Systematic analysis of the antiproliferative effects of novel and standard anticancer agents in rhabdoid tumor cell lines

Henning Lünenbürger, Claudia Lanvers-Kaminsky, Birgit Lechtape and Michael C. Frühwald

Rhabdoid tumors are highly aggressive pediatric malignancies. Although the prognosis of children with rhabdoid tumors has improved, it still remains dismal and long-term survivors suffer from severe side effects of current therapeutic approaches. The objective of our study was to explore the toxicity of standard and novel anticancer drugs against rhabdoid tumors *in vitro* and to prioritize them for future preclinical and clinical studies. Antitumor activity of 10 standard anticancer drugs (doxorubicin, idarubicin, mitoxantrone, actinomycin D, temozolomide, carmustine, oxaliplatin, vinorelbine, methotrexate, thiotepa), five target-specific drugs (sorafenib, imatinib, roscovitine, rapamycin, ciglitazone) and two herbal compounds (curcumin and apigenin) was assessed by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) cell proliferation assay on three rhabdoid tumor cell lines, A204, G401, and BT16, derived from different anatomical sites. Comparable with their high clinical activity, anthracyclines inhibited tumor cell proliferation by 50% (GI_{50}) in the nanomolar range. Actinomycin D exhibited the lowest GI_{50} values overall ranging from 2.8×10^{-6} nmol/l for G401 to 3.8 nmol/l for A204 cells while thiotepa was the only alkylating drug that inhibited tumor cell growth in clinically relevant concentrations. Target-specific drugs, such as sorafenib, roscovitine, and rapamycin, showed promising results as well. In this report, we show for the first time that apigenin and curcumin effectively inhibit rhabdoid tumor cell growth. Supporting earlier reports we conclude that cyclin D1 seems to be an excellent target in the treatment of rhabdoid tumors. Idarubicin or mitoxantrone represent potent alternatives to doxorubicin, and vinorelbine may substitute vincristine in future clinical trials. *Anti-Cancer Drugs* 21:514–522 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Malignant rhabdoid tumors (MRTs) are highly aggressive malignancies occurring almost exclusively in early childhood. The exact incidence is undefined; however, data derived from institutional case series and national cancer registries suggest that in children below the age of 1 year, atypical teratoid AT/RT constitutes up to 50% of malignant brain tumors [1] (Slavc *et al.*, personal communication). RTs of the kidney (RTK) constitute 2% of renal tumors in infants and children. The incidence of RTs of soft tissue is unclear; they may be detected in almost any anatomical localization [2–4]. RTs, regardless of origin, are characterized by mutations in the *SMARCB1* gene (*hSNF5/INI1*) localized in chromosome 22q11.2 [5]. The mutation of this tumor suppressor may affect the expression of the cell cycle regulator $p16^{(INK4a)}$ and activate cyclin D1 [6].

The prognosis of children with RTs has improved but still remains dismal [3,7,8]. Characteristic is an initial response to chemotherapy followed by resistance. Current literature reviews suggest that anthracycline therapy is effective and children should undergo local therapy as early as possible. Current therapeutic philosophies for intracranial RTs suggest either intensive chemotherapy with early radiotherapy or intensive chemotherapy followed by radiotherapy and high-dose chemotherapy for a measure of consolidation. However, because of the young age of most children, radiotherapy has its limitations owing to severe side effects such as endocrine dysfunction and cognitive delay up to mental retardation [9].

Some studies have shown that the combination of ifosfamide, carboplatinum, and etoposide is efficient against AT/RT [10] and RTK [11]. Several current approaches investigate the efficacy of combining chemotherapy and radiotherapy with autologous stem cell rescue (Goldman, NCT00179803; Gajjar, NCT00085202; Reddy, NCT00653068).

Chi *et al.* [7], at the Dana–Faber Cancer Institute, tested a new multimodality therapy regimen (DFCI No. 02–294) with preirradiation, chemoradiation, consolidation, maintenance, and continuation therapy. The chemotherapy

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included vincristine, cisplatinum, doxorubicin, cyclophosphamide, etoposide, actinomycin D, temozolomide and, in individual cases, the cardioprotectant, dexrazoxane. Intrathecal chemotherapy and focal or craniospinal radiation therapy were also administered. This intensive multimodality regimen has increased 2-year overall survival rates up to 70 ± 10% [7]. However, the regimen exhibits significant toxicities with radiation recall, transverse myelitis, and toxic death.

Despite a multitude of case series and single reports, very few reliable data exist with regard to promising unified national or international therapeutic approaches. Owing to the limited number of affected children there is limited interest of pharmaceutical companies in evaluating targeted approaches and the determination of efficacy for single agents is very difficult. Furthermore, the severe side effects of current therapy demand the optimization of current and the development of novel therapeutic strategies.

