A phase II study of epirubicin in colorectal cancer and the use of cyclosporin-A in an attempt to reverse multidrug resistance

J. Verweij1, H. Herweijer1, R. Oosterom1, M.E.L. van der Burg1, A.S.Th. Planting1, C. Seynave1, G. Stoter1 & K. Nooter1,2

1Department of Medical Oncology and 2Department of Clinical Chemistry, Rotterdam Cancer Institute; Daniel den Hoed Kliniek, Groene Hilledijk 301, 3075 EA Rotterdam; 3Institute of Applied Radiobiology and Immunology, Lange Kleiweg 151, 2288 GJ Rijswijk, The Netherlands.

Summary We determined the ability of the multidrug resistance (MDR) reversal agent cyclosporin-A to increase anthracycline drug accumulation in colorectal tumour cells in vitro, using the technique of on-line flow cytometry. Data of four previously untreated patients showed that cyclosporin-A can increase intracellular net-uptake of daunorubicin. A phase II study was initiated in 24 colorectal cancer patients. They received cyclosporin-A at a dose of 3 mg kg\(^{-1}\) over 1 h as i.v. infusion, at 7 h and at 1 h preceding cytotoxic drug administration. At the end of the second cyclosporin-A administration epirubicin 90 mg m\(^{-2}\) was administered as i.v. bolus. Cycles were repeated every 3 weeks. Median cyclosporin-A peak blood levels and levels at 18 h after cytotoxic drug administration appeared to be 6248 ng ml\(^{-1}\) and 1012 ng ml\(^{-1}\) respectively. Only one partial response was observed, despite these high cyclosporin-A levels. Cyclosporin-A did not cause major toxicity, only a 29% incidence of hot flushes was observed. Epirubicin toxicities were as expected but the frequency of severe leucocytopenia was striking. This treatment schedule can not be considered active in colorectal cancer.

Despite the very intensive research for more effective agents for the treatment of colorectal cancer, 5-fluorouracil (5-FU) remains the most effective with a response rate of 5–15\% (Moertel, 1978; Chlebowski et al., 1980; Ehrlichman et al., 1988; Doroshow et al., 1990). A more recent approach with the addition of leucovorin to 5-FU indeed yields a higher response rate, but this is achieved at the cost of increased toxicity and without a meaningful survival benefit (Ehrlichman et al., 1988; Doroshow et al., 1990). A combination of \(\alpha\)-interferon and 5-FU may also improve the response rate (Wadler et al., 1989).

Anthracyclines are considered inactive in this disease, the highest response rates being achieved with 4-epirubicin which yielded an overall response rate of 8% in 272 patients (Falkson & Vorobiof, 1984). The reason of the inactivity of this class of drugs may be due to the classical multidrug resistance (MDR) phenotype, which includes:

1. Cross-resistance to non-related anticancer drugs such as anthracyclines, vinca alkaloids and podophyllotoxins.
2. Decreased intracellular drug accumulation due to enhanced drug efflux, through an activated pump mechanism.
3. Increased intracellular drug accumulation after exposure to a variety of so called MDR reversal agents, such as verapamil (Ozols & Cowan, 1986; Pastan & Gottesman, 1987) and cyclosporin-A (Herweijer et al., 1989; Nooter et al., 1989) resulting in restoration of drug sensitivity.
4. Assumed activity of an energy dependent unidirectional drug efflux pump with broad substrate specificity (Pastan & Gottesman, 1987). This drug pump is composed of a transmembrane glycoprotein (P-glycoprotein) with a molecular weight of 170 kDa and encoded by the mdr1 gene (Pastan & Gottesman, 1987; Shen et al., 1986).

Recently, RNA slot blot analysis revealed a high expression of the mdr1 gene in 35 of 41 previously untreated adenocarcinomas of the colon (Goldstein et al., 1989). In addition, drug resistance in seven colon tumour cell lines could be overcome by verapamil (Kloos & Steinkampf, 1988). Thus, the MDR phenotype may be present in the majority of colon carcinomas.

The current hypothesis on the mode of action of MDR reversal agents is that they restore drug accumulation by competing for efflux through binding to P-glycoprotein. However, clinical trials with verapamil in combination with doxorubicin and vinblastine in several malignancies have been disappointing (Ozols et al., 1987; Cairo et al., 1989; Figueredo et al., 1990).

An explanation may be that verapamil cannot be given at adequate doses because of clinical toxicity. In fact, the documented concentrations required to overcome MDR in vitro are much higher than those which are achieved with daily doses of verapamil in cardiac patients (Fishman et al., 1982). In several in vitro studies cyclosporin-A reversed MDR more effectively than verapamil (Herweijer et al., 1989, Nooter et al., 1989). Moreover, cyclosporin-A concentrations required in vitro (Silberman et al., 1989) can be reached in vivo without toxicity.

