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DNA repair mechanisms involved in gemcitabine cytotoxicity and in the interaction between gemcitabine and cisplatin

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Abstract

The influence of DNA repair mechanisms on the interaction between gemcitabine and cisplatin was studied using a panel of Chinese hamster ovary (CHO) cell lines each deficient in one of the following repair pathways: base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR) and non-homologous end joining (NHEJ). NER and HR are known to be involved in platinum-DNA adduct repair. Single agent experiments demonstrated that each of the repair deficient cell lines had a similar sensitivity towards gemcitabine as the parental cell lines, whereas the NER- and HR-deficient lines showed increased sensitivity towards cisplatin. Furthermore, in the parental cell lines, the administration sequence cisplatin followed by gemcitabine was synergistic, whereas the reversed schedule showed additivity and simultaneous administration revealed antagonistic cytotoxicity. In the repair deficient cell lines, using this synergistic schedule of cisplatin followed by gemcitabine, loss of synergy was observed in the NER- and HR-deficient cell lines. However, the magnitude of the effect in the NER-deficient cells was small. The sensitivity to the combination of cisplatin and gemcitabine shown by the BER- and NHEJ-deficient cell lines did not differ significantly from that of the parental cell line. Cellular accumulation of platinum as well as the formation of GG- and AG-intrastrand adducts in the parental line and in the HR-deficient line were not affected by gemcitabine.

In conclusion, our results indicate that BER, NER, HR, and NHEJ are most likely incapable of modulating the cytotoxicity of gemcitabine, and that HR is involved in the synergistic interaction between cisplatin and gemcitabine in our cell system.

Keywords: Cisplatin; Gemcitabine; Interaction; DNA repair

1. Introduction

Gemcitabine and cisplatin are two anticancer agents increasingly applied in combination therapy in the clinic. Because of their different mechanism of action and non-overlapping toxicity, they are good candidates for combined therapy at full dose. The combination has shown efficacy in a number of malignancies, including non-small cell lung [1,2], head and neck [3], urothelial [4,5], and cervical cancer [6]. Especially in non-small cell lung cancer (NSCLC), the combination of cisplatin and gemcitabine has demonstrated very promising responses [1,2]. Cisplatin exerts its antineoplastic activity by binding covalently to the DNA, thereby forming at least six different types of adducts [7]. The most abundant types of adducts are the intrastrand crosslinks between two adjacent bases (Pt-GG and Pt-AG adducts), which represent approximately 65 and 25%, respectively, of the total number of adducts formed. Minor adducts are monofunctionally bound cisplatin to a guanine base and interstrand crosslinks (ICLs) between two guanines on opposite strands. The ICLs represent approximately 5–10% of the total number of adducts formed [7,8]. Intrastrand adducts are repaired by the nucleotide excision repair (NER) pathway [9], whereas ICLs are repaired by homologous recombination (HR)

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Abbreviations: BER, base excision repair; NER, nucleotide excision repair; HR, homologous recombination; NHEJ, non-homologous end joining; CHO, Chinese hamster ovary.

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[10–12]. However, the repair of ICLs most likely also requires the aid of other pathways, such as NER [13,14]. Increased activity of NER has been associated with cisplatin resistance (reviewed in [15]). Gemcitabine, a structural analogue of cytidine, is phosphorylated three times upon cell entry by the enzyme deoxycytidine kinase (dCK). The triphosphate can be incorporated into the DNA allowing for incorporation of one more nucleotide, after which DNA polymerization stops [16]. This process is called 'masked chain termination'. Because of this, gemcitabine is not easily detected and excised by proofreading exonucleases [17]. However, the incorporated gemcitabine can be recognized by p53 and DNA-dependent protein kinase, which might result in apoptosis [18]. Phosphorylated gemcitabine can also be incorporated into RNA [19], and gemcitabine-diphosphate can inhibit several cellular enzymes, especially ribonucleotide reductase, resulting in decreased cellular nucleotide pools [16,20]. This last effect potentiates gemcitabines activity, because it indirectly inhibits DNA synthesis. In addition, depletion of deoxycytidine will favor gemcitabine incorporation, and the negative feedback of deoxycytidine on dCK will decrease, which in turn enhances gemcitabine phosphorylation [21]. Other cellular effects of gemcitabine that have recently been discovered include an increase in ligase I levels [22]. Thus, gemcitabine exerts its antineoplastic activity through an array of cellular effects on DNA synthesis.

