Cisplatin resistance

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Chapter 8

SUMMARY, CONCLUSIONS, AND PERSPECTIVES

SUMMARY

Cisplatin (cis-diamminedichloroplatinum(II)) is one of the most important agents used in clinical oncology. But, although in germ cell tumours 80% cures are achieved with cisplatin containing chemotherapy, most solid tumours are inherently insensitive or become insensitive after an initial response. In order to improve both the fundamental insight and the clinical treatment results, recent studies focused on relevant *in vitro* models, to elucidate the mechanisms underlying cisplatin resistance, and to try to find a way to (re)gain cisplatin sensitivity. Cisplatin resistance mechanisms frequently found in *in vitro* models include, reduced drug accumulation and increased detoxification of cisplatin in the cellular cytoplasm. In the cell nucleus decreased DNA accessibility and increased DNA repair may play a role.

The numerous attempts made to circumvent cisplatin resistance in *in vitro* as well as in animal models were described and discussed in chapter 1. Treatment of resistant cells with hyperthermic temperatures restored cisplatin accumulation and cytotoxicity. No common role for components of the cell signal transduction system has been found in cisplatin resistance. However compounds that affected cell signal transduction, such as staurosporine and quercetin, increased cisplatin cytotoxicity in some models. Calcium channel blockers and calmodulin inhibitors were no uniformly good modulators of cisplatin resistance. Glutathione, a thiol containing tripeptide, appeared to be one of the most promising targets for modulation of cytoplasmic defense mechanisms. Reduction of glutathione could be achieved by inhibition of its synthesis with buthionine sulfoximine, an agent that entered phase I combination studies with non platinum compounds already some years ago. DNA-repair enzyme inhibitors, such as aphidicolin, a specific inhibitor of DNA-polymerase α, and novobiocin, an inhibitor of DNA-topoisomerase II, potentiated cisplatin toxicity in cell lines with acquired resistance. Modified nucleotides, such as arabinofuranosylcytosine, azidothymidine and 5-fluorouracil were capable of affecting DNA repair, which led to an increased cisplatin sensitivity. Results of modulators varied in several models of cisplatin resistance. It will therefore be important to elucidate not only resistance mechanisms, but also the possible interaction between these mechanisms. This will facilitate the detection and modulation of resistance of tumours, thus making the effects of (combinations of modulators) more predictable. The remaining chapters of this thesis describe the development of cisplatin-resistance models and the search for mechanisms. In addition, attempts performed to modulate cisplatin sensitivity in small cell lung carcinoma and germ cell carcinoma cell lines were reported.

In chapter 2 a study is described, in which a cisplatin sensitive- and resistant small cell lung carcinoma cell line are used to evaluate the possibility of modulation of cisplatin sensitivity by incorporation of polyunsaturated fatty acids in tumour cells. Docosahexaenoic acid was incorporated in the membrane phospholipids of both sensitive and resistant cells and increased accumulation in both cell lines, while cisplatin cytotoxicity was increased only in the resistant cell line. Docosahexaenoic treatment of resistant cells led to an increased formation of cisplatin-DNA interstrand cross links. This could be induced by a change in the fatty acid composition of phospholipids associated with chromatin, as described by others, possibly leading to a change in chromatin conformation and DNA accessibility. However a 4-fold resistance remained in the resistant- compared with the sensitive small cell lung carcinoma cell line, despite the increase in cisplatin-DNA adducts. An increased DNA repair capacity in the resistant cell line might, at least partly, explain this.