The purpose of our study was to explore the activity of standard and novel anticancer drugs against RTs and to compare them with anticancer drugs of recent tumor regimens *in vitro*. Therefore, we reviewed earlier in-vitro studies and tested a variety of compounds from different classes of drugs to identify cytotoxic compounds and to prioritize for future preclinical and clinical studies such as the European Rhabdoid Registry. As RTs are initially responsive to chemotherapy, we tested standard anticancer drugs, such as intercalating antibiotics, alkylating agents such as temozolomide, carmustine and thiotepa, the vinca alkaloid vinorelbine, the platinum compound oxaliplatin, and the antimetabolite methotrexate (MTX) on three tumor cell lines representing the most common locations for RTs: the brain, the kidney, and the liver.

Common to all currently employed therapeutic regimens is the use of intensive anthracycline-based polychemotherapy regimens and aggressive local therapy, in most instances using radiotherapy [12–14,7]. Thus, we tested the anthracycline antibiotics idarubicin and doxorubicin. Mitoxantrone is an anthracenedione, an anthracycline derivate, and acts like the anthracycline antibiotics by DNA intercalation and inhibition of topoisomerase II. Actinomycin D is also an antitumor antibiotic, although structurally different from the anthracyclines and mitoxantrone. The anthracyclines, mitoxantrone and actinomycin D, were grouped as intercalating antibiotics.

We further tested target-specific drugs such as sorafenib, imatinib, rapamycin, ciglitazone, and roscovitine. These have been shown earlier to decrease cyclin D1 in different tumor cell lines. Downmodulation of cyclin D1 has been shown to be an effective means in RT models [15–17].

Furthermore, we tested the herbal products, apigenin and curcumin. Apigenin (4′,5,7-trihydroxyflavone) is a naturally occurring plant flavonoid derived from fruits and vegetables such as parsley and celery [18], whereas curcumin (diferuoylmethane, turmeric) is derived from the rhizome of the perennial herb *Curcuma longa* [19].

Materials and methods

Cell culture

Human cell lines derived from primary RTs of different anatomical locations, BT16 (human brain tumor), A204 (RT of the liver) and G401 (RT of the kidney). A204 and G401 were obtained from ATCC. BT16 was a gift from Dr P. Houghton. All cells were maintained in Dulbecco's modified Eagle's medium high glucose formulation (Invitrogen, Karlsruhe, Germany) supplemented with 2% L-glutamine (Invitrogen) and fetal calf serum to a final concentration of 10% for A204 and G401 or 20% for BT16 (South American, Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Treatment with cytotoxic agents

Oxaliplatin, vinorelbine, mitoxantrone, idarubicin, thiotepa, actinomycin D, MTX, apigenin and curcumin powder were purchased from Sigma-Aldrich (Schnelldorf and Steinheim, Germany). Ciglitazone was obtained from Cayman Chemical (IBL-Hamburg, Germany), temozolomide from Essex Pharma (Munich, Germany), carmustine from Bristol–Myers Squibb (Munich, Germany) and rapamycin from Gentaur (Aachen, Germany). Doxorubicin infusion was provided by HEXAL (Holzkirchen, Germany), roscovitine was provided by Calbiochem EMD Biosciences Inc. (Darmstadt, Germany) and imatinib was purchased from LC Laboratories (Woburn, USA). Sorafenib was kindly supplied by Bayer Healthcare (Leverkusen, Germany).

All drugs (except doxorubicin) were dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 100 mmol/l for thiotepa, temozolomide and carmustine or to a concentration of 10 mmol/l for the other compounds and added to culture medium. The final concentrations were 1% of DMSO at the maximum. Doxorubicin was dissolved in sterile water supplemented with NaCl and acidified with HCl at a concentration of 2 mg/ml and was further diluted with complete cell culture medium to the desired drug concentrations.

Cell viability assay

The antitumor activity of each compound was tested by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) cell proliferation assay (Sigma-Aldrich, Schnelldorf, Germany) [20,21]. Cells in exponential growth were seeded at a concentration of 5000 cells/100 µl for A204 and BT16 and 3000 cells/100 µl for G401 into four 96-well plates and allowed to attach overnight. Cells were subsequently treated in replicates of four with 100 µl of cell culture medium containing each compound at differing concentrations. The highest single DMSO concentration employed had no effect on cell

growth of A204, G401 and BT16. Controls were cultured in medium only. Cell viability was determined after treatment for 24, 48, and 72 h with compounds, by adding 10 µl of MTT reagent (5 mg/ml MTT dissolved in PBS) for 2.5 h, after which the supernatants were removed carefully and the formazan crystals dissolved in 2-propanol. Absorption was measured at 570 nm with a reference of 650 nm using a Mulitiskan Ascent multiplate reader (Labsystems, Helsinki, Finland). Mean, standard deviation (SD) and coefficient of variation were calculated and the concentration at which each drug induced 50% growth inhibition relative to untreated controls (GI_{50}) derived at the respective time points of 24, 48 and 72 h was determined by the equation: {[percentage viable cells (> 50%)] – 50}/[(percentage viable cells (> 50%)] – [percentage viable cells (< 50%)] × (drug concentration above 50% viable cells – drug concentration below 50% viable cells) + (drug concentration below 50% viable cells). The LD_{50} (lethal dose) represents 50% reduction of viable cells compared with untreated controls at the start of drug exposure (day 0). The GI_{50} and LD_{50} data presented in this manuscript refer to day 3. Each experiment was carried out in triplicate [22].

