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Antiangiogenic Potency of Various Chemotherapeutic Drugs for Metronomic Chemotherapy

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Abstract. *From previous preclinical findings continuous low dose (metronomic) chemotherapy is thought to inhibit tumor angiogenesis. This suggests that activated endothelial cells may be more sensitive to chemotherapeutic drugs than tumor cells. Therefore, we assessed the IC_{50} for several relevant chemotherapeutic drugs in different endothelial and tumor cell lines to identify optimal compounds to be used for metronomic therapy in a murine renal cell carcinoma model. Adriamycin, idarubicin, 5-fluorouracil, paclitaxel and etoposide were chosen for our studies because of their oral availability in patients or previous reports on metronomic potential. IC_{50s} were determined by BrdU cell growth assay after short time as well as long term exposure of the following cell lines: human endothelial cells (HdmVEC/HUVEC), human breast cancer (Mcf-7), melanoma (Skmel), liver cancer (Huh7/Alexander), lung cancer (A-549/LXFL), colon cancer (Dld) and murine renal cell carcinoma (RENCA). In addition, FACS analysis was performed to determine the effect on cell cycle. In vivo, doses of 2x12mg/kg, 2x1.2mg/kg and 10x0.24mg/kg adriamycin were applied to 12 RENCA mice each and antitumor as well as antiangiogenic effects were assessed 21 days after tumor cell application. Independent of the exposure time, all chemotherapeutic drugs were more active against the endothelial cell lines. IC_{50s} were significantly lower in endothelial cells (4.02E-06 to 6.16E-14M) as compared to tumor cells (7.44E-02 to 1.9E-11M). Cell cycle analysis of all chemotherapeutic drugs revealed a G1-arrest in endothelial cells. Adriamycin applied in metronomic doses of 10x0.24mg/kg showed significant antiangiogenic activity whereas, in contrast, the application of 2x12mg/kg significantly increased the vessel density in primary tumors. In summary, all chemotherapeutic agents were more active against endothelial cells in comparison to tumor cells. The hypothesis of an antiangiogenic active metronomic therapy could be confirmed in vivo by the use of adriamycin in RENCA.*

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Tumorangiogenesis has been identified as a promising new target for anticancer therapy (1). A first report on the antiangiogenic properties of chemotherapeutic agents appeared more than 15 years ago (2). The mode of action is thought to be explained by the interference of these agents with cycling and proliferating endothelial cells in the process of tumor angiogenesis (3). Conventional application of chemotherapy, targeting the tumor cell directly, is based on the linear dose-efficacy relationship for these drugs resulting in a cyclic treatment to allow for recovery from the side-effects. Any potential damage to the tumor vasculature can be repaired during the long breaks. Continuous, low-dose chemotherapy, *i.e.* metronomic therapy, targeting the endothelial cell directly is thought to have fewer side-effects as well as a lack of drug resistance.

Nevertheless, it still remains unclear which of the chemotherapeutic drugs has the best antiangiogenic potential. Previous *in vitro* studies showed that very low doses of paclitaxel or adriamycin are needed to effectively inhibit endothelial cell proliferation (4). However, for metronomic therapy in patients, availability is limited due to the intravenous application of most drugs. To establish metronomic therapy in patients an oral formulation of the drug is needed. Therefore, we performed proliferation studies of orally-available chemotherapeutic drugs like idarubicin, 5-fluorouracil and etoposide compared to drugs already known to have strong efficacy on endothelial cells. IC_{50} values and changes in cell cycle progression of different endothelial cell lines and tumor cell lines were determined. In addition, one representative compound (adriamycin) was selected to confirm its potential antiangiogenic activity *in vivo*. To investigate novel therapeutic strategies, such as antiangiogenic therapy, the murine renal cell carcinoma (RENCA) is a particularly suitable animal model (5). In this model, primary kidney tumors are induced by

subcapsular renal injection of RENCA cells. Subsequently metastases in the lung, lymph nodes and spleen develop (6).

Materials and Methods

Drugs. All drugs used were provided by the pharmacy of the University Hospital of Freiburg, Germany. To perform *in vitro* studies the drugs were stocked in solutions of 200 to 2x10E-12μM dissolved in aqua dest, with the exception of paclitaxel that was dissolved in cremophor.

