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Apoptosis Induced by (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine in Varicella Zoster Virus Thymidine Kinase-Expressing Cells Is Driven by Activation of c-Jun/Activator Protein-1 and Fas Ligand/Caspase-8

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ABSTRACT

The molecular mode of cell killing by the antiviral drug (*E*)-5-(2-bromovinyl-2′-deoxyuridine (BVDU) was studied in Chinese hamster ovary (CHO) cells stably transfected with the thymidine kinase gene (*tk*) of varicella zoster virus (CHO-VZV*tk*). The colony-forming ability of the cells was reduced to <1% at a concentration of ~1 μM BVDU, whereas for nontransfected cells or cells transfected with *tk* gene of herpes simplex virus type 1 (CHO-HSV*tk*), a 1000-fold higher dose was required to achieve the same response. BVDU inhibited thymidylate synthase in CHO-VZV*tk* but not in CHO-HSV*tk* and control cells. On the other hand, the drug was incorporated into DNA of VZV*tk*- and HSV*tk*-expressing cells to nearly equal amounts. Because coexposure of CHO-VZV*tk* cells to exogenous thymidine protected them from BVDU-induced cell killing, the cells obviously die because of thymidine depletion. At highly cytotoxic BVDU doses (50 μM) and longer exposure times (24–48 h), VZV*tk* cells were blocked to some extent in S and G2/M phase and underwent apoptosis (48–72 h). Not only apoptosis but also necrosis was induced. The findings also show that the drug causes the induction of c-Jun and the activation of activator protein-1 resulting in increased level of Fas ligand (FasL) and caspase-8/-3 activation. Bid and poly(ADP-ribose) polymerase were cleaved by caspases. Expression of Bax increased, whereas Bcl-2/Bcl-x_L remained unchanged. Transfection of dominant-negative Fas-associated death domain and inhibition of caspase-8 by *N*-benzyloxycarbonyl-IETD-fluoromethyl ketone strongly abrogated BVDU-induced apoptosis, indicating Fas/FasL to be crucially involved. Thus, BVDU-triggered apoptosis differs significantly from that induced by ganciclovir, which induces in the same cellular background the mitochondrial damage pathway.

(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU) is a pyrimidine nucleoside analog exhibiting potent antiviral activity especially against herpes simplex virus (HSV) type 1 and varicella zoster virus (VZV) infections. It is licensed (in Germany) for oral therapy of these virus diseases. Similar to most other antiherpetic drugs, the antiviral activity of BVDU relies on selective monophosphorylation by herpesvirus-encoded thymidine kinases (TKs), whereas cellular thymidine kinases, owing to their much more stringent substrate specificities, do not phosphorylate this nucleoside analog (Cheng et al., 1981). In herpesvirus-infected cells BVDU monophosphate (BVDU-MP) is phosphorylated to the diphosphate by a thymidylate kinase that is associated with certain herpesviral thymidine kinases (e.g., those of HSV-1 and VZV) (Cazaux et al., 1998). The thymidylate kinase function of the viral TK is essential for the antiviral activity of BVDU because the drug is inactive in cells infected with herpesviruses in which TKs lack the thymidylate kinase function (e.g., HSV type 2) (Fyfe, 1982; Mayo, 1982). Finally, BVDU diphosphate

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ABBREVIATIONS: BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; HSV, herpes simplex virus; VZV, varicella zoster virus; TK, thymidine kinase; BVDU-MP, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine monophosphate; BVDU-TP, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine triphosphate; CHO, Chinese hamster ovary; TS, thymidylate synthase; GCV, ganciclovir; ACV, aciclovir; AP-1, activator protein 1; fmk, fluoromethyl ketone; PARP, poly(ADP-ribose) polymerase; AIF, apoptosis inducing factor; ERK2, extracellular signal receptor-regulated kinase; pAb, polyclonal antibody; PI, propidium iodide; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; dThd, deoxythymidine; DN-FADD, dominant-negative-Fas-associated death domain; SCGE, single cell gel electrophoresis; BrdU, 5-bromo-2′-deoxyuridine; CREB/ATF, cAMP response element-binding protein/activating transcription factor; MMS, methyl methansulfonate; zXXXX-fmk, *N*-benzyloxycarbonyl-XXXX-fluoromethyl ketone (where X is an amino acid).

is metabolized to the triphosphate by a cellular nucleoside diphosphate kinase. It is generally accepted that BVDU triphosphate (BVDU-TP) is the ultimate antiviral metabolite of BVDU that inhibits the herpesvirus-encoded DNA polymerase or, upon incorporation of the nucleoside analog triphosphate into the viral DNA, destabilizes the newly synthesized virus DNA (Allaudeen et al., 1981; Mancini et al., 1983; Yokota et al., 1984).