Statistical analysis

Statistical analyses were performed using the software package SigmaPlot 11.0 (Systet Software GMBH, Erkrath, Germany) and SPSS. Statistics 17.0 (SPSS Inc., Chicago, Illinois, USA) using one way analysis of variance (ANOVA) with Bonferroni correction.

Results

Effects of standard anticancer drugs

The intercalating antibiotics doxorubicin, mitoxantrone, idarubicin and actinomycin D were the most effective class of drugs inhibiting cell viability. Table 1 summarizes the mean GI_{50} values determined for each drug and each cell line after 72 h. Actinomycin D was the most effective compound of all (Fig. 1). In G401 cells, actinomycin D exhibited the lowest GI_{50} values (mean: 2.8×10^{-9} µmol/l = 2.8 fmol/l). A204 cells were less sensitive but showed a GI_{50} of 3.8 nmol/l and a LD_{50} of 9.8 nmol/l. Moreover, the LD_{50} for actinomycin D in A204 cells was about 5- to 40-fold lower than the GI_{50} for the anthracyclines. Doxorubicin is already a part of many treatment schedules including the DFCI protocol and the one suggested by the European Rhabdoid Registry and showed cytotoxic effects with GI_{50} values ranging from 3.29 to 400 nmol/l. Mitoxantrone inhibited 50% cell growth of all cell lines from 3.53 to 204 nmol/l; however, idarubicin induced the most potent effect of the three anthracyclines tested (mean GI_{50}: 0.04–49 nmol/l) (Fig. 2). With respect to inhibition of proliferation in response to idarubicin, A204 cells were the most resistant to this agent ($P = 0.004$, ANOVA) while G401 was the most sensitive cell line. At the GI_{50} idarubicin was in fact 4-fold more cytotoxic for A204 cells than mitoxantrone and statistical analysis showed a significant advantage for idarubicin compared with doxorubicin ($P < 0.05$, ANOVA). Comparing the effect on G401, idarubicin was about 80 times more cytotoxic than mitoxantrone or doxorubicin.

Table 1 GI_{50} values represent means (µmol/l) after 72 h of incubation according to the MTT assays

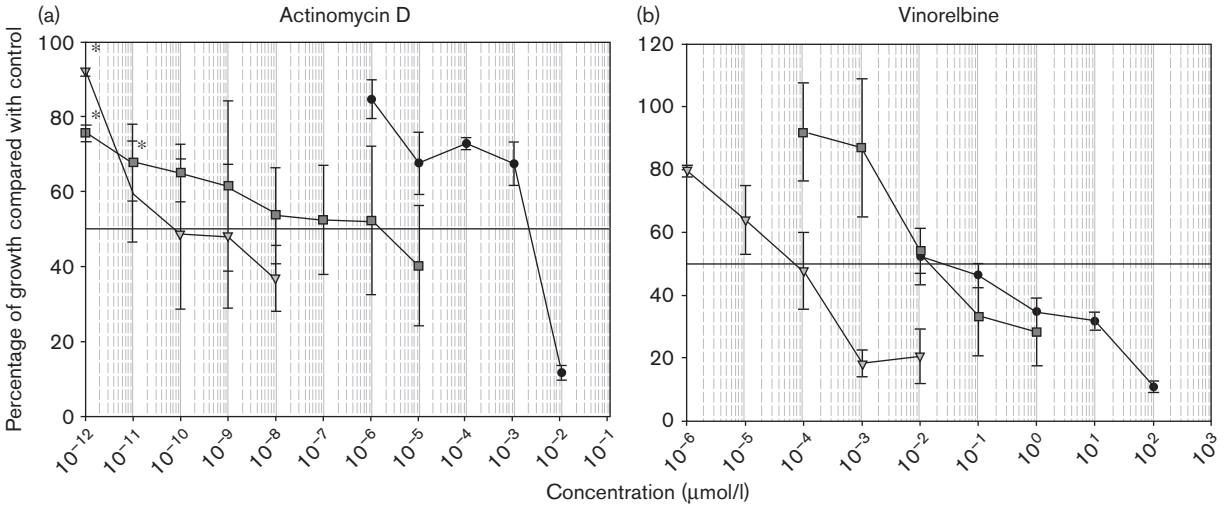
	GI_{50} (µmol/l)		
	Cell line		
	A204	G401	BT16
Standard anticancer drugs			
Intercalating antibiotics			
Doxorubicin	0.40	3.3×10^{-3}	3.8×10^{-2}
Idarubicin	4.9×10^{-2}	3.9×10^{-5}	9.0×10^{-3}
Mitoxantrone	0.20	3.5×10^{-3}	1.3×10^{-2}
Actinomycin D	3.8×10^{-3}	2.8×10^{-9}	4.2×10^{-6}
Alkylating agents			
Temozolomide	903[a]	653	1000[a]
Carmustine	847[a]	88.3	72.1
Thiotepa	64.2	5.57	94.7
Platinum compounds			
Oxaliplatin	70.1	0.91	2.79
Vinca alkaloid			
Vinorelbine	5.8×10^{-2}	1.5×10^{-4}	3.7×10^{-2}
Antimetabolite			
MTX	96.1[b]	7.8×10^{-2}	89.3[b]
Target-specific drugs			
Tyrosine kinase inhibitors			
Sorafenib	3.25	4.88	7.37
Imatinib	34.3	41.4	73.2
PPARg agonist			
Ciglitazone	99.3[b]	58.6	67.0
CDK2 inhibitor			
Roscovitine	44.4	12.8	62.7
mTOR inhibitor			
Rapamycine	38.5	4.90	47.0
Plant compounds			
Flavonoid			
Apigenin	68.0	57.5	36.7
Diferuoylmethane			
Curcumin	6.40	5.21	8.12