Cells and culture conditions. RENCA cells were originally obtained from a tumor that arose spontaneously in the kidney of BALB/c mice. Monolayers of murine RENCA cells were grown in RPMI 1640 with phenol red supplemented with 10 % FCS, 2 mM L-glutamine, 100 U Penicillin/ml and 100 mg Streptomycin/ml. RENCA cells were cultured in a humidified atmosphere of 95 % air and 5 % carbon dioxide at 37˚C. The media were routinely changed every 3 days. The cells were released from the tissue flasks by treatment with 0.05 % (versene) trypsin/EDTA and viability was monitored using the cell analyzer system Casy 1 from Schärfe System (Reutlingen, Germany). For the animal experiments cells were collected during logarithmic growth phase.

All other tumor cell lines were grown as mono layers in a humidified atmosphere of 95% air and 5% carbon dioxide at 37˚C. MCF7, KM20L2, DLD1, SKMEL, LXFL, A 549 and Renca were maintained in RPMI 1640 whereas Alexander and HUH7 were maintained in DMEM. All cell media were supplemented with 10% FCS, 100 U/ml Penicillin, 100 μg/ml Streptomycin and 100 μg/ml L-Glutamine. The endothelial cell line HUVEC was maintained in Endothelial Cell Growth Medium 2 Kit (Promocell) whereas the endothelial cell line HdmVEC was maintained in Endothelial Cell Growth Medium MV 2 Kit (Promocell).

Cell growth assay. Cells were plated onto 96-well plates and incubated overnight at 37˚C in a 5% CO_2 incubator. After the removal of the media, 100 μl of media containing the indicated compound were added to each well. Plates were incubated for an additional 48 or 72 h without subsequent medium changes, and BrdU incorporation was measured using an ELISA-based assay (Roche Diagnostics, Mannheim, Germany).

Cell cycle measurement. Adherent cells were removed after trypsinization and washed in PBS after gentle centrifugation at 610 x g for 5 min. The cell pellets were fixed by resuspending them in 0.5 ml of 70% ethanol for 30 min, centrifuging at 925 x g for 8 min and washing twice with ice-cold PBS to remove residual ethanol. For cell cycle analysis, the pellets were resuspended in 0.5 ml of PBS containing 50 μg/ml of propidium iodide and 100 mg/ml of RNAse, then incubated at 37˚C for 30 min and studied using a FACS Scan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Animal experiments. All experiments were carried out according to the guidelines of the Ethical Committee of the Regierungspräsidium (Freiburg, Germany). Female BALB/c mice were used at 6-8 weeks of age (approximate weight 20 g). The injection of 106 RENCA cells in 0.2 ml aliquots into the subcapsular space of the left kidney was performed through a flank incision after the animals were anaesthetized with 0.5-1.5 volume percent isoflurane with an oxygen flow of 1.5 l/min. The injection of 10^6 RENCA cells in syngenic BALB/c mice induced progressive development of a primary tumor mass in the left kidney. One week after application, the primary tumor was macroscopically visible. At 10 days spontaneous metastases developed in the regional lymph nodes, in the lung, the peritoneum and the liver. The mean survival time of RENCA-bearing mice was approximately 32 days when 106 RENCA cells were injected.

Administration of drug. Therapy with adriamycin or vehicle was initiated on day 10 after tumor cell inoculation in Balb/c mice. Mice received either vehicle on days 10 and 17, 12mg/kg adriamycin on days 10 and 17, 1.2mg/kg adriamycin on days 10 and 17 or 0.24mg/kg adriamycin on days 10-20 as intravenous bolus application *via* the tail vein. All animals were sacrificed on day 21. Twelve animals were included per group.

Evaluation of tumors. On day 21 all mice were sacrificed for determination of the weight and volume of primary tumors, lung weight, number of lung metastases and the number of metastases in the abdominal lymph nodes. Volumes of the primary tumors were evaluated macroscopically by measuring their extensions in three orthogonal dimensions. The number of metastases in the lung and abdominal lymph nodes were counted using a dissection microscope. In the abdominal cavity, all visible lymph nodes were assessed for detection of metastasis, in the knowledge that in healthy animals visible lymph nodes are usually absent. Lymph nodes were inspected randomly with a microscope in order to confirm tumor-bearing tissue.

