Inhibition of Induced Chemoresistance by Cotreatment with BVDU

Inhibition of Induced Chemoresistance by Cotreatment with (E)-5-(2-Bromovinyl)-2'-Deoxyuridine (RP101)

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Abstract

Induced chemoresistance leads to the reduction of apoptoticresponses. Although several drugs are in development that circumventor decrease existing chemoresistance, none has the potentialto prevent or reduce its induction. Here, we present data froma drug that could perhaps fill this gap. Cotreatment of chemotherapywith (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU, RP101) preventedthe decrease of apoptotic effects during the course of chemotherapyand reduced nonspecific toxicity. Amplification of chemoresistancegenes (*Mdr1* and *Dhfr*) and overexpression of gene products involvedin proliferation (DDX1) or DNA repair (UBE2N and APEX) wereinhibited, whereas activity of NAD(P)H: quinone oxidoreductase1 (NQO1) was enhanced. During recovery, when treatment was withBVDU only, microfilamental proteins were up-regulated, and proteinsinvolved in ATP generation or cell survival (STAT3 and JUN-D)were down-regulated. That way, in three different rat tumormodels, the antitumor efficiency of chemotherapy was optimized,and toxic side effects were reduced. Because of these beneficialproperties of BVDU, a clinical pilot Phase I/II study with fivehuman tumor entities has been started at the University of Dresden(Dresden, Germany). So far, no unwanted side effects have beenobserved.

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Introduction

Repeated chemotherapeutic treatment frequently induces, or selectsfor, chemoresistance of remaining cancer cells by altering geneexpression and inducing genomic instability because of mutation, recombination, and gene amplification events. Deregulation ofDNA-repair enzymes is partly involved in this phenomenon (*e.g.p53* gene, *BRCA1/2*, *UBE2N*, *APEX*, and *Rad51*). Furthermore, enzymesthat metabolize and bioactivate drugs [*e.g*. dihydrofolate reductase(DHFR) (1) and NQO1 (2)] or proteins that transport cytotoxicagents (*e.g*. multidrug resitance protein (MDR1, Ref. 3] oftencontribute to chemoresistance.

During the implementation of a long-term screening program forinhibitors of chemoresistance, BVDU (3) was the only substanceof clinical relevance we identified. It inhibited 2-amino-6-mercaptopurine-induced SV40 amplification (4) in Chinese hamster cells, and abrogatedtriethylene-melamine-induced recombination in yeast (5). InFriend mouse erythroleukemia cells, treatment with DOX induced*Mdr1* gene amplification and drug resistance, which was preventedby simultaneous treatment with BVDU (6) . Because the mode ofaction of BVDU in respect to these effects is unknown, thisstudy aimed to elucidate underlying mechanisms. The preclinicaldata we obtained were a prerequisite for the start of clinicaltrials.

We performed *in vitro* experiments with AH13r rat hepatosarcomaand mouse 3T6 cells, and *in vivo* experiments with DMBA-inducedSD-rat fibrosarcomas and adenocarcinomas. To test BVDU in asecond *in vivo* cancer model, we injected AH13r cells into SD-ratsfor tumor induction.

As most antineoplastic drugs eliminate tumor cells by apoptosis,cancer cells can evade cell death by virtue of overactivatedsurvival mechanisms. Thus, chemoresistance mechanisms can involveantiapoptotic traits. Therefore, we investigated several survivalmechanisms, as well as the activated STAT3, and JUN-D. Moreover, we tested the activity of NQO1, an activating enzymefor anticancer drugs like MMC, MXA, or DOX, which is down-regulatedin multidrug-resistant AH130 tumor cells. To identify amore comprehensive spectrum of BVDU-influenced proteins, weperformed a two-dimensional gel electrophoresis and identified proteins that are differentially expressed in response to BVDUusing MALDI-MS.

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Materials and Method

Chemicals.
DMBA, MMC, MTX, and DOX were from Sigma (Deisenhofen, Germany).MXA, cisplatin, glufosfamide, and DOX for *in vivo* tests werefrom Asta Medica (Frankfurt am Main, Germany). BVDU (RP101)was from RESprotect and Berlin-Chemie (Berlin, Germany). RNasewas from Boehringer (Mannheim, Germany), and restriction enzymeswere from New England Biolabs (Schwabach, Germany). All of theother chemicals were purchased from Sigma and Roth (Karlsruhe,Germany).

3T6 Cell Culture and Development of Methotrexate Resistance.
Swiss albino mouse fibroblasts, 3T6, were grown in DMEM supplementedwith 10% fetal bovine serum, penicillin, and streptomycin (Biochrom,Berlin, Germany). Cells (2.8 x 105) were plated into 9 T25-flaskswith MTX, and 9 T25-flasks with MTX and 30 µM BVDU. Assoon as cells approached confluency, they were trypsinized andreplated at the next higher drug concentration. The MTX concentrationwas increased 1.5-fold at 1-week intervals for 60 days startingwith 44 nM MTX. The number of living cells was determined usingthe Cell Counter and Analyser System CASY TT (Schärfe SystemGmbH, Reutlingen, Germany). Cell counting and cell volume determinationwere hereby based on the displacement of conductive electrolyteby dielectric cells. The signals generated by the cells suspendedin an electrolyte were evaluated by pulse area analysis. Thepulse area of the signal was strictly proportional to the volumeof the particle generating the signal. In dead cells, the integrityof the cell membrane is lost. This loss increased the conductivityand reduced the pulse area of the electric signal. Thus, toexclude debris and dead cells, only particles with a size of>7.5 µm were counted as cells.