GI_{50}, growth inhibition by 50%; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; MTX, methotrexate; PPAR, peroxisome proliferator-activated receptor.
[a]GI_{50} was not reached in each experiment; for calculation of the mean GI_{50} in these cases the highest test concentration (1000 µmol/l) was used.
[b]GI_{50} was not reached in each experiment; for calculation of the mean GI_{50} in these cases the highest test concentration (100 µmol/l) was used.

The response of the different RT cell lines to alkylating agents showed large variations. Temozolomide, for instance, was used up to 1000 µmol/l with GI_{50} values of 903 µmol/l for A204 and 653 µmol/l for G401. We had no evidence of any response in BT16 cells. A204 cells were also highly resistant to only carmustine responding to treatment with high concentrations. Conversely, G401 and BT16 were more sensitive to carmustine with similar effects and GI_{50} values achieved in each cell line (mean: 88.3 and 72.1 µmol/l). Thiotepa reduced tumor cell growth by 50% in all cell lines at concentrations ranging from 5.6 to 94.7 µmol/l.

All cell lines were also sensitive to oxaliplatin-induced inhibition of cell viability; however, G401 (GI_{50}: 0.91 µmol/l) and BT16 cells (GI_{50}: 2.79 µmol/l) were more sensitive than A204 cells (70.06 µmol/l) ($P < 0.001$, ANOVA).

Fig. 1



Dose–response curves determined for two drugs suggested for the treatment of soft tissue sarcomas: (a) actinomycin D and (b) vinorelbine after 72 h in A204 (●), G401 (▼) and BT16 (■) cells. The dots represent means, the range bars represent the standard deviations. Each experiment was performed in triplicate, but for some concentrations there were only two values available (*).

The antimitotic vinca alkaloid vinorelbine, an analogue of vincristine, suggested as a treatment option for soft tissue sarcoma, showed GI_{50} values in the nanomolar range from 0.15 nmol/l in G401 cells to 58 nmol/l in A204 cells (Fig. 1). Even for the most resistant cell line A204, the mean LD_{50} in two out of three experiments was 74.2 µmol/l.

G401 cells were more sensitive to the antimetabolite MTX (mean GI_{50}: 0.078 µmol/l, SD = 0.004) than A204 and BT16 cells, in which the GI_{50} was only reached in two of the three experiments at concentrations up to 100 µmol/l. However, in A204 cells MTX inhibited cell growth significantly by approximately 40% at concentrations raging from 0.1 to 100 µmol/l ($P < 0.001$, ANOVA).

Effects of target specific drugs

The tyrosine kinase inhibitors sorafenib and imatinib showed similar results for all cell lines. For sorafenib the GI_{50} ranged between 3.3 and 7.4 µmol/l and for imatinib between 34.3 and 73.2 µmol/l. Imatinib was the only compound in our series that did not show a time-dependent effect. Compared to imatinib all cell lines were slightly, but not significantly, more sensitive to the CDK2 inhibitor roscovitine, which exhibited GI_{50} values from 12.8 to 62.7 µmol/l.

The peroxisome proliferator-activated receptor-γ agonist ciglitazone had the best effect on G401 (mean: 58.6 µmol/l) and BT16 cells (mean: 67.0 µmol/l), whereas in A204 the GI_{50} approximated 100 µmol/l.

The mTOR inhibitor rapamycin inhibited A204 and G401 cell growth to 60% consistently in the nanomolar range and with GI_{50} values ranging from 4.9 to 38.5 µmol/l.

All cell lines were sensitive to the plant flavonoid apigenin, which can be detected in parsley, and to curcumin (diferuloylmethane), which is a spice derived from the rhizome of the plant *C. longa*. Apigenin inhibited RT cell growth by 50% in BT16 cells at a concentration of 36.7 µmol/l and in A204 at a concentration of 68.0 µmol/l. BT16, G401 and the most resistant cell line, A204, showed very similar time- and dose-dependent effects after 24, 48 and 72 h of incubation with curcumin. After 72 h, the GI_{50} derived was 6.4 µmol/l (SD = 1.00) for A204, 5.2 µmol/l (SD = 0.43) for G401 and 8.1 µmol/l (SD = 1.62) for BT16 (Fig. 3).