Immunohistochemistry. For histological examination of the tumor vasculature, tumor tissues and lungs were frozen immediately in liquid nitrogen. Cryosections of tissue with a thickness of 5-10 μm were taken from all groups. For visualizing the blood vessels, immunohistochemical staining for CD31 (Pecam-1 and MEC13.3; Becton Dickinson, San Jose, CA, USA) was performed and vessels were counted microscopically using a defined magnification (x200). Furthermore, immunohistochemical staining for VEGF - receptor 2 (anti-FLK-1, Pharmingen, San Diego, USA) was performed and FLK-1-positive cells were counted using a defined magnification (x200).

Currently, vascular density is investigated in "hot spots" only. This might lead to false high values of vessel density *e.g.* in tumor tissues, because these data are not correlated to the number of "hot spots" found in the tissues. In our studies, a minimum of 3 slides from separate areas of each tumor were used for all analyses. Therefore, sections were representative of the whole tumor. Furthermore, the evaluation was performed by two persons who were blinded with respect to the treatment of the animals.

Statistical analysis. Correlations on IC_{50} values were performed by the Department of Medical Biometry and Informatics of the University of Freiburg, Germany, using the variance-covariance analysis (ANCOVA).

All values reported for the animal experiments are expressed as mean ± SEM. The Mann-Whitney *t*-test was applied to all statistical analyses. *P*-values <0.05 were considered to be significant.

Results

Cell growth. Independently from the exposure time, all chemotherapeutic drugs were more active against the endothelial cell lines. IC_{50s} were reduced in endothelial cells

Table I. *The IC_{50s} for each chemotherapeutic drug on each cell line. Cells were plated onto 96-well plates and incubated overnight at 37˚C in a 5% CO_2 incubator. After removal of the media, 100 µl of media containing the indicated compound were added to each well. Plates were incubated for an additional 72 h without subsequent medium changes and BrdU incorporation was measured using an ELISA-based assay (Calbiochem). Values represent the mean IC_{50} in M whereas n is the number of studies performed.*

		5FU		Adriamycin		Paclitaxel		Idarubicin		Etoposide	
		IC_{50}[M]	n	IC_{50}[M]	n	IC_{50}[M]	n	IC_{50}[M]	n	IC_{50}[M]	n
Endothelial cells	Huvec	3.67E-06	10	5.99E-10	11	1.61E-14	11	1.10E-12	7	2.49E-07	2
	Hdmvec	4.02E-06	7	1.50E-11	9	6.16E-12	10	2.03E-12	8	1.60E-07	4
	Alex	8.40E-04	4	6.25E-08	4	1.35E-08	4	1.11E-07	2	1.26E-05	2
	A549	7.08E-04	6	2.88E-07	5	3.15E-06	5	3.21E-08	5	3.03E-05	7
	Renca	6.71E-03	4	1.01E-08	5	4.45E-06	5	1.30E-08	3	1.20E-07	2
	Dld	1.86E-03	4	7.27E-07	4	5.72E-07	4	1.20E-07	4	5.74E-06	2
	Huh7	2.27E-03	4	3.22E-07	4	2.16E-08	4	1.90E-11	5	2.97E-05	3
	Km20L2	7.74E-03	4	1.27E-06	3	2.81E-09	3	1.75E-07	2	2.12E-02	3
	Lxfl	3.92E-05	4	5.83E-08	5	1.53E-08	7	3.65E-09	4	5.69E-06	4
	Mcf7	1.29E-04	7	5.73E-09	8	5.17E-09	8	5.14E-09	9	5.14E-09	3
	Skmel	7.44E-02	5	1.12E-06	5	1.34E-05	8	1.85E-07	4	4.79E-05	5

