounds are then exported out of the cell via specific transporters. The data are shown in Table 2.

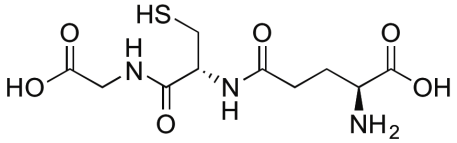

Figure 3. Chemical formula of glutathione.

Table 2 ***Detoxification mechanisms of H69 and its cisplatin-resistant cell lines***

	H69	H69/CDDP$_{0.2}$	H69/CDDP
Relative resistance	1	6.2	10.9
GSH content (nmol/mg protein)	16.6 ± 4.7[a]	14.4 ± 2.9	19.9 ± 3.0
GST activity (nmol/min/mg protein)	11.5 ± 8.7	12.3 ± 4.5	74.3 ± 16.1[b]
MT content (pmol/mg protein)	14.5 ± 1.5	22.3 ± 2.0[c]	32.6 ± 1.6[d]

[a] Mean ± SD.
[b] $P < 0.001$ compared to value for H69 and H69/CDDP$_{0.2}$ by unpaired Student's t test.
[c] $P < 0.001$ compared to value for H69 by unpaired Student's t test.
[d] $P < 0.001$ compared to value for H69 and H69/CDDP$_{0.2}$ by unpaired Student's t test.

9. What are metallothioneins? Which element is particularly represented in this molecule? Cite three biological roles of metallothioneins, with two at least involving metal ions. (0.5 point)
10. Why would GSH or MT interact at all with cisplatin, and through which atom will they do so? Justify. (0.5 point)
11. Are cisplatin-resistant cancer cells be more, less, or equally resistant to $CdCl_2$? Justify using a chemical argument. (0.25 point)
12. Which of the three factors (GSH, MT, GST) is best correlated to cisplatin resistance? Justify. (0.5 point)
13. What is the main factor explaining cisplatin resistance according to these data? (0.25 point)
14. Are cadmium ions present in the environment? Why? (0.5 point)

Part B. Interactions between copper transporters and platinum-based anticancer drugs.

Several studies have provided evidence that platinum-containing anticancer drugs are taken up, shuttled around the cell, and exported by transporters and chaperones belonging to the Cu homeostasis system. In this part of the exam two copper transporters are considered, CRT1 and CTR2. They are two high-affinity plasma membrane Cu transporters with substantial structural homology.

The following *in vitro* studies have been realized with cell lines from either wild type or genetically modified animals. In the genetically modified ones, the gene expressing either CTR1, or CTR2, or both, can be "knocked down" (i.e., gene expression is reduced) or "knocked out" (i.e., the gene becomes fully inoperative), resulting in the corresponding decrease of protein expression. Four mouse embryo fibroblast cells are studied, written $CTR1^{+/+}/CTR2^{+/+}$, $CTR1^{+/+}/CTR2^{kd}$, $CTR1^{-/-}/CTR2^{+/+}$, and $CTR1^{-/-}/CTR2^{kd}$, where $CTR1^{+/+}$ and $CTR2^{+/+}$ means wild-type (protein expressed normally), $CTR1^{-/-}$ means that both alleles of the gene for CTR1 is knocked down (low levels of CTR1), and $CTR2^{kd}$ means that the gene for CTR2 has been knocked out (no CTR2 at all). The authors verified that CTR2 knockdown did not affect CTR1 levels; in the $CTR1^{+/+}/CTR2^{kd}$ subline CTR2 mRNA and protein expression was reduced by 88.5% and 55%, respectively, and in CTR1-/-/CTR2kd cells they were reduced by 81.8% and 33%, respectively.

Metal accumulation in the cells are measured either by mass spectrometry or by radioactive counting using ^{64}Cu sources or ^{14}C-labelled platinum compounds.

Note: In the following cisplatin is noted DDP, and carboplatin CBDCA. They are two of the most widely used chemotherapeutic agents.

15. Do copper ions cross lipid bilayers? Justify. (0.5 point)
16. What are metal ion transporters? What distinguishes them from ionophores? (0.5 point)

The $CTR1^{+/+}/CTR2^{+/+}$, $CTR1^{+/+}/CTR2^{kd}$, $CTR1^{-/-}/CTR2^{+/+}$, and $CTR1^{-/-}/CTR2^{kd}$ cells were exposed to increasing concentrations of DDP for 5 days, and the change in growth rate was quantified by staining the remaining cells with a dye. Table 1 and Figure 5 show the accumulation of DPP, CBDCA, and Cu after incubation, and Figure 4 shows the concentration-survival curves for each of the cell lines.