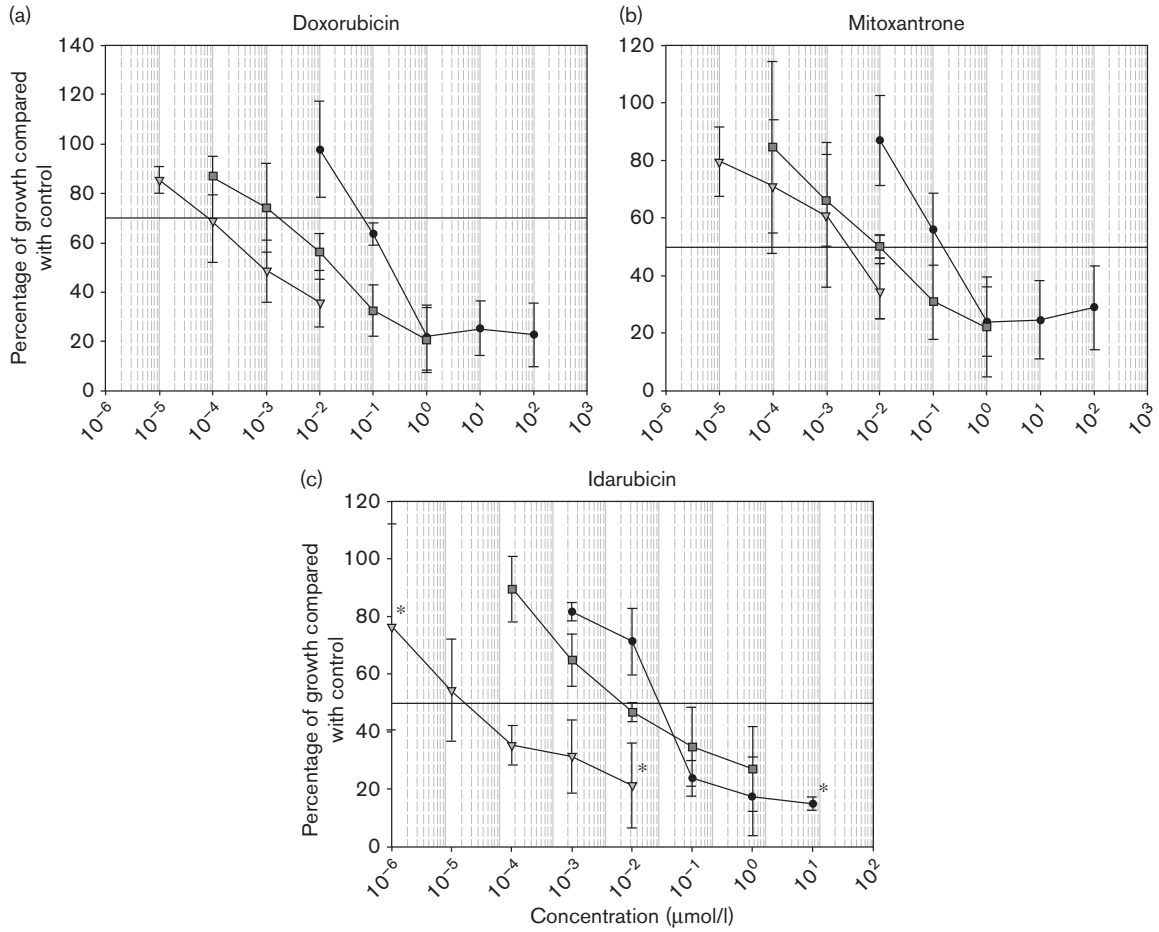
Comparison of the different cell lines

Overall, A204 cells were the most resistant to chemotherapeutic agents (mean GI_{50} calculated for each cell line from all experiments on all drugs: 134 µmol/l) and G401 cells were the most sensitive cell line (mean GI_{50}: 54.9 µmol/l), whereas BT16 cells showed intermediate sensitivity (mean GI_{50}: 91.8 µmol/l). With respect to oxaliplatin and to the anthracycline antibiotics A204 cells were significantly more resistant than G401 and BT16 cells ($P = 0.001$, ANOVA). Figure 4 outlines the cytotoxicity of each compound on the three cell lines. Interestingly, the GI_{50} values of standard anticancer drugs such as oxaliplatin, anthracyclines, alkylating agents, MTX and actinomycin D show large SDs considering all cell lines together, which is explained by the different sensitivity of the three cell lines while the cell cycle targeting compounds induced similar effects in each cell line.

Discussion

Even if no standard therapy for RTs exists at the moment, the molecular biology of these tumors is well characterized

Fig. 2



Dose–response curves determined for the anthracyclines (a) doxorubicin, (b) mitoxantrone and (c) idarubicin after 72 h in A204 (●), G401 (▼) and BT16 (■) cells. The dots represent means, the range bars represent the standard deviations. Each experiment was performed in triplicate, but for some concentrations there were only two values available (*).

[23]. Cytogenetic analysis showed monosomy 22 or loss of a band in 22q11 [24]. Epigenetic analysis from our laboratory showed that aberrant methylation of the tumor suppressor gene *RASSF1A* could be found in the majority of AT/RT [25]. Molecular analysis showed that in most malignant RTs of the kidney and the brain *hSNF5/INI1*, a subunit of the SWI/SNF chromatin remodelling complex, is lost. This leads to a decrease of $p16^{(INK4a)}$ and to an increase of cyclin D1, which is a critical downstream effector of *hSNF5/INI1*. Thus, the cyclin D1/CDK4-pRb-E2F pathway is activated resulting in G1 to S transition [6,16].