Preclinically, the combination of cisplatin and gemcitabine has been investigated in a number of studies. Synergistic cytotoxicity was observed in a number of cell lines, including ovarian, lung, head and neck and colon carcinoma [23–25]. However, this was highly influenced by cell type as well as schedule, because additivity and even antagonism have been described as well [23,26]. Both administration orders, gemcitabine before cisplatin or *vice versa* could result in synergistic cytotoxicity in ovarian, lung, and head and neck tumor cell lines. A stimulatory effect of cisplatin on gemcitabine incorporation into the DNA has been observed in an ovarian cancer cell line, offering a possible explanation for the observed synergism. However, this could not be demonstrated in three other cell lines tested [23]. There is more evidence for effects of gemcitabine on cisplatin pharmacokinetics and/or pharmacodynamics: pretreatment with gemcitabine increased cellular platinum accumulation in one cell line tested, and platinum-DNA intrastrand adduct formation in four [24]. Subsequent *in vivo* studies, however, demonstrated the opposite effect: in mice bearing Lewis non-small cell lung tumors, a decrease of intrastrand platinum-DNA adducts occurred when gemcitabine was given as the first drug [27]. Interestingly, this possible negative effect of gemcitabine on platinum-DNA adduct formation or retention was confirmed in humans. A clinical and pharmacokinetic trial in patients with advanced NSCLC revealed that the intrastrand adduct levels in peripheral white blood cells were decreased with increasing doses of gemcitabine, if this agent was administered first [28]. Thus, the exact interactions between gemcitabine and cisplatin on a cellular level are still highly unexplained.

We have previously investigated the interaction between cisplatin and topotecan in lower and higher eukaryotes. We found that DNA repair, in particular homologous recombination, was required for the synergism occurring with these agents [29]. Because gemcitabine also acts on the DNA, we hypothesized that DNA repair could be involved in the cisplatin–gemcitabine interaction as well. As yet, little is known about the capability of DNA repair mechanisms to reverse gemcitabine-induced DNA damage. It has been shown that the $3' \rightarrow 5'$ exonucleases of the Klenow fragment can not excise gemcitabine from the DNA [18], but to our knowledge, no research has been performed on the more complex DNA repair mechanisms involved at the cellular level. Therefore, we performed cytotoxicity studies of gemcitabine alone, and of gemcitabine in combination with cisplatin in a panel of CHO cell lines, each deficient in a different DNA repair pathway. The CHO cell panel is a good model system to investigate the influence of DNA repair on cytotoxicity, since the CHO DNA repair mechanisms are quite similar to the mechanisms acting in human cells. Furthermore, we investigated the influence of gemcitabine on intracellular platinum pharmacokinetics. The aim of the study was to investigate the interaction between gemcitabine and cisplatin, by determining the schedule giving rise to the strongest synergistic effects in CHO cell lines, and to assess whether the cytotoxicity of this combination is affected by one or more DNA repair mechanisms.

2. Materials and methods

2.1. Cell lines

Chinese hamster ovary cell lines (Table 1) were kindly provided by M. Zdzienicka, Department of Radiation Genetics & Chemical Mutagenesis, University Medical Center. All cells were cultured in nutrient mixture F-10 (HAM), with glutamine, 10% heat inactivated fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin and 25 mM HEPES-buffer at 37° in a humidified atmosphere of 5% CO_2 in air.