Treatment of AH13r Sarcoma Cells in Culture.
AH13r cells, a subline of the rat Yoshida sarcoma, were obtainedfrom the Cell and Tumor Bank of the West German Cancer Center,University Essen, Medical School (Essen, Germany). Cells weregrown in DMEM (FG 0415; Biochrom AG, Berlin, Germany) supplementedwith 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mlpenicillin, and 100 µg/ml streptomycin in a humidifiedatmosphere containing 5% CO2 at 37°C. Logarithmically growingcells were seeded at a density of 100,000 cells/ml and incubatedwith different cytostatic drugs in combination with or withoutBVDU. After 2–4 days (unless otherwise indicated), cellswere counted using the Cell Counter and Analyser System CASYTT (Schärfe System GmbH), and serially passaged.

HOPI Double Staining.
Apoptosis was assayed by HOPI staining as described by Grusch*et al*.. Viable, apoptotic, and necrotic cells werecounted. The Hoechst 33258 dye stains the nuclei of all cells.Nuclear changes associated with apoptosis, such as chromatincondensation and nuclear fragmentation, can be readily monitoredand quantified. Propidium iodide uptake indicates loss of membraneintegrity characteristic for necrotic and late apoptotic cells.The selective uptake of the two dyes allows to distinguish betweenapoptotic and necrotic cell death. Necrosis is characterizedin this system by nuclear propidium iodide uptake into cellswithout chromatin condensation or nuclear fragmentation.

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Treatment of AH13r Sarcomas in SD-Rats.
Ten SD-rats per treatment group were given a single s.c. injectionof ascites Yoshida AH13r hepatoma cells. Five to 7 days aftertumor application, the growth of the resulting tumors (at theinjection site) was suppressed by i.p. treatment of the animalswith 2 or 4 mg/kg DOX (9 times within 3 weeks), 120 or 140 mg/kgglufosfamide (15 times within 3 weeks), and 0.5 or 1.5 mg/kgcisplatin only (4 or 5 times within 3 weeks), and by additionaloral treatment with 15 mg/kg BVDU (15 times within 3 weeks).

Treatment of DMBA-induced Fibrosarcomas and Mammary Adenocarcinomas in SD-Rats.
SD-rats (3.5 weeks old) were purchased from Harlan Winkelmann(Borchen, Germany). The care and use of the animals were inaccordance with institutional guidelines.

At an age of 39 days, a total of 8 male and 8 female rats wereadministered s.c. 10 mg DMBA in 0.75 ml sesame oil (DAB 10)to induce fibrosarcomas at the injection site (neck) and (multiple)mammary adenocarcinomas in female rats. DMBA induces mammarytumors that are comparable with those in humans in terms oftheir long relative latency, histotypes, and endocrine responsiveness (12).

Treatment with DOX or DOX + BVDU.
Beginning at an age of 128 days, 2 male and 2 female rats wereadministered three times a week for 8 weeks s.c. with 1 mg/kgDOX (in 0.9% NaCl solution). Another 3 male and 3 female ratswere administered three times a week for 8 weeks s.c. with 1mg/kg DOX and five times a week p.o. with 15 mg/kg BVDU (in corn oil). The control group of 3 male and 3 female rats received1 ml of a 0.9% NaCl solution i.p., and 5 ml corn oil p.o. fivetimes a week.

Determining Tumor Incidences.
The rats were checked for tumors by palpation regularly twicea week. The rats were killed when the detectable tumor burdendid not allow longer treatment. The surviving animals were killed60 days after beginning the administration of DOX or DOX+BVDU.An autopsy was performed, and tumor samples were fixed in 10%formalin. All of the tumors were embedded in paraffin, sectionedat 4 µm, stained with H&E, and examined histologically.Another sample of each tumor was snap-frozen in liquid nitrogenfor additional analysis.

Southern Blot Analysis.
Analyses were performed using standard procedures (13) .

Western Blot Analysis.
Pelleted cells were suspended in buffer A (20 mM HEPES, 400mM NaCl, 25% v/v glycerol, 1 mM EDTA, 0.5 mM NaF, 0.5 mM Na3VO4,and 0.5 mM DTT) according to Pagano *et al*. (14) , supplementedwith Complete Protease Inhibitor Tablets (Roche, Mannheim, Germany)as described by the manufacturer and then shock-frozen in liquidnitrogen, thawed on ice, and centrifuged (15 min, 4°C, 11,500rpm). The concentration of protein in the supernatant was determinedby the Bradford method using bovine

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-globulin as a standard(Bio-Rad, Munich, Germany).

SDS-PAGE was performed on a 12% polyacrylamide gel with 10 µgof total protein per lane. Proteins were transferred to a polyvinylidenedifluoride membrane (Amersham, Freiburg, Germany) using theMini *trans* -Blot apparatus (Bio-Rad) with transfer buffer (25mM Tris base, 192 mM glycine, and 15% v/v methanol) for 4 hat 50 V on ice. Consistent protein loadings and transfer efficiencywere verified by Ponçeau S-staining.

Destained membranes were blocked with 5% nonfat dry milk inTBST-buffer (20 mM Tris base, 137 mM NaCl, and 0.1% v/v Tween20) followed by incubation with the respective antibodies overnightat 4°C. Antibody dilutions were as follows: cleaved caspase-3(Asp175) antibody (Cell Signaling Technology) 1:2000 in blockingbuffer, STAT3 (NEB, Frankfurt a.M., Germany) 1:500, JUN-D (SantaCruz Biotechnology, Santa Cruz, CA) 1:1000, and anti-P-glycoproteinantibody (Alexis, Grünberg, Germany) 1:3000–1:4000.

After washing, blots were incubated with secondary antibody-horseradishperoxidase conjugates, washed again, and protein bands werevisualized using chemiluminescence reagent plus (NEN, Boston,MA). Images were captured with a Kodak Image Station 440CF andanalyzed with 1d Image Analysis Software (version 3.5; Kodak).