Standard anticancer drugs: the anthracycline antibiotics and actinomycin D show promising results

The alkylating agents cyclophosphamide, temozolomide and ifosfamide are employed in current treatment approaches. In contrast to the promising clinical results, a recent report shows that cyclophosphamide did not inhibit growth up to 383 μmol/l *in vitro* [26]. This is in line with the data of our study. The only exception here was thiotepa which achieved GI_{50} values *in vitro* at concentrations which are clinicalcally achievable *in vivo* during high-dose chemotherapy [27].

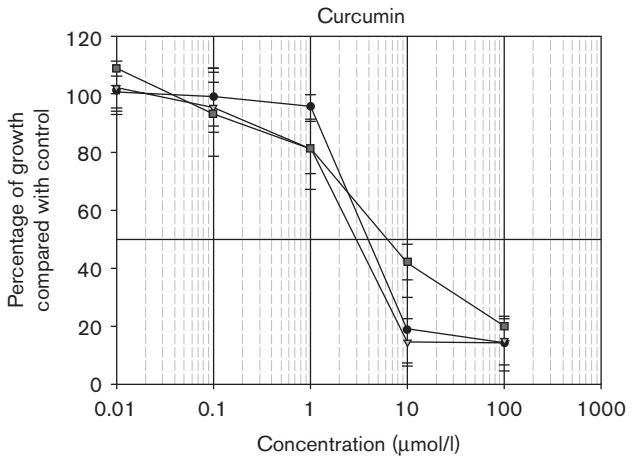
For doxorubicin, which was earlier tested on RT cell lines *in vitro*, Rosson *et al.* [28] measured a mean half maximal effective concentration of 2.9 nmol/l, which is comparable to our results with a GI_{50} of 3.3–400 nmol/l. The half maximal effective concentration calculates the concentration at which 50% of the maximal effect is observed, therefore it can be lower than the GI_{50} because the maximal effect could be less than 100% growth inhibition. In addition, the RT cell lines tested by Rosson *et al.* [28] were different from those tested in our study. In summary, the intercalating antibiotics were the best class of drugs in our screening. Idarubicin was the most cytotoxic anthracycline, but mitoxantrone and doxorubicin also inhibited RT cell growth in the nanomolar range. This

high preclinical cytotoxicity is generally consistent with the clinical activity and effectiveness. Actinomycin D is known to be very effective against rhabdomyosarcomas and Wilms, tumors. It represents a main part in the Wilms, tumor studies SIOP 2001, UKW2 and UKW3 and the recently published DFCI protocol. In our experiments actinomycin D induced the most potent effect of all compounds in concentrations that are clinically achievable. After 0.70–1.5 mg/m^2 of actinomycin D bolus intravenous infusion, Veal *et al.* [29] measured a median peak plasma concentration (C_{max}) of 25.1 ng/ml (which corresponds to 20 nmol/l) at 15 min and a median concentration of 2.4 ng/ml (= 1.9 nmol/l) at 24 h. Thus, our experiments reveal a very low GI_{50} suggesting very high activity in RTs.

Vinca alkaloids are often a part of the treatment of RTs as well. In-vitro tests for vinblastine and vincristine reported half maximal inhibitory concentrations (IC_{50} values) in the low nanomolar range, which supported their important role in chemotherapeutic protocols [30,28]. These tests are comparable with our results for the vinca alkaloid vinorelbine, which has been shown to be less neurotoxic than vincristine. The concentrations that we needed to stop RT cell growth are clinically well achievable; after oral administration the C_{max} of vinorelbine was 137.9 ng/ml = 12 nmol/l, after intravenous infusion even 1877.4 ng/ml = 1740 nmol/l [31,32]. Thus, vinorelbine could represent a potent alternative to vincristine in future therapy regimens. Its oral formulation could be an attractive compound especially for children.