Table 1
Chinese hamster ovary cells

Cell line	Phenotype	Genotype
AA8	Wild type	Wild type
UV5	NER^-	*xpd*(*rad3*)
UV20	NER^-	*ercc1*(*rad10*)
irs1SF	HR^-	*xrcc3*
CHO9	Wild type	Wild type
EM-C11	BER^-	*xrcc1*
XRC1	$NHEJ^-$	*DNA-pkcs*

2.2. Cytostatics

Gemcitabine (dFdC, Gemzar®) powder for injection was obtained from Eli Lilly, and cisplatin (Platosin®) at a concentration of 1 mg/mL was obtained from Faulding Pharmaceuticals. Dilutions were made with 0.9% NaCl.

2.3. Cytotoxicity assay

The sulforhodamine B (SRB) drug cytotoxicity assay was used for single drug and combination experiments as described previously [30]. In brief, 1000 exponentially growing cells per 200 μL per well in 96-well plates were allowed to attach for 24 hr, followed by administration of the first drug. After incubation for 24 hr, the second drug was added. After a final incubation period of another 48 hr, cells were fixed, washed and stained with SRB and the absorption was measured at 540 nm. In the single agent experiments, cells were treated after 24 hr and allowed to incubate for another 72 hr. The following combinations were tested in the parental cell lines: simultaneous administration of cisplatin and gemcitabine, cisplatin followed by gemcitabine, and *vice versa*. In the simultaneous administration schedule both cisplatin and gemcitabine were added after 24 hr and allowed to incubate for 72 hr. In the repair deficient cell lines, only the most synergistic schedule from the three mentioned earlier, was tested. Increasing concentrations of drugs were used: cisplatin was varied from 20.32 to 1.11 mM, and gemcitabine from 0.03 to 2000 nM. Each agent and combination was tested in quadruplicate in three independent experiments. The inhibitory concentration at which 50% of cells was killed (IC_{50}) was determined in each experiment for each drug. Combination data were evaluated according to the median-effect analysis, based on the multiple drug-effect equation described by Chou and coworkers [31,32]. The combination index (CI) for the fractions killed from 10 to 90% was calculated using the following equation: $CI = d_1/D_1 + d_2/D_2 + \{(d_1d_2)/(D_1D_2)\}$, where D_1 and D_2 are the doses of drug 1 and 2 which by themselves result in a given fraction killed and d_1 and d_2 are the doses resulting in the same fraction killed in combination. The CI values were interpreted as an antagonistic effect if $CI > 1.2$, an additive effect if $1.2 > CI > 0.8$ and a synergistic effect if $CI < 0.8$. All of the data were analyzed by use of a Student's *t*-test.

2.4. Cellular platinum pharmacokinetics

To investigate the influence of gemcitabine on cellular platinum pharmacokinetics, 2×10^6 cells were plated in petridishes, and allowed to attach for 24 hr. Subsequently, the first drug was added, followed by the second drug 1 hr later. After an incubation period of an additional 4 hr, cells were washed, harvested and lysed in milli-Q water. The number of surviving cells were calculated by means of the Bradford protein analysis [33]. Total cellular platinum was determined by a validated atomic absorption spectrometry