The solid tumours most sensitive to cisplatin are, without any doubt, germ cell carcinomas. But even germ cell carcinomas become untreatable with cisplatin containing chemotherapeutic regimens in about 20 % of the patients due to development of resistance. Cell lines derived from this tumour type were equally hypersensitive for cisplatin. Therefore feasibility of resistance induction for this tumour was studied in cell lines. In chapter 3 a 3.7-fold cisplatin resistant subline (Tera-CP) of NTera2/D1 (Tera) is described. tera is a previously described, human embryonal carcinoma cell line, derived from a germ cell tumour, with an inherent capacity to differentiate *in vitro*, and *in vivo* when transplanted in nude mice. Karyotypes of both cell lines showed an "overexpression" of the short arm of chromosome 12, which is characteristic for germ cell tumours. In Tera-CP less copies of the short arm of this chromosome were found compared to Tera. Thus, a possible role for the "overexpression" of this chromosome arm as a marker for tumour resistance was not found in our model. Parameters of the detoxifying system, glutathione, glutathione S-transferase activity and glutathione S-transferase-π isoenzyme expression, were increased in Tera-CP. Cellular cisplatin accumulation, and the activity of three DNA repair related enzymes, DNA-topoisomerases I and II, and thymidylate synthase, were the same in both lines. However, cisplatin-DNA binding was decreased in Tera-CP. In vitro Tera and Tera-CP are mainly composed of embryonal carcinoma cells. In contrast Tera and Tera-CP cells in a xenograft in nude mice showed a marked differentiation. Thus, this model enabled us on one hand to study mechanisms underlying cisplatin resistance in originally, extremely sensitive cells, and on the other hand to investigate the effects of differentiation state of cells on cisplatin sensitivity. In vivo Tera-CP tumours showed a 2.8-fold resistance for cisplatin. In new cell lines, derived from the cisplatin treated sensitive and resistant xenografts, the mean resistance factor for cisplatin was 3.6. In these cell lines glutathione and glutathione S-transferase activity were still increased in the resistant cell lines. The Tera/Tera-CP panel seemed to be a good model for in vitro and in vivo cisplatin resistance of germ cell carcinoma, with a reduced binding of cisplatin adducts to DNA probably due to an increased efficacy of the detoxifying system.

The finding of residual mature teratoma after the treatment of germ cell carcinomas with cisplatin containing chemotherapy is well known. This process is associated with primary tumours that have the capacity for spontaneous differentiation. The use of differentiation inducing agents, such as retinoids, in combination with cisplatin-chemotherapy, could be a way to force cisplatin-insensitive tumour cells into a more differentiated and probably less malignant state. In the chapters 4, 5, and 6 differentiation induction in combination with chemotherapy was described, as an alternative strategy for tumour treatment.

In chapter 4, the effects of combination of differentiation induction with cytotoxic chemotherapy as studied in a murine embryonal carcinoma cell line were described. All-trans retinoic acid was used as the differentiation inducing agent. In addition to its combination with cisplatin, this compound was also combined with etoposide, a drug targeted to the nuclear enzyme DNA-topoisomerase II, and a recognized drug in the treatment of germ cell tumours. All-trans-retinoic acid concentrations, capable of differentiation induction, without affecting cell cycle distribution or cell growth, enhanced cisplatin sensitivity. This increased cisplatin cytotoxicity was not attributable to changes in cellular platinum accumulation, or to changes of DNA platination. The cause of the increased cisplatin sensitivity in the differentiated cells remained unclear. All-trans-retinoic acid also enhanced the etoposide sensitivity of the cell line studied. After all-trans-retinoic acid incubation, DNA-topoisomerase II activity was unchanged, DNA-topoisomerase I activity increased 2-fold, and the cellular etoposide concentration increased 2- to 4.5-fold. The latter was most likely the cause of the increased etoposide sensitivity. From this study it was concluded that, as far as the murine embryonal carcinoma cells could serve as a model for germ cell tumours, the rational for combining all-trans-retinoic acid and cisplatin or etoposide was 2-fold: all-trans-retinoic acid induced differentiation and increased sensitivity of murine embryonal carcinoma cells for chemotherapeutic drugs.