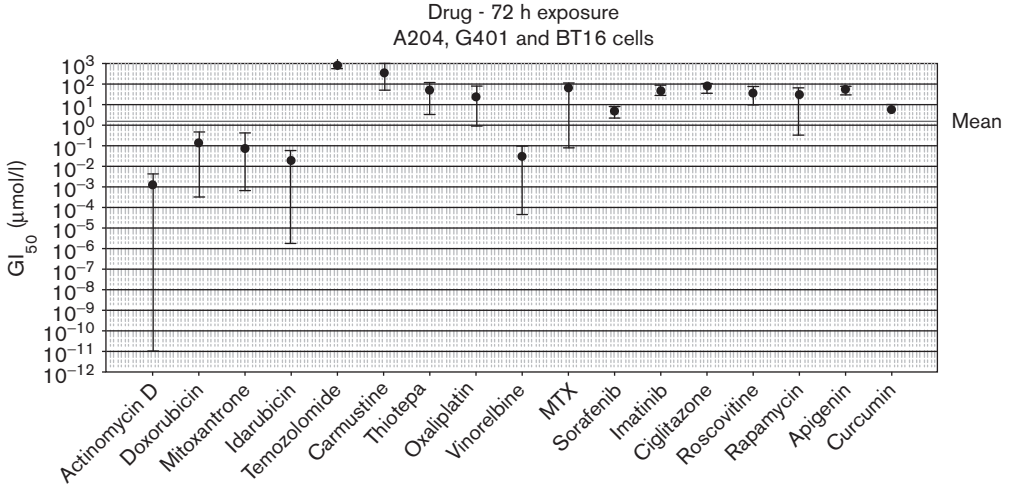
Tajbakhsh *et al.* [33] tested cisplatinum in the screening of the pediatric preclinical testing program and found that cisplatin showed high or intermediate activity in three of three RT cell lines and exhibited an IC_{50} of 0.54 µmol/l *in vitro*. In our panel we tested oxaliplatin, which is a new platinum agent. It seems to be less nephrotoxic but sensory peripheral neuropathy is dose limiting. Previous in-vitro tests performed with three RT cell lines including BT16 reported an IC_{50} of 0.58–2.5 µmol/l [26], which is very close to our GI_{50} of 0.91–2.7 µmol/l for G401 and BT16 cells. A204 cells were significantly more resistant ($P < 0.001$; ANOVA). The cytotoxic concentrations for four out of five cell lines

Fig. 3



Dose–response curves determined for the target specific drug curcumin after 72 h in A204 (●), G401 (▼) and BT16 (■) cells. The dots represent means, the range bars represent the standard deviations. Data performed in triplicate.

Fig. 4



Mean 50% growth inhibitions (GI_{50} values) after drug exposure for 72 h on rhabdoid tumor cell lines. The dots represent the mean GI_{50} values and the range bars represent the lowest and the highest GI_{50}. MTX, methotrexate.

treated with oxaliplatin in earlier studies and for the three cell lines tested in our tests are clinically achievable (mean C_{max}: 2.59–3.22 μg/ml = 6.5–8.1 μmol/l; [34]). Unfortunately in an open-label phase II study, oxaliplatin alone did not prove to be effective in patients with refractory RTs [35].

The role of intrathecal chemotherapy is not yet clear. In the currently most successful therapeutic strategy, Chi *et al.* [7] administered intrathecal therapy to each patient using MTX, cytarabine and hydrocortisone. In contrast to previous in-vitro tests [26], we could confirm the effectiveness of MTX on RT cells with significant inhibition of cell proliferation.

Target specific drugs

Several tyrosine kinase inhibitors have been tested in RT cell lines. Dasatinib and sunitinib have passed the screening of the Pediatric Preclinical Testing Program. Although sunitinib showed high (complete response) or intermediate activity in two of three RT xenografts [36], dasatinib revealed very high activity *in vitro* [37]. Dasatinib also entered a pediatric phase I/II trial in combination with ifosfamide, carboplatin and etoposide in the treatment of metastatic or recurrent pediatric solid tumors (Sato, NCT00788125).

We sought to evaluate the activity of imatinib and sorafenib on our panel of RT cell lines. In a recent report imatinib exhibited an IC_{50} of 13.8 μmol/l for BT12 cells but close to no activity up to 170 μmol/l for KCCF1 and BT16 cells [26]. In contrast to these findings we achieved a GI_{50} in each cell line ranging from 34.3 to 73.2 μmol/l. Sorafenib is a multikinase inhibitor targeting the Raf kinase, vascular endothelial growth factor receptor, platelet derived growth factor receptor, Flt-3 and c-KIT [38]. In a phase I clinical and pharmacokinetic study, Strumberg *et al.* [39] observed a mean C_{max} of 3.42 mg/l (= 7.4 μmol/l) after a single oral dose of 400 mg and a mean plasma concentration of approximately 6 mg/l (= 12.9 μmol/l) after multiple doses of 400 mg of sorafenib. These concentrations were above the doses we employed to decrease RT cell growth by 50%. As sorafenib has been approved for the treatment of advanced renal cell and hepatocellular carcinoma future clinical trials may include sorafenib in the treatment of malignant RTs. Sorafenib also decreases levels of cyclin D1, Rb and phosphorylated Rb consistent with a G1 delay [40].

Owing to the loss of *hSNF5/INI1* in most RTs and the genetic proof of the essential role of cyclin D1 in rhabdoid tumorigenesis *in vivo,* we suggest that targeting the cyclin D1/CDK axis plays a key role in novel treatments [15–17]. Alarcon-Vargas *et al.* [16] reported that downmodulation of cyclin D1 by RNAi treatment or by the compounds *N*-(4-hydroxyphenyl) retinamide and tamoxifen inhibited growth and survival of RTs *in vitro* and *in vivo*. Smith *et al.* [17] found that flavopiridol, a pan-CDK inhibitor, is effective in inducing cytotoxicity in RTs *in vitro* and *in vivo* by downregulation of cyclin D1 and upregulation of p21. Even very low concentrations of flavopiridol inhibited MON and G401 cell proliferation with an IC_{50} of 150–200 nmol/l. Further studies showed that inhibitors of the cyclin D1/CDK4 complex can reduce the expression of antiapoptotic genes and consequently render tumor cells more susceptible to chemotherapeutic agents [41–43].

