

Chapter 8



Summary and general discussion

Anticancer treatment with the anthracycline doxorubicin (DOX) is associated with a dose-dependent cardiotoxicity, which increases rapidly above a cumulative dose of 550 mg/m². As a consequence, most treatment schedules for adult and childhood cancer limit the maximum cumulative dose of DOX to 450–550 mg/m². However, because of the considerable variability in the individual susceptibility to the cardiotoxicity, there is no absolute safe dose. In addition, DOX-induced cardiac damage increases during the length of follow-up.

Different hypotheses have been proposed to explain this cardiac damage and there is a lot of evidence that the generation of free radicals plays an important role. However, several other mechanisms appear to be involved in the development of DOX-induced cardiac damage.

If the incidence of DOX-induced cardiotoxicity resulting in congestive heart failure would drop, the quality and extent of life for patients surviving cancer would improve. Different approaches have been investigated in an attempt to minimize or prevent DOX-induced cardiotoxicity.

In preclinical studies, the protective effect of monoHER against DOX-induced cardiotoxicity has been demonstrated *in vivo*, when monoHER was given as a single i.p. injection (500 mg/kg) once a week 1 hour before DOX (4 mg/kg, i.v.) for a period of six weeks. MonoHER did not affect the antitumor activity of DOX, which was demonstrated *in vitro* and *in vivo*. In addition to its radical scavenging and iron chelating properties, it was found *in vitro* that part of its protective effects may also be due to the anti-inflammatory effect of monoHER. The pharmacokinetic profile of monoHER under protecting conditions (500 mg/kg, i.p.) was characterized and it was found that 5–15 minutes after i.p. administration of monoHER in mice, a mean peak plasma level of about 131 μM was obtained whereas the mean AUC[∞] was 6.3 μM.min. This thesis focuses on more insight in the mechanisms of action of monoHER, optimizing the administration schedule between monoHER and DOX and finally its evaluation and potential cardioprotective effect in a clinical Phase II and I study.

In **chapter 2** it was shown that addition of anti-inflammatory agents during treatment with DOX reduced its cardiac damage in mice. In addition, it was demonstrated that treatment with DOX induces an increase of N^ε – (carboxymethyl) lysine (CML) in intramyocardial arteries in mice. The induced increase in CML, which can be regarded as a biomarker for local endogenous stress, is reduced by these anti-inflammatory agents and monoHER. These results suggested that DOX-induced inflammatory effects are involved in the development of DOX-induced cardiotoxicity and also indicated that monoHER has anti-inflammatory properties besides its radical scavenging and iron chelating properties.

In earlier studies from our group, monoHER showed a strong protection against the cardiotoxic effects of DOX without decreasing its antitumor effects, both *in vivo* and *in vitro*. Because it is known that DOX induces apoptosis, the effect of monoHER hereon was investigated in neonatal rat cardiac myocytes (NeRCaMs), human endothelial cells (HUVECs) and ovarian

cancer cell lines in **chapter 3**. Assessment of the fold anti-apoptotic protection achieved showed that HUVECs and NeRCaMs were stronger protected by monoHER than A2780 tumor cells. Employing the broad caspase inhibitor z-VAD-fmk revealed that DOX triggered caspase-dependent apoptosis in HUVECs and A2780 cells, and caspase-independent cell death in NeRCaMs. Thus, combination treatment with monoHER was effective in suppressing both caspase-dependent and -independent apoptosis. When examining molecular mechanisms that underlie the protective effects of monoHER, it was demonstrated that monoHER strongly reduced the activation of DOX-induced p53 accumulation in these cells. Probably, the radical scavenging properties of monoHER caused the reduction of p53 accumulation, which is a known sensor of ROS-dependent toxicity. The suppressive effect of monoHER on the activation of caspase-9 and -3 and the substrate PARP can also be explained by the neutralization of ROS-dependent triggers of caspase activation. However, the suppressive effect of monoHER on DOX-induced apoptosis in cancer cells raised the question whether monoHER may reduce the antitumor effects of DOX in the clinic. The concentration of monoHER used in this study was much higher than that found in mice under protecting conditions. Thus, it was therefore concluded that besides its potent protective effect against various routes of doxorubicin-induced cell death, it is important not to raise the dose of monoHER above the concentrations that demonstrated protection *in vivo*, because this might adversely affect the antitumor activity of DOX. The tendency of monoHER to protect normal cells more than cancer cells may be attributed to the inherent proliferation capacity of cancer cells. This is supported by earlier investigations showing restricted protection of confluent but not proliferating endothelial cells after treatment with DOX. The different effects of monoHER in terms of apoptosis suppression may reflect the activity of the intrinsic ROS defence systems present in cells, which may also be associated with different mechanisms of cell death activation.