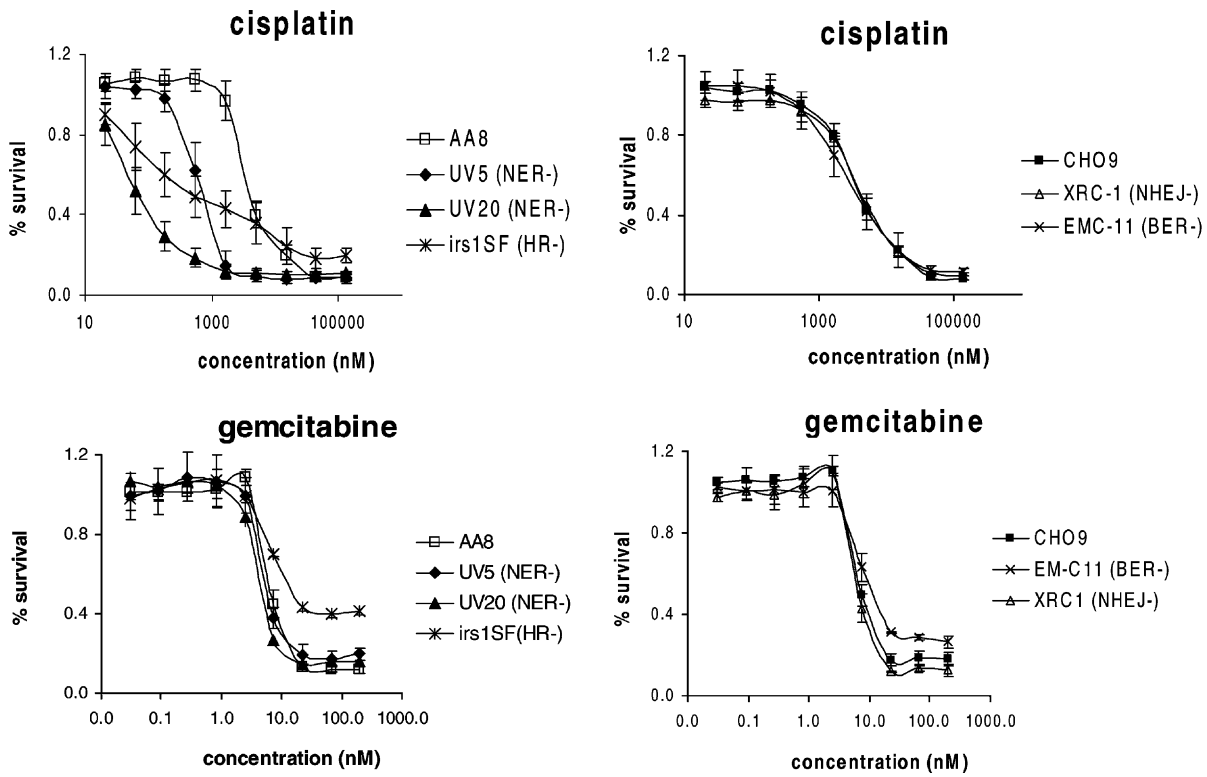

Fig. 1. Survival curves obtained for AA8 and CHO9 cells and their derivatives after treatment with cisplatin or gemcitabine.

assay [34] and platinum-DNA adducts were measured by a validated sensitive ^{32}P-postlabeling assay, enabling the separate determination of GG- and AG-intrastrand platinum adducts [35]. Cells were incubated in duplicate with the following combinations: cisplatin alone, cisplatin followed by gemcitabine and *vice versa*. Three independent experiments were performed.

3. Results

3.1. Single agent experiments

Before combination experiments were performed, cytotoxicity of gemcitabine and cisplatin as a single agent was determined after an exposure of 72 hr. The obtained survival curves are given in Fig. 1, and the IC_{50}'s are outlined in Table 2. As can be seen, for cisplatin both cell lines deficient in NER were more sensitive than the parental cell line, as was the cell line deficient in HR. This confirms the observation that these two DNA repair mechanisms are involved in correcting the DNA damage caused by cisplatin [9–11]. The BER and NHEJ cell lines were equally sensitive to cisplatin as their parental cell line. For gemcitabine, no cell line showed increased sensitivity, in fact, both the HR- and the BER-deficient cell lines were significantly less sensitive to gemcitabine (IC_{50} of 18.5 nM for HR vs. 4.7 nM in the parental cell line and 13.5 nM for BER vs. 7.8 nM in the parental cell line). This indicates that BER, NER, NHEJ and HR are probably

Table 2
The IC_{50} values of cisplatin and gemcitabine after 72-hr exposure (represented as mean ± SD)

	Cisplatin (nM)	Gemcitabine (nM)
AA8 (wt)	4332 ± 422	4.68 ± 1.25
UV5 (NER$^-$)	820 ± 267	6.51 ± 0.52
UV20 (NER$^-$)	80 ± 43	5.54 ± 0.23
irs1SF (HR$^-$)	1326 ± 167	18.54 ± 0.86
CHO9 (wt)	4297 ± 448	7.79 ± 0.96
XRC1 (NHEJ$^-$)	4434 ± 309	7.00 ± 0.74
EMC-11 (BER$^-$)	3954 ± 1155	13.50 ± 0.73