The growth inhibitory effects of peroxisome proliferator-activated receptor-γ agonists have been shown in several tumor cell lines and a variety of studies have shown that ciglitazone inhibits cyclin D1 by transcriptional and posttranscriptional mechanisms [44,45]. Although A204 cells were resistant to incubation with 100 μmol/l of ciglitazone, BT16 cells and G401 cells showed a time and dose-dependent decrease of cell proliferation.

Roscovitine is a potent inhibitor of CDK1, CDK2 and CDK5 undergoing clinical trials. In our experiments roscovitine inhibited cell proliferation in each cell line, but it was less potent than flavopiridol in the studies of Smith *et al.* [17]. One reason might be the difference in the inhibition of cyclin-dependent kinases: roscovitine inhibits CDK1, CDK2 and CDK5, whereas flavopiridol also decreases levels of CDK4 which directly targets cyclin D1.

The natural compound apigenin is one of the most common flavonoids. Apigenin increases the protein expression of several tumor suppressors, whereas it downmodulates the protein expression of the cyclins D1, D2, E and CDK2, CDK4, CDK6. Furthermore, apigenin inhibits the hyperphosphorylation of Rb, induces apoptosis and downmodulates the expression of NF-κB [46,47]. Thus, it seems to be an excellent target-specific drug against RTs. In our experiments apigenin inhibited cell proliferation in each cell line, but the required concentration would not be achievable in serum or tumor of athymic nude mice (approximately 1 μmol/l) [47]. Further preclinical studies are necessary to evaluate the combination of apigenin with standard cancer drugs.

Another mechanism of targeting cyclin D1 can be observed for rapamycin: inhibiting mTOR results in a block of phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1, which reduces the translation of m7GTP cap containing transcripts. Many of these m7GTP cap-containing transcripts encode proteins required for cell cycle progression, including cyclin D1 [48–50]. Rapamycin has already been tested by the PPTP at concentrations from 0.01 to 100 nmol/l, but even the highest concentration showed maximal growth inhibition of only 40% at both rhabdoid cell lines BT12 and CHLA-266 [51]. Arcaro *et al.* [52] showed that rapamycin stopped BT12 and BT16 tumor cell growth at 20 nmol/l. We can confirm these findings, as we measured a GI_{50} above 10 nmol/l but rapamycin significantly inhibited A204 and

G401 cell proliferation in the nanomolar and in the picomolar range ($P = 0.003$, ANOVA). Hence rapamycin must be administered at higher concentrations to inhibit 50% of proliferation but it significantly reduces RT cell growth at very low concentrations. Perhaps rapamycin might be a very potent drug in combination with standard chemotherapy. Interestingly, rapamycin and its analogs are highly lipophilic and may cross the blood–brain barrier [53].

In addition to rapamycin, curcumin targets the PI3K/mTOR pathway and inhibits eukaryotic translation initiation factor 4E-binding protein 1 and mTORC1 activity [54,55]. Curcumin also suppresses NF-κB activity [56] and downregulates cyclin D1 expression through activation of transcriptional and posttranscriptional mechanisms: on the one hand curcumin activates proteases that degrade cyclin D1 and on the other curcumin downregulates cyclin D1 promotor activity and cyclin D1 mRNA, perhaps through the suppression of NF-κB activation [57]. In this study we show, for the first time, that curcumin effectively inhibits RT cell growth *in vitro*. In a phase I study Cheng *et al.* [58] observed no treatment-related toxicity up to 8 mg/day when curcumin was taken orally for 3 months. They measured an average C_{max} of 0.51–1.77 μmol/l after oral administration of 4–8 mg curcumin per day. This shows the poor bioavailability and the C_{max} is lower than the GI_{50} in our experiments. Perhaps short phases of high dose curcumin administration or the combination with drugs that significantly increase the bioavailability like piperin or curcumin formulated with phosphatidylcholine [59,60] could be helpful to reach higher plasma concentrations. A recently published study reported a 5-fold brain-to-plasma ratio for curcumin in mice, suggesting its stability in lipid-rich compartments, where hydrolysis is limited [61]. Thus curcumin could be particularly advantageous against AT/RT.

Conclusion

The anthracyclines and the vinca alkaloid vinorelbine stopped RT cell growth in the nanomolar range. These findings are in line with the high clinical activity. Idarubicin or the less cardiotoxic mitoxantrone may represent potent alternatives to doxorubicin and vinorelbine may as well substitute the highly neurotoxic vincristine in future trials. Actinomycin D was the most cytotoxic with GI_{50} values in the femtomolar and picomolar range for G401 and BT16 cells and it will certainly be used in future therapeutic regimens. The cell cycle targeting compounds sorafenib, roscovitine and rapamycin showed promising results, especially for the highly resistant RT cell line A204. In this study, we show for the first time that the target-specific drugs apigenin and curcumin effectively inhibit RT cell growth. Supporting earlier studies, we conclude that targeting cyclin D1 seems to be a key in the treatment of RTs [15–17].

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