Table 1. Accumulation of DDP, CBDCA, and Cu

	$CTR1^{+/+}/CTR2^{+/+}$	$CTR1^{+/+}/CTR2^{kd}$	$CTR1^{-/-}/CTR2^{+/+}$	$CTR1^{-/-}/CTR2^{kd}$
DDP uptake at 5 min*	1.05 ± 0.16	2.17 ± 0.20	0.51 ± 0.12	1.43 ± 0.03
DDP uptake at 1 h*	4.17 ± 0.11	8.78 ± 0.20	2.01 ± 0.08	7.08 ± 0.43
CBDCA uptake at 1 h †	99.5 ± 17.7	128.7 ± 27.8	55.5 ± 5.6	63.9 ± 11.8
Cu uptake at 1 h*	3.60 ± 0.18	4.96 ± 0.17	2.52 ± 0.26	2.98 ± 0.03
DNA adduct formation, pmol/L Pt/μg DNA	0.14 ± 0.02	0.30 ± 0.01	0.10 ± 0.01	0.32 ± 0.03
Vesicle accumulation, pmol/L Pt/100 ng sulfur	0.70 ± 0.04	0.73 ± 0.05	0.70 ± 0.04	0.70 ± 0.08

*pmol/L/100 ng sulfur.
† cpm/μg protein.

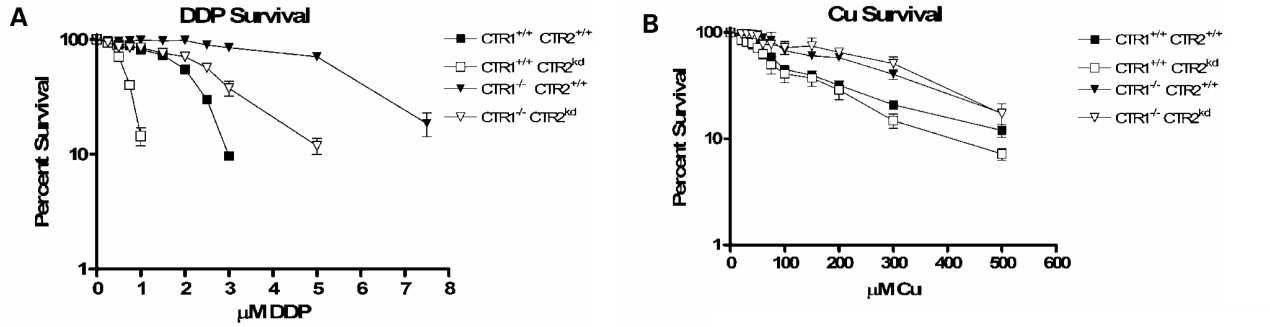

Figure 4. Inhibition of growth as a function of concentration. A, DDP. B, $CuSO_4$.

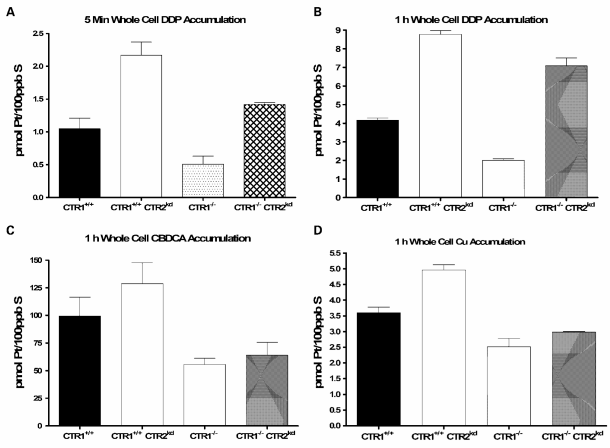

Figure 5. Whole-cell accumulation of DDP in $CTR1^{+/+}/CTR2^{+/+}$, $CTR1^{+/+}/CTR2^{kd}$, $CTR1^{-/-}/CTR2^{+/+}$, and $CTR1^{-/-}/CTR2^{kd}$ cells. Whole-cell Pt accumulation following 5-min (A) and 1-h (B) exposure to 30 μmol/L DDP as measured by ICP-MS. C, whole-cell [^{14}C]CBDCA accumulation following 1-h exposure to 50 μmol/L [^{14}C]CBDCA as measured by scintillation counting. D, whole-cell ^{64}Cu accumulation following 1-h exposure to 2 μmol/L $^{64}CuSO_4$ as measured by g counting. Bars, SE.