Earlier, the protection of monoHER against DOX-induced cardiac damage was shown in mice when monoHER was given once as an i.p. dose of 500 mg/kg 1 hour before DOX. Because of the relatively short final half-life of monoHER (about 30 min), it was expected that the time-interval between monoHER and DOX might be of influence on the cardioprotective effect of monoHER i.e. becomes better with a shorter time interval and worse when lengthening the time interval. Our data described in **chapter 4**, did not indicate a significant change in protection against DOX-induced cardiac damage over the time interval between monoHER and DOX from 2 h to 10 min.

It is known that the long-term effect of DOX on cardiac tissue may progress in time to more severe myocardial injury resulting in cardiomyopathy or even chronic heart failure. Previous studies showed the protective effect of monoHER against DOX-induced cardiotoxicity within the first 8 weeks of treatment. Our data in **chapter 5** showed that the cardioprotective effect

of monoHER is lasting for a longer period of time thereafter, however towards the end of 26 weeks of observation the cardioprotection by monoHER is not present anymore and toxicity becomes comparable to that in DOX-treated animals. Continuation of weekly injections of monoHER (after 6 weeks of DOX administration) for another 26 weeks even seems to aggravate the development of DOX-induced cardiotoxicity. It has been demonstrated before that flavonoids, which can act as antioxidants, may display pro-oxidant action at higher doses and/or when administered for an extended period of time. Because of these properties, it is possible that monoHER was administered at a too high dose in the animals receiving monoHER just before DOX and especially in the animals receiving monoHER also once every week during the observation period. Therefore, it could be that the assumed overdose of the antioxidant monoHER started to behave as a pro-oxidant as shown for other flavonoids before. A possible explanation for the pro-oxidant activity may be that monoHER is converted into a reactive oxidation product which subsequently depletes the already small amount of cardiac antioxidants. Thus, the right balance between the dose of monoHER and its anti- or pro-oxidant properties has not been established yet.

In **chapter 6** the possible side effects and the pharmacokinetics of monoHER were evaluated in a clinical phase I study with healthy volunteers. Up to the highest dose of 1,500 mg/m², monoHER was well tolerated and no serious side effects were observed. It was not attempted to establish the maximal tolerated dose (MTD) of monoHER, because the pharmacokinetic end-points were obtained i.e. a mean peak plasma concentration of 360 ± 69.3 μM and a mean AUC[∞] of 6.3 ± 2.1 μmol.min/ml at a dose of 1,500 mg/m². At this dose level the solubility of monoHER in the i.v. infusion fluid was also reached. MonoHER was rapidly distributed and eliminated from the plasma compartment, which corresponds with the rapid uptake in and elimination from heart tissue as found before in mice. Our conclusion was that 1,500 mg/m² of monoHER would be a potential effective dose which could be administered safely.

This dose was used in a clinical phase II study in which the cardioprotection of monoHER was investigated in cancer patients receiving DOX as described in **chapter 7**. Eight patients with metastatic cancer were treated with DOX preceded by a 10 min i.v. infusion of 1,500 mg/m² monoHER. Of them, five patients received a cumulative dose of ≥300 mg/m² and underwent an endomyocardial biopsy. Three patients were treated with a time-interval (ΔT) of 1 hour between monoHER and DOX, one patient with ΔT = 10 min and one patient with ΔT = 2 hours. No difference in biopsy score was found between the patients. The mean biopsy score of the five patients was higher (2.7) than the mean score (1.4) from historical data of patients who received a similar cumulative dose of DOX. Although there is a considerable variability in the few investigated patients, it was indicative that monoHER enhanced DOX-induced cardiotoxicity.