In the study described in chapter 5 the human embryonal carcinoma cell lines Tera and Tera-CP are used to determine differentiation induction, cisplatin sensitivity, and the amount of cisplatin induced apoptosis after pre-treatment of the cell lines with three modulators. All-trans-retinoic acid was used as a known differentiation inducing agent. In this study, the effects of docosahexaenoic acid and hexadecylphosphocholine on the parameters mentioned above were also evaluated. Both agents are membrane composition modulators that could affect cell signal transduction, and therefore might have an impact on processes such as differentiation and apoptosis. Apoptosis is a physiological form of cell death that also occurs after treatment of cells with several chemotherapeutic drugs. The role of the susceptibility of cells to apoptosis in the sensitivity of tumour cells for drugs is still unclear. It has been postulated that higher amounts of unrepaired DNA damage in a cell correlate with increased drug sensitivity, but a number of publications indicated that especially drug sensitive cells are more susceptible to apoptosis. It was found that, without drug treatment, both cell lines consisted of embryonal carcinoma and Tera-CP showed less apoptosis than Tera. After treatment with the modulators only, still more apoptosis was found in Tera than in Tera-CP. Equitoxic cisplatin concentrations

led to a comparable percentage of apoptotic cells in both lines. Treatment with all-trans-retinoic acid resulted in differentiation, a decreased cisplatin sensitivity, and a decreased percentage of apoptosis after cisplatin treatment in both cell lines. Docosahexaenoic acid enhanced cytotoxicity and apoptosis caused by cisplatin without affecting the embryonal character of both cell lines. Cytotoxicity and differentiation state of both cell lines were not affected by hexadecylphosphocholine. Effects of the modulators on differentiation, cisplatin induced cytotoxicity and apoptosis were not different for Tera and Tera-CP. Thus, there might be a decreased propensity of untreated Tera-CP cells to go into apoptosis. This was also found for Tera-CP cells treated with the modulators, of which doses inducing less than 10 % cell kill were used. After treatment of Tera and Tera-CP with equitoxic cisplatin concentrations this difference in apoptosis was no longer observed. Docosahexaenoic acid potentiated cisplatin induced cytotoxicity and apoptosis in both lines, which made it unlikely that a specific cisplatin resistance mechanism was affected by this drug. Although all-trans-retinoic acid induced differentiation, it strongly prohibited cisplatin induced cytotoxicity and apoptosis in this model of human embryonal germ cell carcinoma.

In murine- and human embryonal carcinoma cell lines all-trans-retinoic acid induced comparable morphological differentiation, but its effects on cisplatin sensitivity were contradictory. This might be due to the fact that all-trans-retinoic acid was previously shown to differently induce, the expression of genes, growth factors, and growth factor receptors in murine and in human embryonal carcinoma cell lines (1). For instance, epidermal growth factor receptors increased in murine (2), and decreased in human (3) embryonal carcinoma cells after all-trans-retinoic acid treatment. For epidermal growth factor receptors it was shown, in ovarian carcinoma cell lines, that their expression and functionality correlated with cisplatin sensitivity (4). The difference in cisplatin sensitivity in murine and human embryonal carcinoma cells after retinoid treatment could be due to this mechanism.

In the study presented in chapter 6 the potential of the addition of differentiation induction by a retinoid to cisplatin chemotherapy is studied in vivo. The human embryonal carcinoma cell line Tera and its cisplatin resistant subline Tera-CP, both with an inherent capacity to differentiate in vivo, were transplanted in nude mice. Instead of all-trans-retinoic acid as a differentiation inducing agent, 13-cis-retinoic acid was used because it was described to have higher and more predictable plasma levels than all-trans-retinoic acid, and comparable biological effects. On the other hand nuclear retinoic acid receptor affinity was described to be lower for 13-cis- than for all-trans-retinoic acid. Mice were treated with 13-cis-retinoic acid on 5 consecutive days (30 mg/kg), or cisplatin in a single dose (7 mg/kg), or cisplatin followed by, or preceded by 13-cis-retinoic acid. To study tumour histology all mice were sacrificed at day 12. All treatments had an acceptable toxicity as determined by loss of body weight. Toxicity was less pronounced than toxicity reported for all-trans-retinoic acid in previous studies. The size of Tera-CP but not of Tera tumours was reduced by 13-cis-retinoic acid alone. Cisplatin reduced the tumour size in Tera tumours to 20 % of control values (day 1),

and no regrowth was found. The tumour size in Tera-CP was reduced by cisplatin to 70 % of control values (day 1), after which a regrowth followed. In Tera and Tera-CP tumours 13-cis-retinoic acid, before or after cisplatin, did not affect cisplatin anti-tumour activity. Differentiation after cisplatin alone, and after combined treatments was more extensive in Tera than in Tera-CP. Cisplatin combined with 13-cis-retinoic acid induced more differentiation in Tera-CP, than cisplatin only.