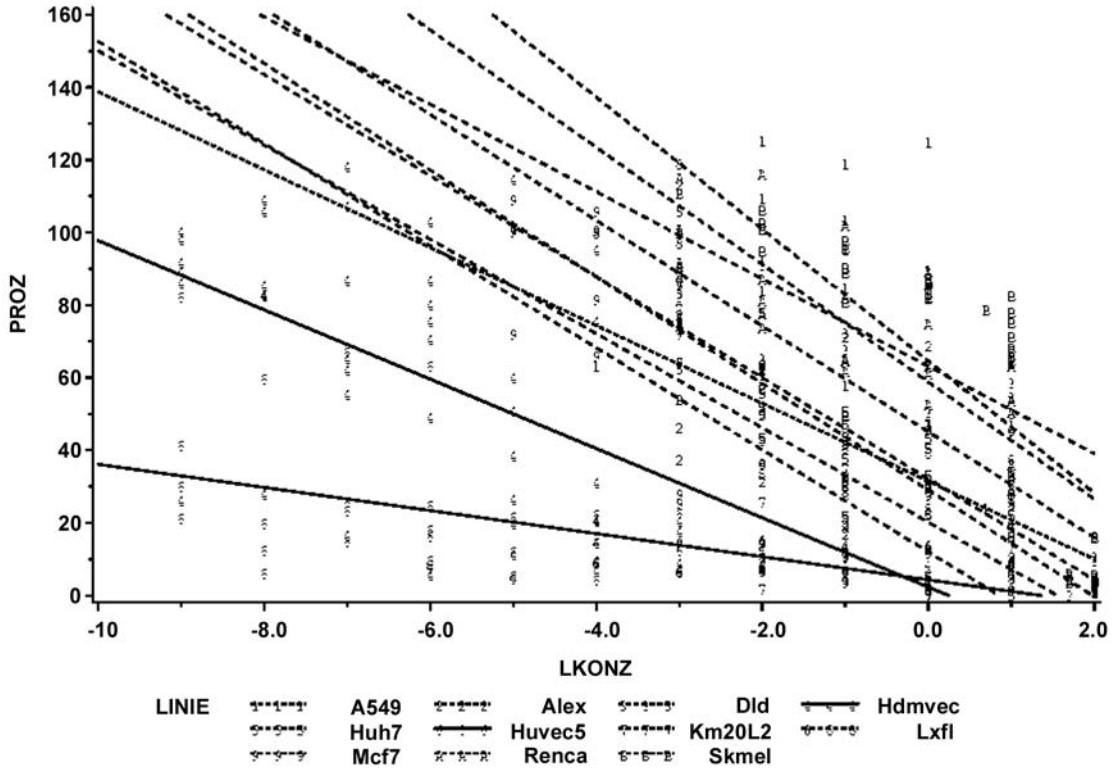

Figure 1. *Concentration against the percent of inhibition curve for paclitaxel (Taxol) on all cell lines. Cells were plated onto 96-well plates and incubated overnight at 37˚C in a 5% CO_2 incubator. After the removal of the media, 100 µl of media containing the indicated compound were added to each well. Plates were incubated for an additional 72 h without subsequent medium changes and BrdU incorporation was measured using an ELISA-based assay (Calbiochem). Results for endothelial cell lines are given in plain lines whereas results for tumor cell lines are given in interrupted lines.*

(4.02E-06 to 6.16E-14M) compared to tumor cells (7.44E-02 to 1.9E11M). Table I gives the IC_{50s} for each drug on each cell line. The differences in IC_{50s} between endothelial cells and tumor cells became significant for all compounds. The highest activity on the endothelial cell lines was found for paclitaxel with an IC_{50} of 1.61E-14M (Figure 1) followed by idarubicin with an IC_{50} of 1.10E-12M and adriamycin with an IC_{50} of 1.5E-11M.