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CGH.
DNA labeling and CGH on normal rat chromosomes obtained fromfetal rats were performed as described previously (15). Briefly,equal amounts of digoxigenin- and biotin-labeled genomic DNAfrom drug-treated and from BVDU + drug-treated tumors, respectively,were hybridized to normal metaphases obtained from fetal ratcells. Hybrid molecules were detected with one layer of avidin-FITC(Sigma, Munich, Germany) and rhodamine-antidigoxigenin Fab fragments(Roche Diagnostics, Mannheim, Germany). Slides were mounted and counterstained with antifade solution containing 1 µg/ml4,6-diamidino-2-phenylindole and actinomycin D (both Serva,Heidelberg, Germany) and analyzed using a cooled CCD camera(Hamamatsu Photonics, Herrsching, Germany) mounted on a ZeissAxioskop epifluorescence microscope (Carl Zeiss, Göttingen, Germany). The ISIS Digital Image Analysis System (MetaSystems,Altlussheim, Germany) was used for ratio profile analysis, whichwas based on >7 well-hybridized metaphases.

Real-Time PCR.
Genomic DNA was isolated from each cell subline at various timepoints using the DNeasy Tissue kit (Qiagen, Hilden, Germany)in accordance with the manufacturer's instructions. Real-timePCR was performed using the ABI Prism 7700 Sequence DetectionSystem (Applied Biosystems, Foster City, CA). For the targetgene, *Dhfr*, amplification mixes (30 µl) contained 0.8ng genomic DNA, 10x SYBR-Green buffer, 200 µM dATP, dCTP,dGTP, and 400 µM dUTP, 3 mM MgCl2, 0.75 units of AmpliTaqGold, 0.3 units of AmpErase uracil *N*-glycosylase (Applied Biosystems),and 200 nM of each primer (Thermo Hybaid, Ulm, Germany). Forthe reference gene,18S rRNA, 10x TaqMan buffer, 5 mM MgCl2,400 nM of each primer, and 200 nM TaqMan fluorogenic probe (ThermoHybaid) were used. Thermal cycling consisted of 40 cycles at 95°C for 15 s and 60°C for 1 min, and contained an initialstep of 2 min at 50°C and 10 min at 95°C. We prepareda reference calibration curve with stepwise dilutions (1:5)starting with 100 ng genomic DNA. Each 96-well microplate includedthe standard curve and the sample genomic DNA in triplicate.The normalized *Dhfr* gene copy number was derived from the ratioof the *Dhfr* gene copy number to the 18S rRNA reference genecopy number. The amplification of the *Dhfr* gene was calculatedrelative to the *Dhfr* gene copy number of an untreated 3T6 cellline.

Assay of NQO1 Enzyme Activity.
DT-D was assayed as dicoumarol-inhibitable NAD(P)H: dichlorophenolindophenolreductase essentially as described by Hodnick and Sartorelli (16). Equal numbers of exponentially growing AH13r cells were removed from the medium by centrifugation and resuspended in1 ml of ice-cold PBS. Cells were kept on ice and homogenizedtwice for 30 s with an Ultra-Turrax T8 homogenizer (IKA, Staufen,Germany). Dicoumarol-sensitive NADPH:2,6-dichloroindophenol(DCPIP) reductase was assayed in a reaction mixture containing0.05 M potassium phosphate (pH 7.5), 0.3 mM NADPH, 0.04 mM DCPIP,0.07% BSA, and 0.1 ml of homogenized cells in a final volumeof 1 ml. The reaction was initiated by the addition of NADPH,and the rate of DCPIP reduction was determined at a wavelengthof 600 nm. Dicoumarol (1 µM final concentration) was thenadded, and the rate was measured again. The rate of dicoumarol-sensitiveNADPH:DCPIP reductase was determined as the difference betweenthe uninhibited and dicoumarol-inhibited rates.

Two-Dimensional Gel Electrophoresis.

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AH13r cells were treated for 17 days (36 h recovery for samples5 and 6) as follows: (*a*) DMSO (untreated control); (*b*) BVDU;(*c*) MMC; (*d*) MMC + BVDU; (*e*) recovery after MMC treatment; and(*f*) recovery with BVDU after MMC + BVDU treatment. Proteinsof each group were separated by two-dimensional gel electrophoresis.Differentially expressed proteins were identified by MALDI-MS.

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Results

In Vitro Experiments.
We first investigated 3T6 cells treated with increasing dosesof MTX ± BVDU. 3T6 cells were chosen because of theirability to amplify the *Dhfr* gene forming double minutes duringtreatment with MTX (17). BVDU inhibited induction of *Dhfr* amplification.Whereas treatment with MTX induced amplification of *Dhfr* to give 4–27 copies (mean 14) after 39 days, cells cotreatedwith 10 µg/ml BVDU amplified the *Dhfr* gene only 3–7times (mean 4). Additional treatment sustained this effect (Fig.1*A*).