An interesting finding was that all four patients with metastatic soft tissue sarcoma (STS) responded (3PR, 1SD) to the combination therapy, which is much higher than expected. Although these effects of monoHER are in contrast to the earlier animal studies, they show a certain correspondence with the results of chapter 5. The differences may be explained by a possible difference in monoHER metabolism between mice and patients. In both patients (this chapter) and mice (chapter 4) it was found that the time interval between monoHER and DOX was not relevant. On the other hand, the dose of monoHER may be crucial as found in mice (chapter 5) and in patients (chapter 7). A possible explanation may be that during scavenging of reactive oxygen species, the antioxidant monoHER is converted into an oxidation product, which is reactive with thiols. Such a reaction may lead to toxicity in two ways. First, to a reduction of the antioxidant status of the cardiomyocyte and thus to a decrease in the protection against cardiotoxicity. Secondly, the oxidation product of monoHER may react with other thiols, such as protein thiols. These adducts may accumulate in the cell and cause additional toxicities. Previous studies have demonstrated that the concentration of the antioxidant glutathione may play a role in the antitumor effect in soft tissue sarcoma cells, and thus the same mechanism may play a role as that hypothesized for the cardiomyocytes. It was concluded that the dose of monoHER may play a crucial role: at a dose of 1500 mg/m² there was no protection against DOX-induced cardiotoxicity in patients with metastatic disease, but it may have an enhancing effect on the antitumor activity of DOX in patients with metastatic STS.

Conclusion

The semisynthetic flavonoid 7-monohydroxyethylrutoside (monoHER) has besides its radical scavenging and iron chelation properties, anti-inflammatory capacity which is indicated by inhibiting DOX-induced neutrophil adhesion of HUVECs and DOX-induced VCAM and E-selectin overexpression *in vitro* and its reducing effect on DOX-induced CML increase *in vivo*. In addition, anti-apoptotic properties of monoHER were demonstrated. This effect is probably also due to the ability of monoHER to neutralize ROS.

Although, considering the relatively short half-life of monoHER, it was expected that the time interval between monoHER and DOX might be of influence on the cardioprotective effect of monoHER, no influence was observed. In contrast, data in this thesis indicate that the dose of monoHER may be important. High concentrations of monoHER (> 7 times of the maximal plasma concentration found in mice under protecting conditions) demonstrated protection of ovarian cancer cells *in vitro*. This observation indicates that the dose of monoHER may not lead to a concentration above that showing protection *in vivo* (131 µM). In addition,

repeated dosing of monoHER after the treatment period with DOX, tended to aggravate the development of DOX-induced cardiac damage in mice. It is supposed that the created overdose of the antioxidant monoHER starts to behave as a pro-oxidant as shown before for other flavonoids. Therefore, it seems that fine-tuning of dose and frequency of monoHER administration is crucial in obtaining an optimal and desired effect (anti-oxidant activity or pro-oxidant activity) of monoHER. These conclusions are in agreement with the findings in patients during the Phase II study.

Implications and further research

The present research indicates that the dose and frequency of monoHER administration are crucial in the protection against DOX-induced cardiotoxicity. There may be a dose-dependent transition in the effect of monoHER i.e. a high dose (≥ 1500 mg/m²) for obtaining a potentiating effect of the antitumor effect for at least soft tissue sarcomas and a low dose (somewhere below 1500 mg/m²) for obtaining cardioprotection.

Several interesting research lines are open for the future. The first is to investigate the potentiating effect of monoHER on the effect of DOX on human soft tissue sarcoma cell lines. If these effects are positive, its mechanism of action has to be evaluated. If possible the findings have to be extrapolated to other tumor types. In the mean time a clinical Phase II study can be started to investigate the antitumor effect of monoHER in combination with DOX in patients with STS.

A second question to be answered is whether differences in metabolism of monoHER in mice and patients are responsible for (part) of the differences in cardiac response between the species.

A third area of special attention is the critical dose of monoHER necessary for obtaining long-term cardioprotection. After obtaining more insight in the changes of the intracellular metabolism due to monoHER, new *in vivo* experiments can be started.