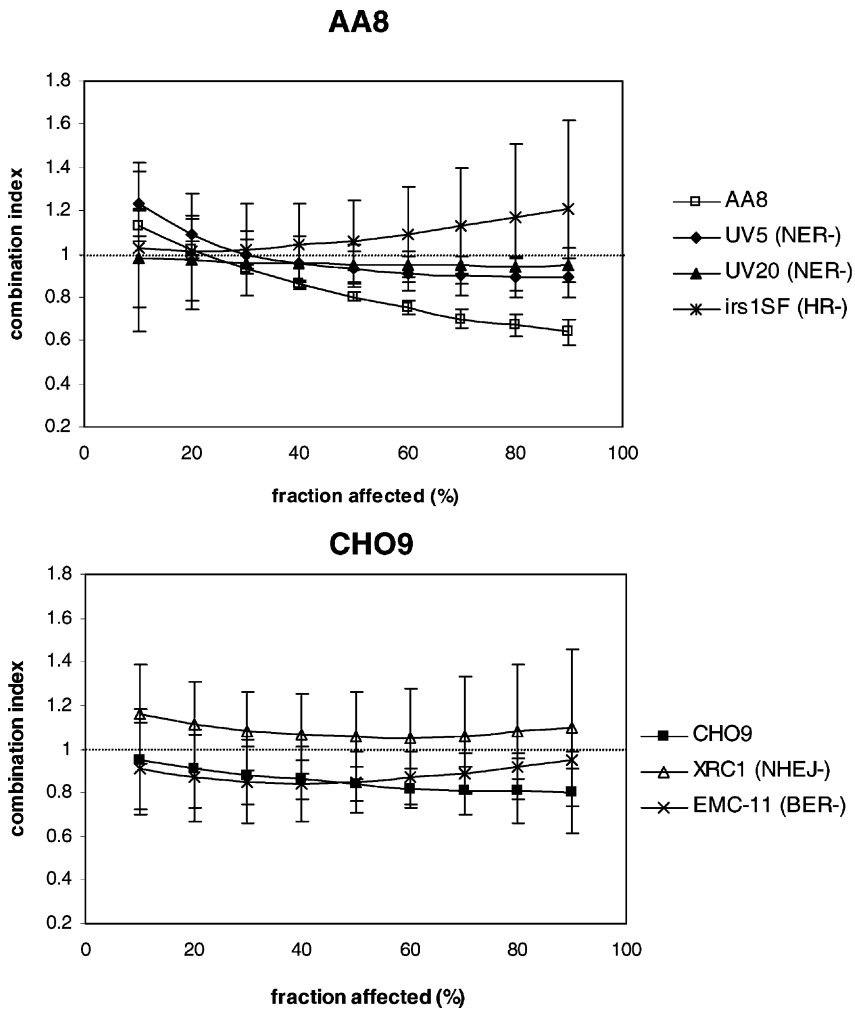

Fig. 2. Combination index vs. fraction affected for cisplatin followed by gemcitabine in AA8, CHO9 and daughter cells. The repair deficient cells derived from the AA8 cell line (top panel), differed statistically significant from the parental cell line above a fraction affected of 50%.

incapable of influencing the effects of gemcitabine on the DNA.

3.2. Combination experiments

In the parental cell lines AA8 and CHO9, combination experiments were performed to determine the highest synergistic interaction schedule. In both cell lines, simultaneous administration was antagonistic, and gemcitabine followed by cisplatin was additive (data not shown). In the AA8 cells, cisplatin followed by gemcitabine was synergistic from a fraction affected of 50% (Fig. 2, top panel). In CHO9 cells, cisplatin followed by gemcitabine was most active and strongly tended to synergy, especially at affected fractions >60% (Fig. 2, bottom panel). Hence, the schedule of cisplatin followed by gemcitabine was chosen for further testing in the repair deficient cell lines. These results are given in Fig. 2. For AA8 derived cells lines (NER and HR), statistically significant loss of synergy was observed at fractions affected >50%. Both NER-deficient cell lines demonstrated a shift towards additivity ($P < 0.009$), whereas the HR-deficient cell line demonstrated a shift to antagonism at the highest fractions affected ($P < 0.02$). The CHO9 derived repair deficient cell lines also demonstrated some loss of synergy when compared to the parental cell line. The effect was largest for the NHEJ-deficient cell line. However, in both cases the effects were smaller than