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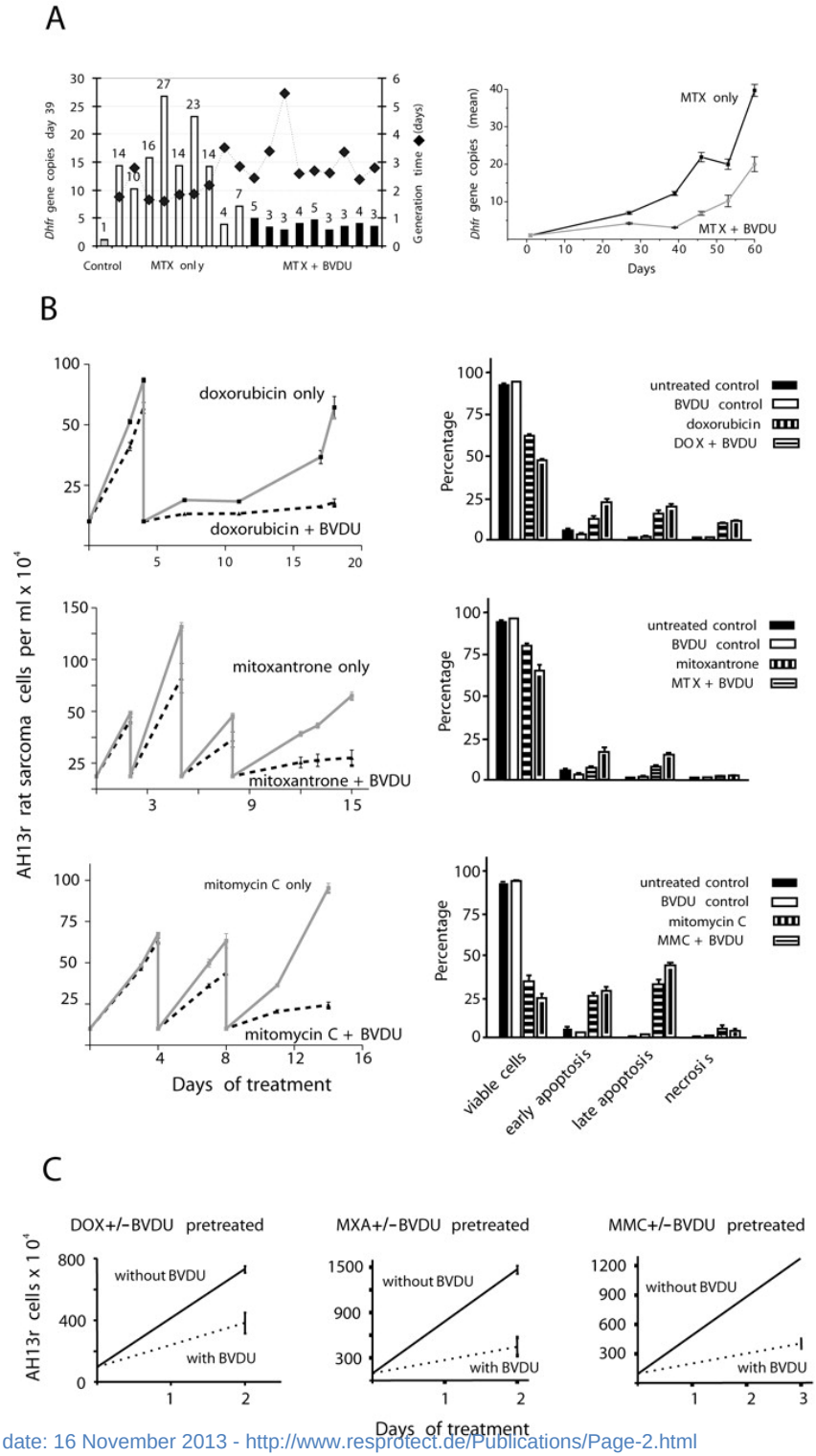

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Fig. 1. *In vitro* experiments. *A*, *Dhfr* gene amplification in mouse 3T6 cells (nine independent sublines each). *B*, effect of BVDU on cell numbers and apoptosis in AH13r rat sarcoma cells. *Left*, treatment with cytostatic drugs only, or in combination with BVDU (three independent experiments). DOX, 1.33 ng/ml; MXA, day 0: 0.1 ng/ml, day 2: 0.1 ng/ml, day 5: 0.1 ng/ml, day 8: 0.15 ng/ml; MMC, day 0: 35 ng/ml, day 4: 50 ng/ml, day 8: 75 ng/ml; 10 µg/ml BVDU. *Right*, HOPI stain evaluation (mean value ± SD, nine independent experiments). *C*, effect of BVDU on the recovery of AH13r cells (three independent experiments).

Furthermore, BVDU cotreatment sensitized AH13r sarcoma cellsfor chemotherapy-induced apoptosis (Fig. 1*B*). BVDU cotreatmentsignificantly reduced cell numbers. BVDU itself was nontoxic(data not shown). We detected increasing numbers of pyknoticcells (7.5–11.5 µm diameter) as a result of BVDU combinatorial treatment (Fig. 2*A*) . This observation indicatedan induction of apoptosis.