Beside its use as an antiviral agent, BVDU gained considerable interest as a potential prodrug for anticancer gene therapy in combination with transduction of herpesvirus *tk* (HSV*tk*) as a suicide gene because of the observation that low BVDU concentrations may selectively kill HSV*tk*-transfected mammalian cells in vitro (Balzarini et al., 1985, 1994). We tried to reproduce these findings in CHO cells transfected with the *tk* gene of HSV-1 but did not observe dramatic sensitivity differences between HSV*tk*-transfected and nontransfected cells. On the other hand, BVDU was highly cytotoxic in CHO cells transfected with the *tk* gene of VZV, which confirms data obtained in VZV*tk*-transfected human and rodent tumor cell lines (Degrève et al., 1997; Grignet-Debrus et al., 2000). The data indicate that the antiviral and the cytotoxic activity of BVDU relies on two different modes of action of the agent: the inhibition of herpesvirus-encoded DNA polymerases together with diminished stability of BVDU-substituted virus DNA caused by the incorporated BVDU-TP and, on the other hand, the inhibition of thymidylate synthase (TS) caused by BVDU-MP, respectively (Balzarini et al., 1987). The TS is an essential enzyme of the de novo pathway of deoxythymidine monophosphate (thymidylate) synthesis. Herpesvirus TK-expressing cells when treated with BVDU presumably die because of thymidylate depletion. Although such a mode of cytotoxicity is common among certain anticancer drugs (e.g., 5-fluorodeoxyuridine or methotrexate), it is different from that induced by other antiviral nucleoside analogs that are used in combination with suicide gene transduction [e.g., ganciclovir (GCV) and aciclovir (ACV)]. Although several studies have been published regarding the mode of cell killing and genotoxicity caused by purine nucleoside antivirals ACV, GCV, and penciclovir in "metabolically competent" target cells (i.e., in cells expressing herpesvirus-encoded TK) (Rubsam et al., 1998; Beltinger et al., 1999; Thust et al., 2000a,b; Tomicic et al., 2002), the process leading to cell death upon BVDU treatment is essentially unknown. This is an important issue, however, because insights into the molecular mechanism of cell killing by BVDU might be helpful for improvement of suicide gene therapy using the drug in combination with VZV*tk* gene transduction. Here, we report that BVDU induces both necrosis and apoptosis in VZV*tk*-transfected cells. Induction of apoptosis is related to activation of c-Jun/AP-1 and the Fas-triggered pathway. This is essentially different from the induction of apoptosis by GCV in the same cell system expressing HSV*tk*, as recently reported (Tomicic et al., 2002).

Materials and Methods

Reagents and Antibodies. BVDU was a gift of Berlin Chemie (Berlin, Germany). 2′-^{3}H-Labeled BVDU and [5-^{3}H]2′deoxyuridine 5′-monophosphate were purchased from Moravek Biochemicals (Brea, CA). Thymidine (dThd) was from Sigma-Aldrich (München, Germany). Irreversible cell-permeable fluoromethyl ketone (fmk)-modified caspase inhibitors zVAD-fmk, zIETD-fmk, and zDEVD-fmk were products of R & D Systems (Wiesbaden, Germany). Mouse anti-Bcl-2 monoclonal antibody and rabbit anti-Bax, anti-Fas, anti-Fas ligand (FasL), anti-PARP, anti-apoptosis-inducing factor (AIF), anti-cytochrome *c*, anti-Jun, and anti-ERK2 pAb were from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Rabbit anti-Bid and anti-Bcl-x_L pAb were from BD Transduction Laboratories (Heidelberg, Germany). The anti-VZV-TK antiserum was a generous gift of Dr. J. Piette (Liège, Belgium). Horseradish peroxidase-coupled secondary anti-mouse and anti-rabbit IgG antibodies were from Amersham Biosciences Inc. (Freiburg, Germany).