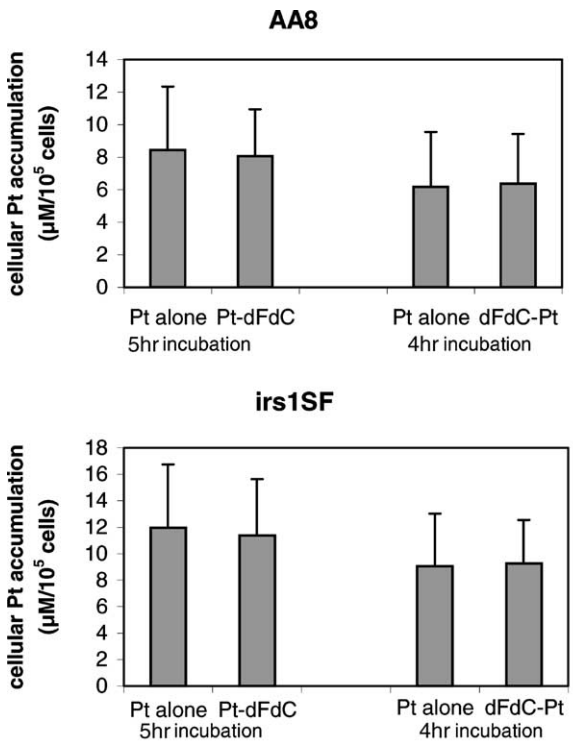

Fig. 3. Cellular accumulation of platinum in AA8 and irs1SF cells. In the administration sequence cisplatin followed by gemcitabine, cisplatin had an incubation time of 5 hr, and in the reversed sequence, gemcitabine followed by cisplatin, an incubation time of 4 hr. Differences were not statistically significant; dFdC: gemcitabine, Pt: platinum.

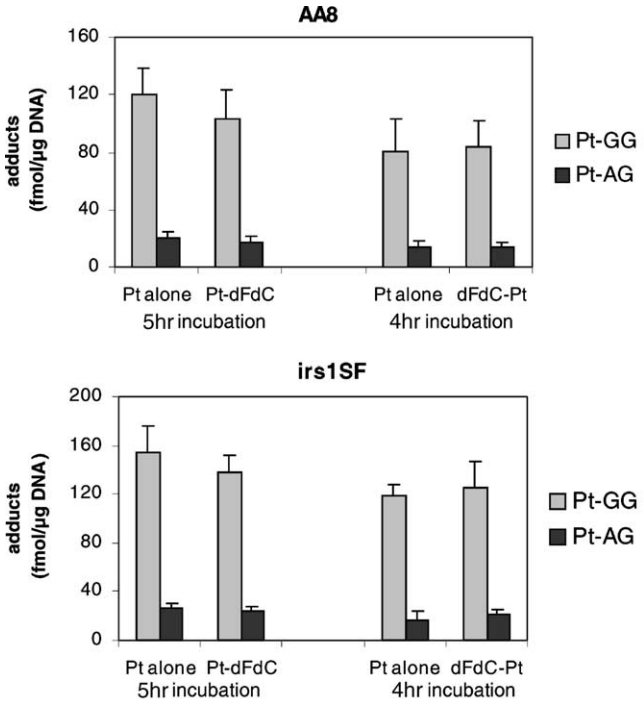

Fig. 4. Pt-GG and Pt-AG intrastrand adducts in AA8 and irs1SF cells. In the administration sequence cisplatin followed by gemcitabine, cisplatin had an incubation time of 5 hr, and in the reversed sequence, gemcitabine followed by cisplatin, an incubation time of 4 hr. Differences were not statistically significant; dFdC: gemcitabine, Pt: platinum.

observed for the HR line in the AA8 derived panel, and did not reach statistical significance. Thus, it can be concluded that HR most likely plays a major role in the synergistic cytotoxicity observed with the combination of cisplatin followed by gemcitabine, while NER may be involved as a minor player.