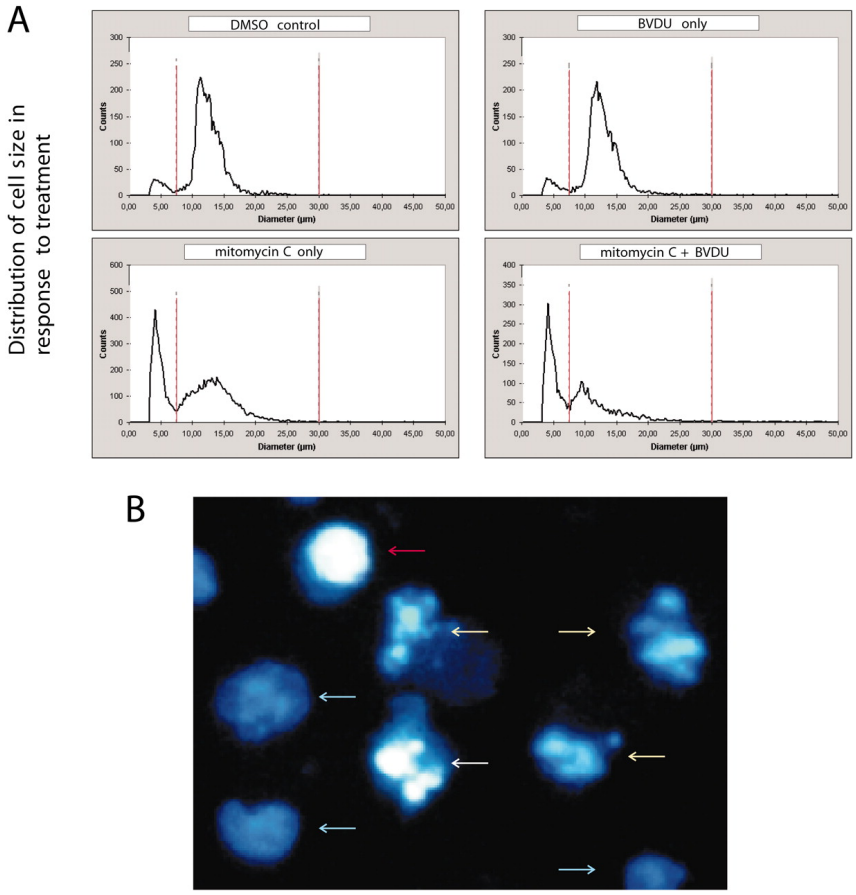

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Fig. 2. Assessment of apoptosis. *A*, treatment of AH13r rat sarcoma cells. Cell size distribution profiles obtained on day 14 of MMC treatment, using the CASY Cell Counter and Analyzer. AH13r cells were treated with 0.05% DMSO (*control*), 10 µg/ml BVDU, increasing doses of 35–75 ng/ml MMC only, and MMC+BVDU. *B*, apoptosis assay. HOPI staining image, example. *Blue arrows*, viable cells; *yellow arrows*, early apoptosis; *white arrow*, late apoptosis; *red arrow*, necrosis.

These results were confirmed by HOPI analysis (10) ; Fig. 2*B*).BVDU cotreatment increased the number of apoptotic cells onaverage by 15% (Fig. 1*B*, *right*).

We next investigated several survival pathways using Westernblot analysis. This included the Akt/forkhead-related transcriptionfactor pathway, the Raf/extracellular signal-regulated kinase,MDM2, p14, p53, p38, and survivin pathways. Neither of thoseappeared to be affected by BVDU cotreatment. Also, the expressionpatterns of several cell cycle regulators such as p27, p16,cyclin-dependent kinases, and cyclins remained unchanged (datanot shown).

However, BVDU in combination, but not by itself, reduced theamount of the oncogene protein STAT3 to up to 50% (Fig. 3*A*).Moreover, in combination with DOX or MMC, this reduction ofSTAT3 expression by BVDU was maximal during recovery, when thecytostatic drug was omitted after previous treatment, but BVDUwas still present (see Fig. 1*C*). Additionally, during MMC recovery,the oncogene protein JUN-D was overexpressed, but remained atcontrol level in the presence of BVDU. Treatment was also accompaniedby activation of caspase-3 (Fig. 3*A*).

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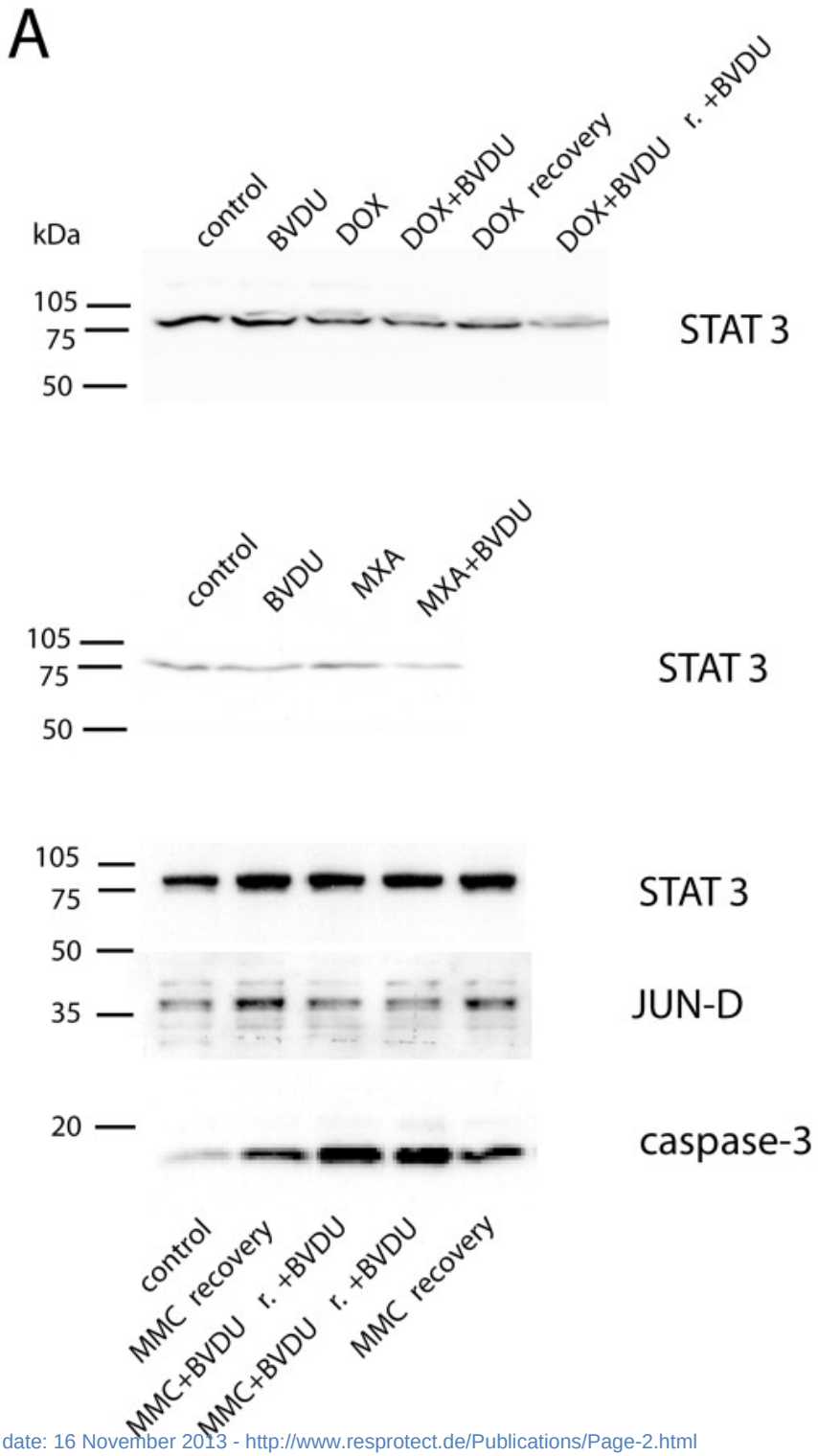