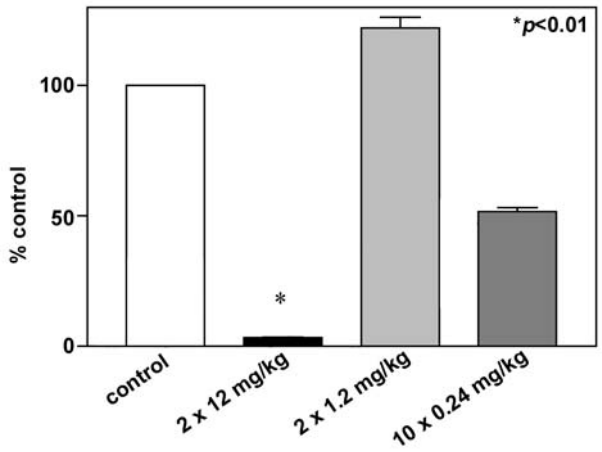

Figure 2. *Effect of different therapeutic schedules of adriamycin on primary tumor volume in murine renal cell carcinoma. Adriamycin was administered with 12mg/kg on days 10 and 17, 1.2mg/kg on days 10 and 17 or 0.24mg/kg on days 10 to 20 as intravenous bolus application via the tail vein. Animals were sacrificed on day 21 after inoculation of RENCA cells into the subcapsular space of the left kidney of syngeneic BALB/c mice and primary tumor volumes were assessed. Values are % of control; bars, SEM. Ps were calculated by comparing means of the treated group and means of the control group using the Mann-Whitney t-test. *p, significant.*

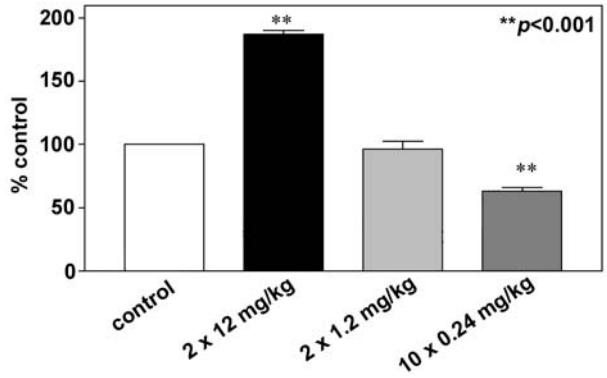

Figure 3. *Effect of different schedules of adriamycin on vessel density in primary tumors of murine renal cell carcinoma (magnification x200). Primary tumor tissues were quickly frozen in liquid nitrogen 21 days after subcapsular inoculation of the RENCA cells into the left kidney. Cryosections of the tissues were taken from both the control group (vehicle) and treatment groups receiving 12mg/kg adriamycin on days 10 and 17, 1.2mg/kg adriamycin on days 10 and 17 or 0.24mg/kg adriamycin on days 10 to 20 as intravenous bolus application via the tail vein. For the visualization of the blood vessels, immunohistochemical staining for CD31 was performed. Values are % of control; bars, SE. Ps were calculated by comparing means of the treated group and means of the control group using the Mann-Whitney t-test. **p, significant.*

Cell cycle. Additional cell cycle analysis of all chemotherapeutic drugs revealed a G1-arrest in endothelial cells (data not shown). No analysis was performed on the tumor cells since the influence on cell cycle is well published for each chemotherapeutic drug.

Tolerability. Animal weights were increased by 3.8% in the control group. A decrease of animal weight by 2.3% in the adriamycin group scheduled with 12mg/kg on days 10 and 17 is representative of a limited tolerability, whereas an increase in animal weight by 3.1% in the adriamycin group scheduled with 1.2mg/kg on days 10 and 17 and an increase of animal weight by 0.6% in the adriamycin group scheduled with 0.24mg/kg on days 10-20 (data not shown) reflects an increased tolerability in the low dose groups. No drug-related death was observed.

Antitumoral activity. Compared to primary tumors of animals treated with vehicle, a significant reduction of 97% ($p<0.01$) was observed in primary tumors of animals treated with 2x12mg/kg adriamycin (Figure 2). No effect could be observed in primary tumors of animals treated with 2x1.2mg/kg adriamycin, whereas a reduction of 47% ($p<0.1$) was observed in primary tumors of animals treated with metronomic doses of 10x0.24mg/kg adriamycin (Figure 2).

Antimetastatic activity. Compared to the number of lung metastases of animals treated with vehicle, a significant reduction of 98% ($p<0.01$) could be observed in the number of lung metastases of animals treated with 2x12mg/kg adriamycin. No effect could be observed on the number of lung metastases of animals treated with 2x1.2mg/kg adriamycin. The reduction in number of metastases of animals treated with metronomic doses of 10x0.24mg/kg adriamycin became not significant (data not shown).