3.3. Intracellular platinum pharmacokinetics

The effect of gemcitabine on platinum accumulation and intrastrand DNA-adduct formation was studied in the HR repair deficient cell line, irs1SF, which showed loss of synergism in the combination experiments, and in the parental cell line AA8. First, the total cellular accumulation of platinum was measured in the following experiments: cisplatin followed by gemcitabine, gemcitabine followed by cisplatin and cisplatin alone as a reference (Fig. 3). No influence of gemcitabine on cellular accumulation of platinum could be observed, nor was there a statistically significant difference in accumulation between the parental cell line AA8 and the HR-deficient irs1SF (Fig. 3). Secondly, the platinum-DNA intrastrand adducts were measured in the same experiment. Both Pt-GG and Pt-AG adducts showed no change if cisplatin was combined with gemcitabine, in both administration schedules vs. cisplatin alone (Fig. 4). Hence, the observed loss of synergism in the HR-deficient cell line is not the result of a change in intracellular accumulation of platinum. Nor is

the loss of synergy reflected in a change in the number of intrastrand adducts at 4–5 hr post-treatment.

4. Discussion

Using a panel of CHO cell lines, each deficient in a specific DNA repair mechanism, we investigated the role of DNA repair in gemcitabine cytotoxicity, and in the synergistic cytotoxic interaction between cisplatin and gemcitabine. To our knowledge, this is the first study which addresses the involvement of DNA repair pathways in the cytotoxicity of gemcitabine, although it has previously been demonstrated that the $3' \rightarrow 5'$ exonuclease activity of the Klenow fragment is relatively incapable of excising gemcitabine from the DNA [14]. Our panel comprised cell lines deficient in BER, NER, HR and NHEJ. The first of these, BER, can repair small chemical changes of the DNA bases, such as uracil incorporated instead of thymidine, damage caused by oxygen radicals or damage caused by X-rays. NER repairs bulky adducts such as cisplatin intrastrand adducts and damage caused by UV-light. The recombination repair pathways HR and NHEJ repair double strand breaks and/or interstrand crosslinks (HR only), caused for example by radiation or cisplatin (reviewed in [36]). None of the repair deficient cell lines in our panel showed increased sensitivity towards gemcitabine, indicating that these repair mechanisms are not capable of protecting cells against gemcitabine induced cytotoxicity. However, to confirm this hypothesis, experiments measuring gemcitabine incorporation into the DNA in these repair deficient cell lines are warranted. Unfortunately, no CHO-derived mismatch repair (MMR)-deficient cell line was available for testing, so a possible role for that particular mechanism can not be ruled out. We observed a modest but statistically significant gemcitabine resistance in the HR- and BER-deficient cell lines. Possible explanations for this phenomenon could be less incorporation of gemcitabine into the DNA in these two cell lines, or activation of another (recombination) repair pathway. For example, in the HR-deficient cell line, single strand annealing or break induced replication (recombination subpathways) could still be active. In the case of cisplatin, the NER-deficient cell lines as well as the HR-deficient line were more sensitive towards treatment. This was expected, because these mechanisms repair the different platinum-DNA adducts [9–11,29,37,38]. When comparing the two different NER-deficient lines, one mutated in *XPD*, a helicase involved in the unwinding of the DNA during NER, and the other in *ERCC1*, required for the excision of the damage, the latter was most sensitive to cisplatin. This observation might be explained by the recent observations that *ERCC1* is not only involved in NER, but also in HR [39,40]. The increased sensitivity of the *ERCC1* mutated cell line to cisplatin has been reported in other studies as well [29]. Moreover, a recent clinical study identified *ERCC1* expression as a prognostic factor in lung cancer patients treated with cisplatin and gemcitabine [39].