Summarizing, this study indicated that 13-cis-retinoic acid did not affect the cisplatin sensitivity of both tumour types. Induction of differentiation after combined treatment with 13-cis-retinoic acid and cisplatin seemed possible in resistant tumours. For future experiments the simultaneous addition of 13-cis-retinoic acid and cisplatin might be a way to achieve better results.

In the animal model it appeared that cisplatin combined with 13-cis-retinoic acid was as active as cisplatin alone. This is in contrast with the in vitro loss of cisplatin sensitivity after retinoid treatment in Tera and Tera-CP. The presence of growth factors and the interactions with the extracellular matrix could play an important role in in vivo retinoid response (for review: 1). The absence of these interactions *in vitro* might cause the different retinoid response in that setting. 13-Cis-retinoic acid pre-treatment did not reduce the cisplatin-sensitivity of the tumour cells, while spontaneously differentiated Tera cells seemed more resistant to cisplatin. Therefore, retinoid induced alterations that cause morphological differentiation of embryonal carcinoma cells, are different from those that underlie its effect on cisplatin sensitivity.

In addition to the relevance of studies on models of in vitro acquired resistance even more clinically relevant information about resistance mechanisms might be found in models of intrinsic resistance. Such a model is described in chapter 7, in which cisplatin sensitivity and possibly responsible cellular parameters in three germ cell- and three colon carcinoma cell lines, as models of intrinsically cisplatin sensitive and resistant tumours were compared. Cisplatin sensitivity of the cell lines mimicked the clinical situation. Glutathione levels of the cell lines correlated with cisplatin concentrations inhibiting cell survival by 50 % (IC50). Total cellular sulfhydryl content was inversely correlated with IC50. These higher amounts of total cellular sulfhydryl in the most cisplatin sensitive cell lines were in contrast with the higher levels of metallothionein found in some cell lines with acquired resistance. This discrepancy could be (partly) explained if the sulfhydryl containing proteins in germ cell carcinomas were different from those in colon carcinomas. This may be the case as for rat testicular tissue, in contrast to other tissues, the metal-binding proteins were found not to be metallothioneins (5). IC50 of the cell lines correlated neither with glutathione S-transferase activity nor with glutathione S-transferase-π expression, DNA-topoisomerase I or II activity. Immediately after a 4-h cisplatin incubation, cellular platinum accumulation and platinum bound to DNA did not correlate with IC50, but after another 24-h drug-free culture, platinum bound to DNA in germ cell-, but not in colon, carcinoma cell lines correlated with IC50. With exception of in vitro sensitivity and total cellular sulfhydryl content none of the parameters studied discriminated between both groups of cell lines. Correction of cisplatin sensitivity parameters for phenotypical

differences between the cell lines did not influence statistical correlations. Analysis of variance revealed a correlation between IC50 and the combination of glutathione, glutathione S-transferase activity and platinum bound to DNA. But at other cisplatin cytotoxicity levels sensitivity was also correlated with platinum accumulation, DNA-topoisomerase II activity and total cellular sulfhydryl content in various combinations. This model of intrinsic cisplatin resistance showed that multiple parameters ought to be studied to explain cisplatin resistance. But the model did not elucidate the cause of the unique sensitivity of germ cell carcinoma, although the unexpected values of total cellular sulfhydryl content deserve further attention.