Patients and methods

Drug uptake studies

Colon carcinoma cells were obtained from ascites. The presence of tumour cells was assessed by cytology. Cells were spun down (200 g, 15 min), washed once, and resuspended in RPMI-1640 medium. To determine the drug uptake kinetics of these cells, they were incubated with daunorubicin at a final concentration of 2 \(\mu\)M. Daunorubicin was chosen over the clinically applied epirubicin because of its faster uptake kinetics. However, the same type of accumulation curves can be obtained with all anthracycline drugs (unpublished data).

A modified flow cytometer was used that enables uninterrupted monitoring of the scattering and fluorescence signals, from the moment of drug addition up to a few hours thereafter (Herweijer et al., 1989; Nooter, 1989). The cells (2 \(\times\) 10\(^5\) m\(^{-1}\) in RPMI-1640 medium without phenol red) were kept at 37°C in a reaction vessel surrounded by a thermostated water jacket and connected to the flow cuvette of the flow cytometer. By means of air pressure the medium containing the cells is forced through the flow cuvette. An extra inlet in the reaction vessel allows for the addition of drugs while monitoring the cells. After reaching steady state at \(\pm\) 90 min, either cyclosporin-A at a final concentration of 3 \(\mu\)M, or medium was added to the incubation medium. Drug
accumulation was then measured for another 30 min. For each cell, three parameters were recorded upon excitation which 0.6 W of 488-nm laser light: forward and perpendicular light scattering (through 488-nm band pass filters), and daunorubicin fluorescence (through a 550-nm long pass filter). The microcomputer controlling the flow cytometer stored the data of 2,000 cells at 35 preselected time points before and after the addition of daunorubicin or MDR reversal agents. The collected data set can be used to select for specific subpopulations of cells, and to determine accumulation of daunorubicin in cells from these subpopulations (Noote et al., 1983). The carcinoma cells were distinguished from contaminating hemopoietic cells by gating on the scattering parameters. Dead cells were identified by counterstaining with the non-vital dye Hoechst 33258 and were excluded during the analysis. In the drug accumulation curves, the mean daunorubicin fluorescence of the tumour cell subpopulation (calculated at each of the 35 time points of data collection) is plotted vs the time after addition of the drug. The difference in net uptake is calculated by the equation

\[
\text{FID}_{\text{AS}} = \text{FID}_{\text{CSA}} - \text{FID}_{\text{CYA}}
\]

were \(\text{FID}_{\text{AS}}\) = relative daunorubicin fluorescence at steady state after cyclosporin-A addition, and \(\text{FID}_{\text{CSA}}\), \(\text{FID}_{\text{CYA}}\) = relative daunorubicin fluorescence at the start of cyclosporin-A addition.

Clinical study

A phase II study of the combination of epidoxorubicin and cyclosporin-A was performed. Patients were eligible if they had a histological or cytological diagnosis of colorectal cancer with measurable metastatic lesions. Age limits were 18–75 years; WHO performance score of \(\leq 2\) was required. One previous line of chemotherapy was allowed. WBC had to be \(> 4 \times 10^9\) platelets \(> 100 \times 10^9\), serum creatinine \(< 120 \mu\text{mol}\) and serum bilirubin \(< 25 \mu\text{mol}\) at the start of the study treatment. Oral informed consent was obtained from all patients. Treatment consisted of cyclosporin-A \(3 \text{ mg kg}^{-1}\) in 100 ml of dextrose 5% as an i.v. infusion of 1 h given twice on day 1, at 7 and 1 h before epidoxorubicin. Epidoxorubicin \(90 \text{ mg m}^{-2}\) was administrated by i.v. bolus. All patients received antiemetics. Cycles were repeated every 3 weeks. Retreatment was to be postponed for 1 week if at day 21 WBC was \(< 3 \times 10^9\) and/or platelets \(< 100 \times 10^9\). If hemotological recovery was not complete after 1 week of delay, dose reductions were prescribed. For the first two cycles weekly follow up, including hematology parameters and blood biochemistry, was done.

Responses were evaluated after two cycles of treatment and defined according to standard WHO criteria.

The given dose of cyclosporin-A had been shown in a pilot study (unpublished data) to yield peak cyclosporin levels of more than 3,000 \(\mu\text{g}\) and more than 1,000 \(\mu\text{g}\) 18 h later. In vitro, even in the most resistant cells MDR could be overcome by these concentrations of cyclosporin-A (Silbermann, 1989). Cyclosporin-A blood levels were monitored by Cyclo-TRAC radioimmunoassay (IncStar Corporation, Amsterdam, The Netherlands) (Holt et al., 1988).

Results

In vitro drug uptake studies

Single cell suspensions of colon carcinoma cells were obtained from ascites from four patients, later entered into the phase II study.

Drug accumulation curves are shown in Figure 1. In this figure, the mean drug accumulation of the carcinoma cells is plotted vs the time after addition of daunorubicin. Upon addition of cyclosporin-A an increase in daunorubicin accumulation could be measured in cancer cells from all four patients. Increases measured were 58, 37, 33 and 14%, respectively.