Cell Lines and Transfection Experiments. CHO-9 cells were grown in Ham's F-12/Dulbecco's minimal essential medium (1:1) containing 5% fetal bovine serum and 50 μg/ml gentamicin. HSV*tk* transfectants were generated by cotransfection of CHO-9 cells with the plasmids pMCI-tk harboring the *tk* gene of HSV-1 and pSV2neo as described previously (Thust et al., 2000a). VZV*tk*-transfected clones C1E and C1-9 were generated by electroporation of linearized vector pRc-VZTK (generous gift of Dr. T. Suzutani, Asahikawa, Japan; for description, see Koyano et al., 1996). In brief, ~7.5 μg of DNA (in 30 μl of Tris-EDTA, pH 8.0) were mixed with 750 μl of CHO-9 cells in electroporation buffer (272 mM sucrose, 7 mM sodium phosphate pH 7.4, 1 mM $MgCl_2$) and incubated for 10 min on ice. The mixture was subjected to electroporation (25 μFD, 0.4-kV impulse, Gene Pulser; Bio-Rad, München, Germany) and afterwards incubated for 10 min on ice. A portion of 260 μl of electroporated cell suspension was seeded per 10-cm dish in triplicates and, after 48-h recovery at 37°C, fresh medium was added. The transfected cells were selected with 480 μg/ml G418. G418-resistant clones were picked in 24-well plates and tested upon expansion for sensitivity to increasing concentrations of BVDU. Transfectant clones were routinely cultured in medium containing 1.5 mg/ml G418 (Sigma-Aldrich) that was omitted during the experiments. Cytotoxicity experiments using BVDU in combination with dThd were performed with 10% dialyzed fetal bovine serum. For transient transfection [e.g., with dominant-negative FADD (DN-FADD)], 3×10^5 CHO-VZV*tk* cells were transfected with 1 μg of the test vector using Effectene transfection kit (QIAGEN, Hilden, Germany). After overnight transfection, the fresh medium containing serum and 5 μM BVDU was added to cell culture, and 60 h later CHO-VZV*tk* cells were subjected to Western blot or to flow cytometric analysis to determine apoptosis.

Colony Formation Assay. Cytotoxicity was determined by colony-forming assay. Cells (150) were seeded per well in six-well clusters with 3 ml of complete culture medium per well. Five hours later BVDU was added from a 10-fold stock solution and left with the cells for the duration of the experiment. Colony formation assays using BVDU and 20 μM dThd were performed in the same way as described above (i.e., cells were continuously exposed to both substances). After 1 week the colonies were fixed in methanol and stained with crystal violet. Relative cell survival (plating efficiency) was calculated from the number of colonies per well (at least three wells per condition) in relation to those in untreated controls.

Incorporation of Radioactively Labeled BVDU. A total of 2×10^5 CHO-VZV*tk* or CHO-HSV*tk* cells, respectively, were seeded on 6-cm dishes, grown for 24 h, and subsequently treated for 14 h with 0.1 to 1 μM [2′-^{3}H]BVDU (specific activity, 14.3 Ci/mmol). Alternatively, cells were exposed to 0.1 μM [2′-^{3}H]BVDU for different time periods up to 14 h and harvested by trypsinization. DNA was isolated using the QIAamp blood kit (QIAGEN) according to the manufacturer's protocol. The amount of BVDU incorporated into DNA was determined in a liquid scintillation counter and expressed as counts per minute per microgram of DNA.

Thymidylate Synthase Assay. Inhibition of TS activity by BVDU was measured basically as described by Kawai et al. (1993). In brief, 2×10^5 cells were seeded per well in six-well clusters and 24 h later the cells were treated with 2, 20, or 100 μM BVDU for 3 h. Thereafter, the cells were washed with medium, trypsinized, and collected by centrifugation. Cell pellets were resuspended in buffer

(50 mM Tris-HCl pH 7.5, 10 mM dithiothreitol, 0.1% Triton X-100) and sonified (Branson sonifier; 30 kHz, 3 × 10 s). After centrifugation, supernatants were used in the TS assay. A 50-μl fraction of the cell extract was mixed with 30 μl of reaction mixture (0.15 M Tris-HCl pH 7.5, 0.8% bovine serum albumin, 0.1 mM tetrahydrofolate, 0.05% formaldehyde, 50 mM sodium fluoride, 5 mM dithiothreitol, 0.06 mM deoxyuridine-5′-monophosphate, and 1 μCi of deoxy[5-^{3}H]uridine-5′-monophosphate) and incubated at 37°C for 30 min. Thereafter, 0.5 ml of 2% activated charcoal (Sigma-Aldrich) was added and the mixture was vigorously mixed and incubated at 4°C for 30 min by mixing several times. After 10-min centrifugation (14,000 rpm, 4°C), radioactivity released into an aliquot of supernatant was measured in a liquid scintillation counter. Relative TS activity was expressed as ratio between BVDU-treated cells and the corresponding untreated control.