CONCLUSIONS AND PERSPECTIVES

Models. Induction of *in vitro* resistance to cisplatin had been shown to be possible in cell lines that originated from tumours that frequently became unresponsive to therapy with this drug. In this thesis resistance induction in a cell line derived from an extremely sensitive tumour type, namely germ cell carcinoma was described. The mechanisms found to underlie this resistance, an increased detoxifying capacity and a reduced DNA platination, were not different from those found in other tumour types. The study of a model of inherent sensitivity and resistance to cisplatin, using germ cell carcinoma and colon carcinoma cell lines, made clear that, although detoxifying capacity also seemed to be important, the cause of cisplatin resistance could be related to several mechanisms, of which the contribution might vary at different cytotoxicity levels. Remarkably is the fact that in this model correlation between DNA platination and cisplatin sensitivity was found for the germ cell carcinoma, but not for the colon carcinoma, cell lines. These new models of cisplatin resistance did not reveal new resistance mechanisms. This stressed the importance of elucidating the relations and interactions between the known causes of cisplatin resistance.

Mechanisms. A role for the detoxifying capacity of tumour cells in cisplatin sensitivity is repeatedly found in the models described in this thesis. Glutathione levels were related to cisplatin sensitivity in the small cell lung carcinoma model (6), the germ cell carcinoma model, and in the germ cell-/colon-carcinoma model. Glutathione S-transferase activity and its π-isoenzyme levels were increased in the resistant germ cell carcinoma cell line. The total cellular thiol content was inversely related to cisplatin sensitivity in the germ cell-/colon-carcinoma model.

However, cellular thiol availability is a dynamic process that is dependent on synthesis of thiol containing compounds in relation to their utilization. Depletion of glutathione was previously shown to increase the sensitivity of several tumour cell lines to cisplatin, while in others no sensibilisation was found. This might be tumour type dependent. Glutathione has a role in many processes important for normal cell functioning, it is conceivable that cell types

vary in their dependence of this compound. They therefore might react differently to glutathione depletion either or not in combination with cytotoxic treatment. Also protein bound thiols, the other component of total cellular thiol content seemed to function in a tissue specific way. Metallothionein is a thiol containing protein of which high levels coincided with reduced cisplatin sensitivity in some models. But in our germ cell-/colon-carcinoma model a high total cellular thiol content was found in the most cisplatin sensitive cells. A phenomenon that might be related to the different nature of protein bound thiol in testicular tissue compared with other tissues, as already found in rats. Glutathione S-transferase was shown by others to have, in testicular tumours a decreased expression, and in colon tumours an increased expression of its π-isoenzyme compared to normal adjacent tissue (8,9). This probably indicated a difference in regulation and dependency between these tissues. It remains difficult to draw firm conclusions about the role of the detoxifying system based on the study of the models described. In order to be able to better define the significance of this system in cisplatin sensitivity, the relation between the various components, the kinetics of the components, as well as the relation between normal tissues and their respective tumours need further study.

DNA-platination was reduced in the resistant small cell lung carcinoma cell line and the resistant germ cell carcinoma cell line when measured immediately after cisplatin incubation. DNA-platination 24 hours after cisplatin treatment correlated with cisplatin sensitivity in the germ cell carcinoma, but not in the colon carcinoma, cell lines, in the germ cell-/colon-carcinoma model. The reduced DNA-platination in the cell lines with acquired resistance and in the germ cell carcinoma cell lines is in accordance with the concept that the final amount of cisplatin induced DNA damage determines the cytotoxicity of cisplatin. The lack of correlation between DNA damage and sensitivity in the colon carcinoma cell lines of the germ cell-/colon-carcinoma model could be an indication that cellular sensitivity to DNA damage might vary between different cell types. This sensitivity could be related to the triggering of apoptosis. The mechanisms that do initiate this process are still unclear but the disturbance of normal cell cycle progression and its regulation seem to be of importance. An increased knowledge of this process could certainly shed light on many cases of tumour cell sensitivity and resistance that are still inexplicable now.