17. What is the effect of knocking out the CTR1 gene on the resistance to DDP, and on DDP uptake? What do these data suggest to you about the uptake mechanism of cisplatin? (1 point)

18. Did deletion of both CTR1 alleles in mouse embryo fibroblasts completely eliminate the accumulation of cisplatin? What does this fact suggest to you on the uptake mechanism? (0.5 point)
19. What is the effect of knocking down CTR2 on DDP sensitivity? Does this depend on the expression of CTR1? (0.5 point)
20. What is the effect of knocking down CTR2 on DDP uptake? Can you make a hypothesis on the role of CTR2 for DDP? (0.5 point)
21. Is an excess of copper in the growing medium toxic to these cells? Give two reasons why intracellular free copper is dangerous for a cell. (0.5 point)
22. What is the effect of knocking out CTR1 on cell resistance to Cu? And on Cu accumulation? How do you interpret this? (0.5 point)
23. What is the effect of knocking down CTR2 on cell resistance to Cu? How do DDP and Cu compare regarding the role of CTR2? (0.5 point)

End of the exam.

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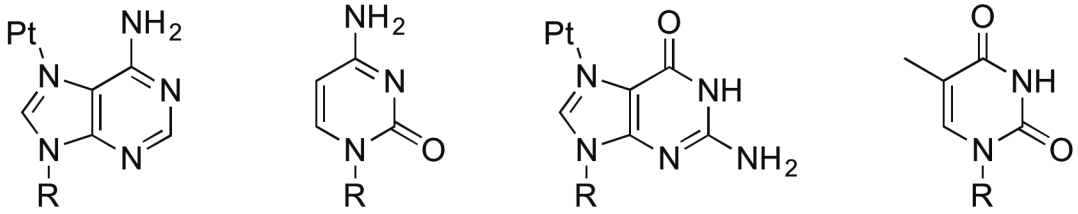
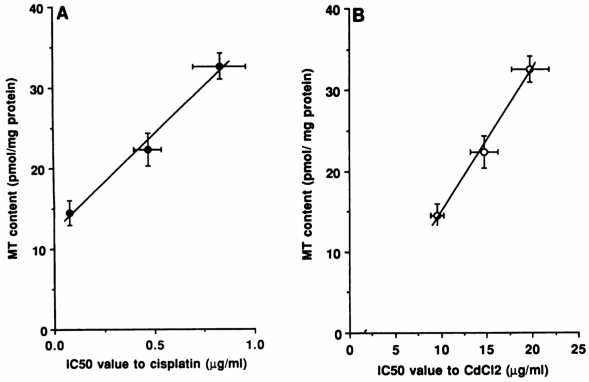
Answers to Part A.
See Kasahara et al, Cancer Res. ***1991****, 51,* 3237-3242

1. Cigarette smoking and asbestos.
2. Pt(II).
3. the metal coordinates to nitrogen-based ligands on DNA, whereas other organic drugs methylate or intercalate into DNA, or inhibit enzymes (fluorouracil).
4. Two, the other one is trans-platin. They do not have the same biological activity, as trans-platin has no anticancer properties.
5. Cisplatin binds to the N7 atoms of guanine and adenine, thus



6. yes, the accumulation increases as a function of exposure time.
7. No: all three cell lines take up the drug similarly, whereas they have very different sensitivities to the drug (see Table 1).
8. No: the efflux is comparable for all three cell lines, whereas they have different sensitivies to the drug (figure 2).
9. MT are small proteins involved in metal detoxification, zinc homeostasis, and oxidative stress protection. It is a cistein-rich, thus sulfur-rich protein able to coordinate up to seven Zn^{2+} ions via thiol groups of cysteine residues. The strength of the seventh metal binding site is poor which allows transporting zinc ions within the cell.
10. They are both sulfur-based molecules. According to the HSAB theory the softness of sulfur allows it for binding with a high affinity to heavy metal ions such as Pt(II) or Cd(II).
11. Because both are toxic to human cells, and because both are soft metal compounds that will be detoxified by similar mechanisms.
12. The best correlation is found with MT levels. The easiest justification is to draw the evolution of this factor as a function of IC_{50} . Below the graphs for cisplatin and CdCl2.