Determination of Apoptosis and Necrosis. To detect drug-induced apoptosis and necrosis within the same cell population, annexin V/PI double staining combined with flow cytometry was used (Vermes et al., 1995). Exponentially growing cells were chronically treated with different doses of BVDU and analyzed 72 h later. The cells were trypsinized, combined with the floating cells in the supernatant, washed with cold PBS, and subjected to annexin V-FITC according to the manufacturer's instructions (BD PharMingen, Heidelberg, Germany). For detection of internucleosomal fragmentation, DNA was isolated as described previously (Ioannou and Chen, 1996). Briefly, 5 × 10^5 cell were seeded per 10-cm dish and grown for 24 h. Cultures were treated with BVDU for 72 h and harvested by trypsinization. Cells (8 × 10^6) per sample were lysed in a hypotonic solution (5 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.5% Triton X-100). For selective precipitation of genomic DNA, 2.5% polyethylene glycol and 1 M NaCl were used. After phenol-chloroform extraction, nucleic acids in the aqueous phase were precipitated with 2 volumes of ethanol and treated with 0.1 mg/ml RNase for 30 min at 37°C. The samples were loaded onto a 1.5% agarose gel.

Single Cell Gel Electrophoresis (SCGE, Comet Assay). Exponentially growing cells were exposed to different BVDU concentrations and after certain time periods rinsed with ice-cold PBS and trypsinized. Cold medium was added and the cells were centrifuged and washed. The alkaline cell lysis was modified after Singh et al. (1988) and Klaude et al. (1996) as described previously (Tomicic et al., 2001).

Cell Cycle Analysis. To follow progression of cells through the cell cycle, exponentially growing cells were pulse-labeled with 10 μM BrdU for 20 min (pulse-chase experiment) and thereafter the thymidine analog was washed out and cells were nontreated or treated with BVDU. After treatment, cells were fixed, incubated with FITC-coupled anti-BrdU antibody, and stained with PI as described by the manufacturer (BD Biosciences). Alternatively, cells were trypsinized, washed with PBS, and fixed by the addition of 70% ethanol overnight at −20°C. Before flow cytometric analysis, cells were treated with RNase (0.1 mg/ml PBS) and stained with 20 μg/ml PI. In parallel to cell cycle analysis, apoptosis was determined as sub-G_1 fraction. Flow cytometric analysis was performed using CellQuest (BD Biosciences).

Gel Retardation Analysis. Isolation of nuclear protein extracts for gel retardation analysis ("bandshift assay") was performed as described previously (Grösch and Kaina, 1999). The AP-1 oligonucleotides (collagenase AP-1 site) were as follows: 1) 5′-AGTGGTGACTCATCACT-3′ and 2) 5′-AGTGATGAGTCACCACT-3′ as well as the CREB/ATF oligonucleotides with a sequence as follows: 1) 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′ and 2) 5′-CTAGCTCTCTGACGTCAGGCAATCTCT-3′ were annealed and labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (Roche Diagnostics, Mannheim, Germany).

Determination of Caspase Activity. Caspase colorimetric assay (R & D Systems) was used according to the manufacturer's protocol. Briefly, at various postexposure times, cells were trypsinized, counted, and collected by centrifugation (10 min, 1500 rpm, 4°C). The cell pellet was lysed on ice and centrifuged (5 min, 14,000 rpm, 4°C). The supernatant was used for the enzymatic reaction that was carried out in 96-well microtiter plates (405 nm, 1–2 h, 37°C) upon addition of reaction buffer and appropriate caspase substrate supplied with the kit.