In our combination experiments, we found a schedule dependency of the interaction between cisplatin and gemcitabine. In the CHO cell lines, cisplatin followed by gemcitabine was the most cytotoxic sequence. However, contradictory results have been reported in previous preclinical studies, and the schedule resulting in synergism for this combination is most likely cell-type specific. Cisplatin followed by gemcitabine was synergistic in human ovarian and lung cancer cell lines, but simultaneous administration or the reversed order have also been reported as most cytotoxic [24,27]. Using the repair deficient cells, we found a loss of synergism, although not complete, in the NER-deficient cell lines. Inhibition of NER activity by gemcitabine has recently been described in another study [40]. It was shown that gemcitabine prevented chain elongation in repair patches during nucleotide excision repair. Moreover, blocking NER abrogated the repair activity and simultaneously reversed the synergism between cisplatin and gemcitabine in a mismatch repair deficient cell line [40]. In our study, the effect of NER-deficiency on the loss of synergy between gemcitabine and cisplatin was far less than the effect of HR-deficiency. In fact, HR has been implicated as well in the interaction between cisplatin and other antimetabolites, as established in studies that revealed increased numbers of interstrand adducts in cells cotreated with cisplatin and fludarabine or cytarabine [41,42]. The mutant cell line used was deficient in *xrcc3*, indicating that the gene conversion subpathway of homologous recombination is involved in the synergism between cisplatin and gemcitabine. The NHEJ-deficient cell line showed a shift from low additivity (CI of 0.8) to high additivity (CI of 1.1) when compared to the parental cell line (Fig. 2). However, this phenomenon did not reach statistical significance and requires further testing before conclusions can be drawn.

In the present study, we have found that synergism between cisplatin and gemcitabine involves HR and in a minor way NER, but no changes in cellular accumulation of platinum or numbers of intrastrand platinum adducts were observed. In future studies, it would be useful to measure not only the intrastrand adducts, as we have done, but also the ICLs. Hypothetically, involvement of HR and NER might be explained by incorporation of gemcitabine at the gaps left behind after platinum-DNA adduct removal. In cisplatin interstrand-DNA adduct repair (performed by HR) two guanine bases on opposite strands are excised. Hence, in the subsequent repair steps this must always be followed by incorporation of cytidine, which is the nucleotide gemcitabine competes with. Moreover, the length of the newly synthesized DNA can stretch out up to a few kilobases in the HR process. In repair of intrastrand adducts (performed by NER) cytidine incorporation will not necessarily be required in each case, because the strand opposite the adduct, which has a cytidine paired with the guanine to which cisplatin is bound, is left intact.

Moreover, only short stretches of DNA, of <30 base pairs, are synthesized during NER. Thus, in addition to the incorporation of cytidine opposite the cisplatin bound guanines, the far larger stretch of DNA synthesis in HR also increases the possibility of gemcitabine incorporation as compared to NER. This mechanism could explain our observation that the HR driven repair influences the gemcitabine–cisplatin interaction stronger than the NER driven repair. However, to prove this hypothesis, more detailed investigations of both platinum and gemcitabine pharmacology on the DNA are required. Furthermore, the influence of enzymatic reactions, for example, a possible effect of cisplatin on the phosphorylation of gemcitabine, or an effect of gemcitabine on the enzymes involved in the respective DNA repair mechanisms, can not be ruled out. Complete elucidation of the interaction between cisplatin and gemcitabine is warranted, for it may aid in designing the optimal administration schedule in the clinic.

In conclusion, we have demonstrated that BER, NER, HR and NHEJ are probably not capable of efficiently modulating the effects of gemcitabine on the DNA. Furthermore, the present study showed that HR and NER are involved in the synergistic interaction between gemcitabine and cisplatin, and that cellular accumulation of platinum and formation of platinum-DNA intrastrand adducts are not affected by gemcitabine.

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