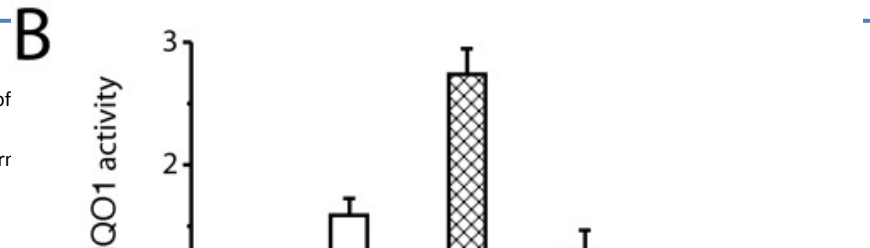

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Fig. 3. Effect of BVDU combinatorial treatment on protein expression. *A*, Western blot analysis, expression of STAT3, JUN-D, and caspase-3 (proteolytically activated form) in response to cytostatic drug treatment. The expression levels were determined densitometrically (*r*. = recovery, see Fig. 1*C*). *B*, NQO1 enzyme activity (mean value ± SD, six independent experiments).

We investigated NQO1 activity in cell extracts (16) after treatmentwith the cytostatic drugs ± BVDU (Fig. 3*B*). BVDU cotreatedcells showed higher NQO1 activity than untreated control cellsor cells treated with cytostatic drugs only. Interestingly,cells treated with MMC+BVDU, which caused the strongest antiproliferativeeffect, did not enhance NQO1 activity.To elucidate the effects of BVDU, we performed a two-dimensionalgel electrophoresis and identified differentially expressedproteins by MALDI-MS (Table 1). During combinatorial MMC+BVDUtreatment, or during recovery (MMC omitted, BVDU present) from combinatorial treatment, the expression of three major "clusters"of protein classes was affected: (*a*) microfilamental (or regulatory)proteins were up-regulated during recovery (actins, tubulin,myosin, and tropo-modulin); (*b*) proteins involved in ATP generationwere down-regulated (succinate dehydrogenase, pyruvate dehydrogenase,and malic enzyme; however, malate dehydrogenase was up-regulated);and (*c*) proteins regulating DNA repair were suppressed (APEXand UBE2N). One protein with oncogenic potential, DDX1, wasaffected by BVDU alone. In total,

75% of the spots were identifiedby MALDI-MS.

Table 1 Effects of cotreatment of MMC with BVDU on protein expression

Proteins Fold change
[illegible] BVDU

[illegible], 116-KD

[illegible] E1-subunit A, Flavoprotein; SDHA

[illegible] 2; PCBP2

[illegible] deficient 7; mitotin, cell division cycle-like 1; CDCL1

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In Vivo Experiments.
In vivo, BVDU enhanced anticarcinogenic effects on AH13r sarcomasin rats. Three cytostatic drugs of different mode of action(DOX, glufosfamide, and cisplatin) were tested in two independentexperiments (Fig. 4*A*, *panels 1* and *3*). On the basis of previousresults with rats (17), we used a daily dose of 15 mg/kg togain peak plasma levels of

~

25 µg/ml 20 min after application.After cotreatment, BVDU was additionally administered in therecovery phase for 4 days (Fig. 4)

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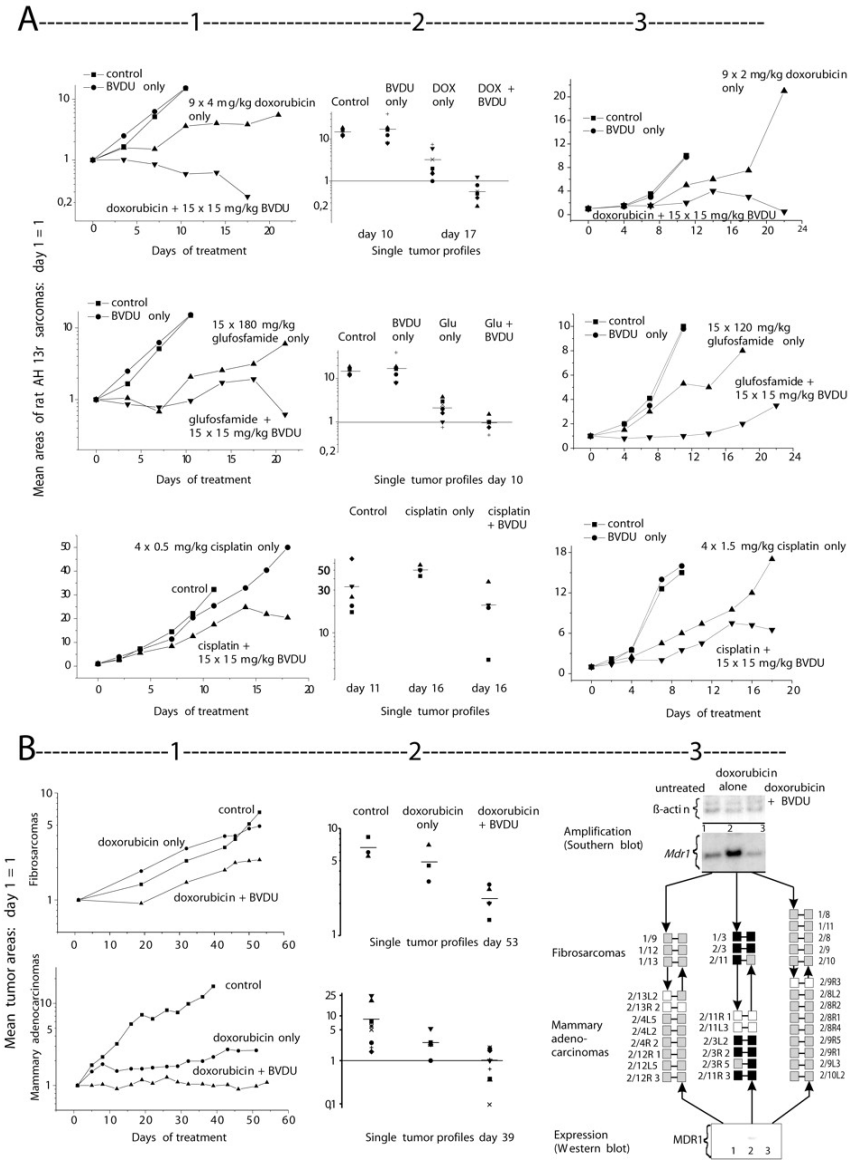