Preparation of Cell Extracts and Immunoblotting. Whole-cell extracts were prepared by lysis in ice-cold sample buffer (25 mM Tris-HCl pH 6.8, 5% glycerol, and 2.5% 2-mercaptoethanol; phenylmethylsulfonyl fluoride was added freshly) followed by sonification (Branson sonifier; 30 kHz, 3 × 10 s) on ice. Aliquots of 20 to 30 μg of protein extract were separated by 10 to 12% SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany), and incubated with antibodies (dilution up to 1:500) in 5% nonfat dry milk, 0.2% Tween-PBS. Protein-antibody complexes were visualized by enhanced chemiluminiscence (Amersham Biosciences Inc.). Mitochondrial and cytosolic extracts for expression of AIF and cytochrome *c* were isolated by differential centrifugation as described previously (Tomicic et al., 2002).

Results

CHO Cells Expressing VZV-TK but not HSV-TK Are Hypersensitive to BVDU. To gain stable transfected CHO clones expressing VZV thymidine kinase, cells were transfected by electroporation using the linearized expression plasmid pRc-VZTK (Koyano et al., 1996). To examine the level of expression of VZV-TK in the transfected CHO-VZV*tk* cells (clones C1E and C1-9), Western blot experiments were performed. Both clones expressed VZV-TK protein (~35 kDa), which was not detectable in CHO-HSV*tk* cells serving as negative control (Fig. 1A). A strong band appeared irrespective of the cell type at ~30 kDa, which seems to be an unspecific signal. Expression of HSV-TK protein (~40 kDa) is shown in Fig. 1A, right. CHO cells transfected with VZV*tk* became highly sensitive to BVDU. Upon chronic exposure to BVDU, the colony-forming ability of CHO-VZV*tk* cells (clones C1E and C1-9) was almost abolished at a drug concentration of ~1 μM, compared with 1 mM for nontransfected cells. Thus, VZV*tk*-expressing cells were about 1000-fold more sensitive to BVDU than nontransfected cells. HSV*tk* expressing CHO cells were only slightly more sensitive to BVDU than the nontransfected control (Fig. 1B). For further experiments, if not otherwise indicated, the C1E clone was used, being designated as VZVtk.

BVDU Induces Both Apoptosis and Necrosis. BVDU concentrations that reduced clonogenic survival of CHO-VZV*tk* cells (≥1 μM) were positive in the annexin V assay simultaneously detecting apoptosis and necrosis. No significant increase in apoptosis and/or necrosis within the same dose range of BVDU was found in CHO-9 control and CHO cells expressing HSV*tk*, respectively (data not shown). It is noteworthy that almost equal proportions of necrotic and apoptotic cells were induced in CHO-VZV*tk* cells using different BVDU concentrations (Fig. 2A) and different exposure times (Fig. 2B). Maximum induction was seen after ≥72 h of chronic exposure to BVDU. Moreover, BVDU induced significant internucleosomal fragmentation ("DNA laddering") in CHO-VZV*tk* cells (Fig. 2C). No such effect was detected in CHO-9 and CHO-HSV*tk* (data not shown) cells exposed to BVDU.

Incorporation of BVDU into Genomic DNA of VZVtk and HSVtk Cells. To see whether BVDU becomes incorporated into genomic DNA, dose- and time-dependent incorpo-

ration of radioactively labeled BVDU into DNA of VZV-TK and HSV-TK-expressing cells was determined. The results are shown in Fig. 3. Somewhat more incorporated drug was found in the DNA of CHO-HSV*tk* cells after 14-h exposure to 1 μM BVDU than in CHO-VZV*tk* cells. BVDU incorporation was not detected in nontransfected CHO-9 cells (data not shown).

Analysis of DNA Breakage upon BVDU Exposure. To determine whether treatment with BVDU causes DNA breakage in CHO-VZV*tk* cells, alkaline SCGE experiments were performed. Although BVDU was incorporated, there was no significant increase in the frequency of DNA single-strand breaks in VZV*tk* cells exposed to BVDU either for a short (2-h) or a long (24-h) period, even at the drug concentrations as high as 50 μM (Fig. 4). Similarly, using the neutral SCGE assay, no DNA double-strand breaks were observed (data not shown).