Antiangiogenic activity. For histological examination of tumor vasculature, primary tumor tissues of all groups were stained for CD31. Initial examination of all tissue sections at low magnification showed homogeneous vessel density with lack of hot spots. Comparison of vessel density in primary tumors of untreated animals with those of animals treated with 2x12mg/kg adriamycin revealed a significant increase of vessel density by 90% ($p<0.001$). No effect could be observed in vessel density in primary tumors of animals treated with 2x1.2mg/kg adriamycin, whereas a significant reduction of 44% ($p<0.001$) in vessel density in primary tumors of animals treated with 10x0.24mg/kg adriamycin could be observed (Figure 3).

Comparison of the number of FLK-1-positive cells in primary tumors of untreated animals with all treated animals revealed no difference (data not shown).

Discussion

Our results highlight the antiangiogenic potential of metronomic therapy with cytotoxic agents. *In vitro*, cytotoxic

agents had a significant decrease in IC_{50} values of endothelial cells compared to all investigated tumor cell lines, indicating antiangiogenic doses far below the optimal doses for direct antitumor activity.

Furthermore, our studies showed strong differences in the antiangiogenic potency between the investigated cytotoxic agents, indicating a pivotal role of the mode of action of each compound. In contrast, our studies on cell cycle inhibition by FACS analysis showed a G1-arrest of endothelial cells, similar for all investigated cytotoxic agents. Therefore, the reason for the superior activity of tubuline inhibitors like paclitaxel as well as anthracyclines on endothelial cells remains unclear. Previous publications support the high potency of tubuline inhibitors like paclitaxel (4,7) without comparison against other drugs.

Although the application of oral cytotoxic agents like 5-fluorouracil or etoposide might be more feasible in patients, our *in vitro* data indicate less antiangiogenic activity. Ongoing clinical studies, which are mainly using oral 5-fluorouracil derivatives, should take that into account (8).

In vivo, our study is the first to show the antitumoral and antiangiogenic activity of a metronomic chemotherapy with adriamycin in the murine RENCA model. Adriamycin was chosen because of its strong activity against endothelial cells, as shown in our *in vitro* data, as well as previous experience with conventional doses in the RENCA model (9). The reduction of the sum dose of adriamycin from 24mg/kg to 2.4mg/kg was only effective if given continuously in single doses of 0.24mg/kg (metronomic) whereas the application of 2 doses of 1.2mg/kg did not show any effect. In contrast to some specific antiangiogenic agents, the antitumor activity of adriamycin applied in metronomic doses became not significant for all tumor sites, although vessel density detected by immunohistochemistry was significantly reduced. This finding indicates a partial angiogenesis-independent growth of the fast growing RENCA tumor. This hypothesis is supported by earlier findings (5), where vessel density declined in the period between 2 and 3 weeks after inoculation without slowing down tumor growth, indicating that the proliferation rate of tumor cells was higher than that of endothelial cells. Furthermore, tumors may have the possibility of infiltrating vessels for a blood supply in murine models (6).

The potency of the antiangiogenic effects observed under treatment with adriamycin applied in metronomic doses is comparable with the results from previous studies on specific VEGF-receptor tyrosine kinase inhibitors (6). In contrast, metronomic therapy with adriamycin did not affect the expression of the VEGF-receptor 2 in primary tumors, indicating a different mode of action.

Interestingly, the conventional therapy of adriamycin significantly increased the vessel density in primary tumors of RENCA mice when compared to the vessel density in primary tumors of the control group. This finding supports the hypothesis of endothelial cell rescue under cyclic chemotherapy resulting in increased endothelial cell proliferation.

In summary, our results confirm the superior antiproliferative activity of cytotoxic agents on endothelial cells compared to tumor cells with the highest activity for tubulin inhibitors and anthracyclines. The activity on endothelial cells was characterized by a G1-cell cycle arrest for all agents. *In vivo*, metronomic doses of adriamycin showed antitumoral and antiangiogenic activity, whereas conventional dosing of adriamycin caused increased vessel density in primary tumors of RENCA mice.

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