Modulation. Modulation experiments in this thesis focused on two agents: docosahexaenoic acid and retinoids. Docosahexaenoic acid increased cisplatin sensitivity in the resistant small cell lung carcinoma, and in both the sensitive and resistant germ cell carcinoma cell line. In the lung carcinoma model increased DNA damage was specifically seen in the resistant line, suggesting a role for docosahexaenoic acid in processes associated with DNA-accessibility or -repair. Although not determined, comparable mechanisms could play a role in the germ cell carcinoma model. On the other hand unsaturated fatty acids, such as docosahexaenoic acid affect membrane composition and might affect cell signal transduction. Thus, despite the fact that sensitizing was achieved with this, *in vivo* mildly toxic modulator, the mechanisms behind

this phenomenon need further study.

Retinoid treatment in combination with cisplatin incubation was studied in three models. Firstly, a murine embryonal carcinoma cell line, secondly a panel of two human embryonal carcinoma cell lines with different cisplatin sensitivities *in vitro*, and finally the human embryonal carcinoma model transplanted in nude mice, were used. In the murine embryonal carcinoma model differentiation induction with all-trans-retinoic acid led to an increased cisplatin sensitivity. In the human embryonal carcinoma model *in vitro*, all-trans-retinoic acid induced differentiation was accompanied by a decreased cisplatin sensitivity in both the initially cisplatin sensitive and resistant cell line. In the human embryonal carcinoma model xenografted in nude mice, neither a positive, nor a negative influence of 13-cis-retinoic acid on tumour cisplatin sensitivity was found.

In all three models morphological differentiation was induced. The effects of retinoid treatment on cisplatin sensitivity were different between murine- and human embryonal carcinoma cells, and between human embryonal carcinoma cells *in vitro* and *in vivo* in tumours transplanted in nude mice. A difference in cisplatin sensitivity was also observed between cells that differentiated spontaneously and cells with a retinoid induced differentiated phenotype. It must therefore be concluded that effects of retinoids that were not directly related to morphological differentiation, were responsible for the alterations in cisplatin sensitivity of the models. In this context, the role of epidermal growth factors receptors, and possibly others, is intriguing and deserves further attention. Response to treatment with retinoids in combination with chemotherapy varied between different cell types and may depend on the scheduling of the drugs. As long as mechanisms underlying the interactions are not clear, clinical use of this combination must be discouraged.

Interactions and relations between the various compounds of the detoxifying system currently deserve much attention in the study of cisplatin resistance. The germ cell/colon carcinoma panel provides a good model for these studies. In addition to the elements described in this thesis, there is a revival of interest for the role of the cell membrane in cisplatin resistance (10). Recently, in one model, a membrane pump for glutathione-complexes has been described (11). Demonstration of membrane factors in other models of cisplatin resistance could mean an important contribution to the understanding of resistance mechanisms.

Elucidation of the role of apoptosis in drug sensitivity could be an important contribution to the knowledge of drug resistance. Therefore experiments that contribute to the unravelling of this process are needed. Apoptosis is a controlled process that leads to self-elimination of cells. It is, among others, thought to be triggered in cells that suffered an amount of DNA damage that can not be repaired before entrance in the next phase of the cell cycle (7). It is most likely that certain nuclear proteins play a role in the recognition of this damage and the subsequently induced cascade of events. Retinoids are known to cause alterations in the nuclear protein

composition of cells (1). In addition in this thesis it was shown that retinoids strongly prohibited the cisplatin induced cytotoxicity and apoptosis in the human embryonal carcinoma cell lines Tera and Tera-CP. Therefore this panel might be an excellent model for attempts at elucidation of the role of nuclear proteins in the processes of DNA damage recognition and apoptosis induction.

In conclusion, in addition to the study of the role apoptosis in drug resistance, the study of the possible interactions between the mechanisms of cisplatin resistance in suitable, already existing models deserves attention. Existing models could also be used in the search for other, until now unknown factors that might play a role in cisplatin resistance. Both seem to be relevant ways to come to a facilitated detection, and a more successful treatment, with combinations of drugs, of resistant tumours in the clinic.

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