Inhibition of Thymidylate Synthase. In the next step, the extent of TS inhibition in the VZV*tk*-expressing cells was assayed. After 3-h exposure to 2 μM BVDU, TS activity was reduced by ~50% in VZV*tk*-expressing cells. Obviously, BVDU-MP is a very potent inhibitor of TS in VZV*tk* transfectants. Much higher BVDU concentrations were required to inhibit TS in HSV*tk*-expressing cells. Thus, 100 μM BVDU

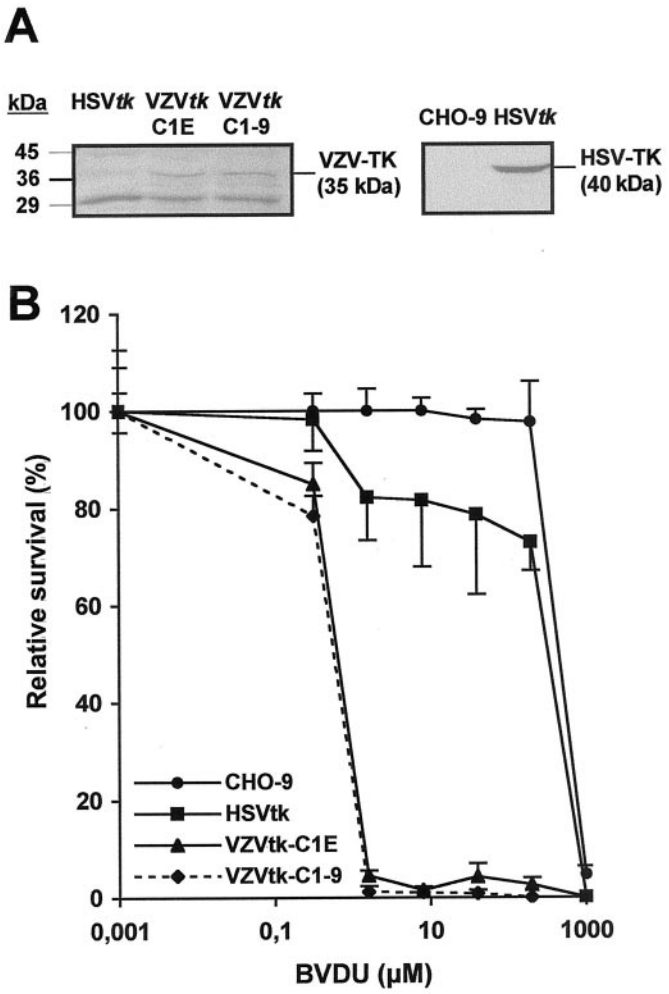

Fig. 1. Expression of viral thymidine kinases and clonogenic survival. A, expression of VZV-TK and HSV-TK in total cell extracts of stable CHO-VZV*tk* transfectants (clones C1E and C1-9) and CHO-HSV*tk* transfectants, as determined by incubation with anti-VZV-TK and anti-HSV-TK serum. Left, a distinct band at ~35 kDa corresponds to VZV-TK. CHO-HSV*tk* transfectants were used as a negative control. A strong band at ~30 kDa is unspecific signal used to show equal protein loading. Right, expression of HSV-TK protein of ~40 kDa. B, relative clonogenic survival as a function of BVDU concentration (plotted logarithmically) after chronic exposure of CHO-9, CHO-HSV*tk*, and CHO-VZV*tk* cells (clones C1E and C1-9) to the drug. The results are the mean of two independent experiments in triplicates ± error bars.