13. Resistance is not due to decreased uptake or increased efflux of the drug, it is correlated to the amounts of MT, so the most plausible resistance mechanism for this particular cancer cell line is detoxification of the cell by metallothioneins, leading to less DNA lesions.

14. Cadmium is one of the main heavy metal pollutant in the environment, and considered as carcinogenic. It is produced by the chemical industry (Ni-Cd batteries, metal mines), but also present in paint pigments or in natural ores (zinc sulfides, phosphates).

Answers to Part B.

See Blair et al, *Clin. Cancer Res*. **2009**, *15*, 4312-4321

15. no, because Cu^{2+} is too polar for the hydrophobic part of the lipid membrane.
16. They are transmembrane proteins specialized in ion transport through the membrane. The difference with ionophores is that the transport requires energy and/or gating to occur, whether ionophores simply increase the solubility of metal cations in the lipophyllic part of the membrane.
17. On the one hand, Figure 4A shows that the survival of the $CTR1^{-/-}/CTR2^{+/+}$ cells increases significantly compared to $CTR1^{+/+}/CTR2^{+/+}$. Thus, knocking out the CTR1 increases resistance to cisplatin. On the other hand, Table 1 indicates that such effect is correlated on a decreased (~50%) uptake of DDP in $CTR1^{-/-}/CTR2^{+/+}$ cells, compared to the wild type. Thus, the CTR1 copper transporter must be involved in cisplatin uptake, and removing them decreases the amount of drug that penetrates in the cell, thus increasing resistance.
18. No: $CTR1^{-/-}/CTR2^{+/+}$ cells still take up DDP. This fact suggests that CTR1 is not the only entrance door of the drug; typically, passive diffusion through the membrane is possible as the DDP compound is a small neutral molecule.
19. Knockdown of CTR2 rendered cells hypersensitive to DDP irrespective of whether CTR1 was expressed or not. Knockdown of CTR2 in the $CTR1^{+/+}/CTR2^{+/+}$ cells reduced the IC_{50} by 69% lacking CTR1 to 0.7 μmol/L. Likewise, knockdown of CTR2 in the $CTR1^{-/-}/CTR2^{+/+}$ cells reduced the DDP IC_{50} by 51% to 2.7 μmol/L. Stated another way, loss of CTR2 expression caused a 3.2-fold increase in DDP sensitivity in wild-type cells and a 2.0-fold increase in cells lacking CTR1. Note: a similar effect is seen with CBDCA.
20. Knocking down CTR2 double the amount of DDP accumulated in the cell (Table 1). As this is correlated to a decreased survival (see question 19), and knowing that DDP is toxic once inside the cell one can hypothesize that CTR2 is involved in effluxes of DDP: less CTR2 means more DDP inside the cell, thus less survival.
21. Yes: as shown in Figure 4B cell survival is reduced when the cells are grown in presence of increasing amounts of Cu. Free copper induces Fenton chemistry, and copper binds usually better than other metals so it will displace other metals from metalloproteins.
22. loss of CTR1 function rendered the cells 2.6-fold resistant to Cu, while Cu accumulation is only reduced by 31% (2.52 vs. 3.60). If a copper influx transporter is not expressed anymore less Cu will be taken up, thus increasing cell resistance with a growing medium containing an excess of Cu. However, the slightly lower Cu accumulation indicates that there are other copper transporter than CTR1.
23. reduction in the expression of CTR2 had no discernable effect on the sensitivity to Cu in either the $CTR1^{+/+}$ or $CTR1^{-/-}$ background (Figure 4B). The mean IC_{50} values for Cu were as follows: $CTR1^{+/+}/CTR2^{+/+}$ cells, 243.9 μmol/L; $CTR1^{+/+}/CTR2^{kd}$ cells, 309.6 μmol/L; $CTR1^{-/-}/CTR2^{+/+}$ cells, 93.1 μmol/L; and $CTR1^{-/-}/ CTR2^{kd}$ cells, 95.8 μmol/L.
    Thus, there was a clear difference in the effect of knocking down CTR2 expression on sensitivity to DDP and the effect on the sensitivity to Cu.