Fig. 4. SD-rats treated with DOX, glufosfamide, and cisplatin. *A*, rats with AH13r sarcomas. *A1* and *A3*, comparison of control animals, BVDU-control animals, cytostatic drug-treated animals, and cytostatic drug+BVDU-treated animals (calculation of the mean of all individual tumor areas). *A2*, tumor areas of single tumors 10–17 days after treatment start (—mean). *B*, rats with DMBA-induced fibrosarcomas

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and mammary adenocarcinomas. *B1, top*, SD-rats with DMBA-induced fibrosarcomas, calculation of the mean tumor area. Three control animals, three DOX-treated animals, and five DOX+BVDU-treated animals with fibrosarcomas. *Bottom*, DMBA-induced mammary adenocarcinomas, calculation of the mean of all individual tumor areas. Within the control animals, 8 tumors, within the DOX-treated animals, 6 tumors, and within the DOX+BVDU-treated animals, 9 tumors could be observed. *B2*, area of the individual tumors 39 days after treatment start (—mean). *B3*, *Mdr1* gene amplification and expression patterns of DMBA-induced fibrosarcomas and mammary adenocarcinomas in SD-rats. Representative tumor of 1) rat treated with solvent, 2) rat treated with DOX, and 3) rat treated with DOX+BVDU. Amplification of the *Mdr1* gene was detected by Southern blot analysis and expression of the MDR1 protein by Western blot analysis using the murine anti-P-glycoprotein monoclonal antibody JSB-1. The densitometrically determined *Mdr1* gene dosages (amplification levels) were subdivided into two categories.

▒

, indicate normal copy number,

■

, amplification.

□

, indicate that no analysis was performed because the whole tumor probe was used for histological analysis. Western blot analysis gave a yes (

■

) or no (

▒

) result in respect to *Mdr11* = male, *2* = female/animal number, position of mammary adenocarcinomas). gene express patterns. The numbers indicate different tumors examined (1 male, 2 female/animal number, position of mammary adenocarcinomas).

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The BVDU cotreated groups showed significantly less tumor growth.The tumor areas of these groups were significantly smaller thanthat of controls or the groups which were treated with cytostaticdrugs only (Fig. 4*A*, *panel 2*), with the differences being significantat the 5% level (*t*-test/Mann-Whitney test).Whereas the treatment with cytostatic drugs (DOX, cisplatin,and navelbine+ifosfamide+cisplatin) led to a defined loss of body weight, cotreatment with BVDU partly inhibited loss ofbody weight (Table 2). This may indicate reduced nonspecifictoxicity and optimized antitumor efficiency of the BVDU cotreatment.If cytostatic treatment led to a gain of body weight, BVDU cotreatmentdid not additionally support this effect.

Table 2 BVDU reduced unspecific toxic effects in cytostatic drug-treated rats (mean of the data of six to seven rats)

Treatment	Change of mean area of AH13r rat sarcomas (day 1 = 1) Day 14	Change of mean net body weight Day 14
[illegible]	[illegible]	[illegible]

Interestingly, in AH13r tumors or tumor cells, we could neitherobserve gene amplification or genome-wide changes (data notshown). We used *in situ* CGH (18) and PCR to test for amplificationof genes that are frequently amplified in tumors, *i.e*. ß-actin(control), *Erbb2*, *Gstt1*, *Mdr1*, *c-Myc*, *n-Myc*, and topoisomerase IIa/GST-

1 encoding gene (*Top2a*).

In additional *in vivo* studies, BVDU cotreatment enforced growthretardation of DMBA-induced fibrosarcomas and mammary adenocarcinomasin SD-rats. The DMBA-induced fibrosarcoma growth of controlanimals surpassed the fibrosarcoma growth of the DOX-treatedrats only at the end of the treatment period. In contrast, DOX+BVDU-treatedanimals showed an inhibited tumor growth over the whole time period analyzed (Fig. 3*B*, *panel 1*). When the areas of individualtumors were compared 53 days after treatment, the mean tumorarea of the DOX+BVDU group was significantly smaller than thatof the DOX or control group (Fig. 4*B*, *panel 2*).

We observed similar, but much more pronounced, effects withmammary adenocarcinomas (Fig. 4*B*, *panel 1*). DOX- (6 tumors)or DOX+BVDU-treated animals (9 tumors) showed an inhibited tumorgrowth over the whole treatment period in comparison with thecontrol group (8 tumors). The areas of the individual tumors (Fig. 4*B*, *panel 2*) showed clear differences 39 days after treatmentstart. In 4 of 9 tumors of the DOX+BVDU group, a clear regressionwas observed. The overall tumor area of the DOX+BVDU group wassignificantly smaller than that of the DOX group or of controls.All of the differences were significant at the 5% level (*t* -test/Mann-Whitneytest).

Tumors of rats treated with DOX showed amplification and/oroverexpression of the *Mdr1* gene, whereas tumors of DOX+BVDU-treatedor control rats showed neither amplification nor overexpression(Fig. 4*B*, *panel 3*).