Phase II study

Twenty-four patients entered the study and were eligible and fully evaluable. Median age was 56 years (range 27–74), 13 patients were males, 11 were females, median WHO performance score was one (range 0–2). Eleven patients had received prior chemotherapy, but none of them had received drugs implicated in MDR. The median number of treatment cycles given was three (range 1–7). The median cyclosporin-A peak level was 6,248 ng ml⁻¹ (range 3,340–13,510) and the median 18 h level was 1,012 ng ml⁻¹ (range 301–5,020). Cyclosporin-A infusion caused flushing in seven patients (29%), assumed to be related to the vehicle. No other cyclosporin-A related toxicities were noted. Chemotherapy related side effects are tabulated in Table I. Of note, an unexpected 33% of grade 3–4 leucopenia was observed. One partial response (4%), lasting 6 months, was achieved in a non-pretreated patient; 14 patients had stable disease (median duration 3 months, range 3–8 months) and nine showed tumour progression (including respectively two and two patients with positive in vitro findings).

Discussion

In a study from Goldstein et al. (1989) 85% of 41 non-pretreated colorectal tumours were found to have high mdr1 mRNA levels, suggesting that at least a part of the clinical resistance of these tumours might be explained by an intrinsic multidrug resistance phenotype. By on-line flow cytometry we are able to test the activity of the P-170 glycoprotein drug-efflux pump, the presence of which is increased in MDR (Pastan & Gottesman, 1987).

Anthraccline drug accumulation can be accurately measured using the technique of flow cytometry (Herweijer et al., 1989; Noote et al., 1983; Noortet et al., 1989). The major advantages of the use of flow cytometry to measure anthracline drug accumulation are the ability to discriminate between several subpopulations of cells present in a sample, and the possibility to quantitate drug accumulation in large numbers of cells, resulting in more accurate figures. The extension of this technique in on-line flow cytometry enables accurate measurement of complete drug accumulation curves over long periods of time (up to a few hours), and enables monitoring of effects of the addition of reversal agents on the drug accumulation of MDR cells (Herweijer et al., 1989; Noote et al., 1989; Silbermann et al., 1989). Using various methods, it has been shown that cyclosporin-A can restore the defective drug accumulation characteristic of MDR cells.
in vitro (Herweijer et al., 1989; Nooter et al., 1989; Silberman et al., 1989). It has also been shown the cyclosporin-A can effectively restore the cytotoxicity of anthracycline drugs in MDR cells in vitro (Twentyman et al., 1987) and in vivo (Slater et al., 1986). The efficacy of reversal agents can be studied by comparing the drug accumulation of MDR cells in the presence and absence of specific reversal agents. In this study we determined the effects of reversal agents on the daunorubicin accumulation characteristics of colon carcinoma cells in vitro.

For such studies cell suspensions of intact tumour cells are required, that can be obtained in case of pleural fluid or ascites. Although the number of experiments is limited due to the low occurrence of colorectal cancer patients with pleural fluid and/or ascites, our data with on-line-flow cytometry in four patients suggest that adequate concentrations of cyclosporin-A can increase the intracellular drug concentration of anthracyclines in vitro. In an in vitro study (Silberman et al., 1989) a dose-response relationship was found for cyclosporin-A to circumvent MDR in cell lines with different levels of resistance. Projecting these in vitro data to the in vivo situation we felt that for the clinical study the achievement of a peak cyclosporin blood level of 3,000 μg l⁻¹ and a level of 1,000 μg l⁻¹ after approximately one half-life of the chosen cytotoxic drug would suffice, also in view of a positive pilot experiment in a leukemia patient (Sonneveld & Nooter, 1990). Another pilot experiment had indicated that the aimed blood levels could be achieved by the infusion schedule used. A longer exposure to cyclosporin-A was deliberately avoided in order not to have immunosuppressive effects. The toxicity observed from cyclosporin-A was restricted to hot-flushes, presumably related to the cremaphore and not to the actual drug. The number of patients with severe leucocytopenia is remarkably higher than expected with this dose of epipodoxorubicin (van Oosterom et al., 1984) and suggests some influence of the addition of cyclosporin-A. As normal white blood cells are known to lack increased P-170 glycoprotein expression, other mechanisms may be involved in this increase of toxicity. However, in vitro nor in vivo data to explain this observation are available. The reasons for the failure of the given treatment remain speculative, such as: (1) intrinsic inactivity of epipodoxorubicin in colorectal cancer precluding cytotoxicity even at increased intracellular accumulation, (2) inadequate cyclosporin-A concentrations at the site of the tumour cells, despite high blood levels (this could for instance be due to the ± 80% binding of cyclosporin-A to plasma lipoproteins in man, if the MDR reversing effect is only related to unbound drug, but no data on this topic are available, (3) cyclosporin-A may not increase intracellular epipodoxorubicin levels in vivo in contrast to in vitro, (4) MDR may just be a minor part of the many possible causes for clinical resistance of colorectal cancers. Indeed the amount of p-170 glycoprotein differs from cell to cell in a single tumour (Fojo, 1989). Several of these postulations can only be studied in an in vivo model, which development is awaited. The presence clinical data also suggest that the interpretation of the in vitro data should be cautious.

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