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Discussion

Our results indicated that BVDU cotreatment enhanced chemosensitivity.This might have been because of: (*a*) inhibition of oncogenicand DNA repair-associated enzymes; (*b*) induction of NQO1 activity;(*c*) suppression of chemotherapy-induced *Mdr1* or *Dhfr* gene amplification;or (*d*) inhibition of the overexpression of survival pathwaysand reduced expression of ATP-generating enzymes in the recovery phase.

Three rat tumor models gave evidence that BVDU cotreatment contributedsignificantly to tumor regression *in vivo*.

DDX1, which was down-regulated by BVDU alone, seems to be ofspecial importance. *DDX1* is coamplified with *MYCN* and overexpressedin a subset of neuroblastoma and retinoblastoma cell lines/tumors (20,21) . Preliminary studies have shown that neuroblastomapatients with amplification of both *DDX1* and *MYCN* have a worseprognosis than patients with only the *MYCN* gene amplified (21).Thus, DDX1 seems to have oncogenic potential, and it is predictedto function by RNA binding and modulation of RNA secondary structure.

Of the five genes affected by BVDU cotreatment with MMC, twoare linked to DNA repair. BVDU reduced the expression of UBE2Nand APEX to

30% of control level. The *UBE2N* gene encodes a ubiquitin-conjugatingenzyme, which is thought to be involved in protein degradation.The protein complex containing UBE2N seems to be involved inthe assembly of novel polyubiquitin chains for signaling inDNA repair and, through differential ubiquination of PCNA, affectsresistance to DNA damage (22,23) .

Apurinic sites result from treatment with cytostatic drugs.The resulting abasic sites can block the progress of the DNAreplication apparatus. These sites must be corrected to restoregenetic integrity. Silencing of APEX expression by RNA interferencenearly doubled specific cell lysis, showing enhanced DNA nicking (24).

BVDU induced NQO1. This is in accordance with the observationthat a multifactorial multidrug resistance phenotype of tumorcells involves a decrease and not an increase in NQO1 expression (9) . NQO1 enzyme activity was enhanced in response to BVDUcombinatorial treatment with DOX or MXA, respectively. Hence, it can be speculated that enforced NQO1-mediated bioactivationof DOX and MXA could increase the cytotoxic potential of thesedrugs. On the other hand, MMC, its effects being strongest dependingon NQO1 enzyme activity, showed no enhancement of NQO1 activity.Therefore, the sensitizing effect of BVDU does not seem to beimplicitly NQO1-dependent.

Many of the drugs used in anticancer therapy, such as DOX andMXA (25), perturb the redox state and the mitochondrial respirationof the target cancer cell, which leads to the production ofROS. However, a

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subsequent burst of ROS will indiscriminatelyaffect not only tumor cells but also normal tissue, which causesunwanted systemic side effects during therapy. NQO1 is a scavengerof ROS, and that way, induced NQO1 activity can protect cellsfrom nonspecific ROS and electrophile attack (26). This mayexplain the improved therapeutic outcome against experimentaltumors *in vivo* with no systemic toxicity and gain of body weightin response to BVDU cotreatment, as was observed in our animalmodels.

The first direct evidence that in some tumor cells overexpressionof genes because of amplification gives cells a selective advantagein the presence of a cytostatic drug derives from analysis oftumor cells taken from patients treated with MTX, an inhibitorof the enzyme DHFR (1). Resistance to MTX in human tumors hasin many cases been shown to be associated with amplificationof the gene encoding DHFR (27). Furthermore, expression ofthe *DHFR* gene has been implicated in resistance to a varietyof chemotherapeutic agents and has been detected in human ovarianand colon tumors (28).

In our experiments, treatment with DOX for 50 days caused *Mdr1*gene amplification and overexpression in DMBA-induced rat tumors.Cotreatment with BVDU inhibited this cytostatic drug-inducedeffect. Beyond that, *Dhfr* gene amplification was inhibited in3T6 mouse cells.

A comprehensive effect of BVDU was observed in the recoveryphase. Gene products linked to survival, microfilament formation,differentiation, signal transduction, and ATP generation wereaffected.

BVDU inhibited survival pathways and enforced apoptotic response.BVDU cotreatment might have promoted apoptosis by blocking anantiapoptotic survival pathway involving STAT3 and JUN-D. Additional mechanisms are quite likely, but still under investigation.

Constitutively activated STAT3 is oncogenic (7) and contributesto the development of various human cancers (29) by inhibitingapoptosis (30). Thus, STAT3 promotes cell survival and renderscancer cells resistant to chemotherapy. Accordingly, the inhibitionof STAT3-signaling induces apoptosis specifically in tumor cells,and increases sensitivity to chemotherapeutic agents (7). Alongthis line, it was demonstrated that dominant-negative *Stat3*-expressionsensitizes melanoma cells to FAS-L-induced apoptosis (30). Therefore, STAT3-signaling in human tumors has been proposedas a novel molecular target for therapeutic intervention toreduce resistance of tumor cells to apoptosis (29). JUN-D isan essential and ubiquitously expressed component of the activatingprotein-1 transcription factor complex. *Jun-D*(-/-) primary fibroblasts exhibit premature senescence and increased sensitivity to p53-dependentapoptosis on UV-irradiation or tumor necrosis factor

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treatment (8), which suggests that JUN-D may activate the nuclear factor

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B survival pathway. Moreover, p202, which is directly regulatedby JUN-D, renders fibroblasts more refractory to apoptosis (31).In support of this reasoning, we demonstrated that BVDU cotreatment down-regulated the STAT3 and JUN-D survival pathways, therebylimiting chemoresistance.

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Acknowledgments

We thank Clemens Dasenbrock (Hannover, Germany) for supervisingthe *in vivo* experiments and Volker Rehse (Hannover, Germany)for performing them. Furthermore, we thank Christian Scheler,Proteome Factory (Berlin, Germany) for performance of the twodimensional gel-electrophoresis and protein identification byMALDI-MS.

Footnotes

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1 Supported by the "Sächsische Aufbaubank" (Dresden/Germany)Project 6261, and the Fraunhofer-Society (München/Germany).

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