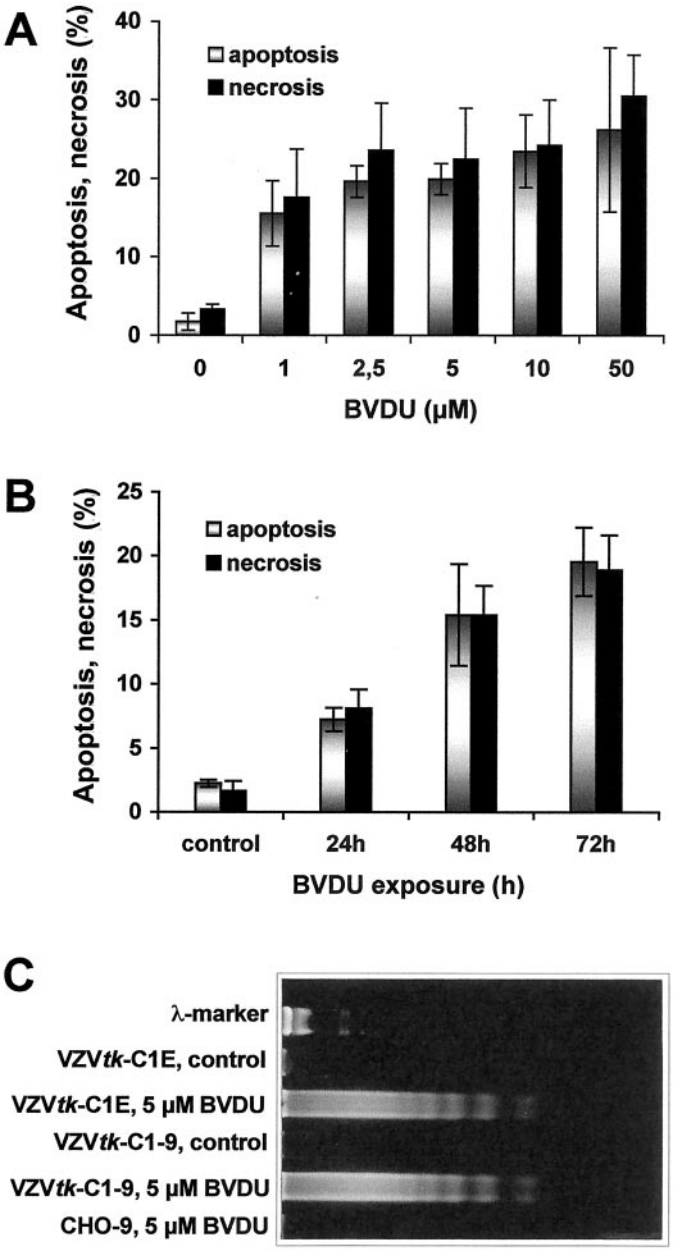

Fig. 2. Induction of apoptosis and necrosis. Apoptosis and necrosis were determined after chronic exposure of CHO-VZV*tk* cells to increasing BVDU concentrations (A) and after different exposure periods (B), as determined by flow cytometry using annexin V-FITC/PI staining. Apoptotic cells, annexin V-FITC (FL1); necrotic cells, annexin V-FITC + PI (FL1 versus FL3). The dot plots were analyzed using quadrant statistics: lower left quadrant, viable cells; lower right quadrant, apoptotic cells; upper right quadrant, necrotic cells. Cells were clearly separated in the three populations. C, nuclear DNA fragmentation. CHO-VZV*tk* cells (clones C1E and C1-9) and CHO-9 cells were not treated or chronically exposed to 5 μM BVDU; internucleosomal DNA fragmentation (DNA laddering pattern) was determined as described under *Materials and Methods*. λ-Marker was used as a determinant of DNA length.

caused only a slight decrease of TS activity. No TS inhibition was observed in CHO-9 controls at any BVDU concentration tested (Fig. 5A). Inhibition of TS by BVDU is expected to cause depletion of dThd that might be involved in BVDU-induced cell killing of VZV*tk* cells. To examine this, cells were cultivated in medium containing dialyzed (dThd-free) serum. The VZV*tk* cells were sensitized to dThd depletion, as shown in clonogenic survival after continuous treatment with BVDU (without exogenous dThd), whereas addition of 20 μM dThd significantly protected cells from BVDU-induced cell killing in concentrations of up to 1.5 μM BVDU (Fig. 5B). Similar results were obtained with the second clone (data not shown).

Cell Cycle Distribution. To elucidate whether transient thymidylate depletion caused by inhibition of TS activity in CHO-VZV*tk* cells in turn causes cell cycle alterations, a BrdU pulse-chase experiment was conducted. It revealed that during the BVDU exposure period from 3 to 9 h both HSV*tk*- and VZV*tk*-expressing cells progress through the cell cycle without significant delay, even at a highly toxic concentration of 50 μM BVDU (Fig. 6A). With respect to longer BVDU exposure periods (24–72 h) to 50 μM BVDU, cells were blocked in S phase (24-h exposure) and G2/M (48-h exposure) as shown in histograms of the cell cycle progression (Fig. 6B). Apoptotic cells are visible as sub-G_1 fraction. Later ($\geq$48 h), cells started undergoing apoptosis. With lower BVDU concentrations (5 μM), being used to monitor all molecular events of apoptosis, only a slight S-phase arrest in VZV*tk* cells after a 24-h treatment was observed (data not shown). Interestingly, HSV*tk* cells accumulated in G2 without any delay in S phase during the treatment with 50 μM BVDU.

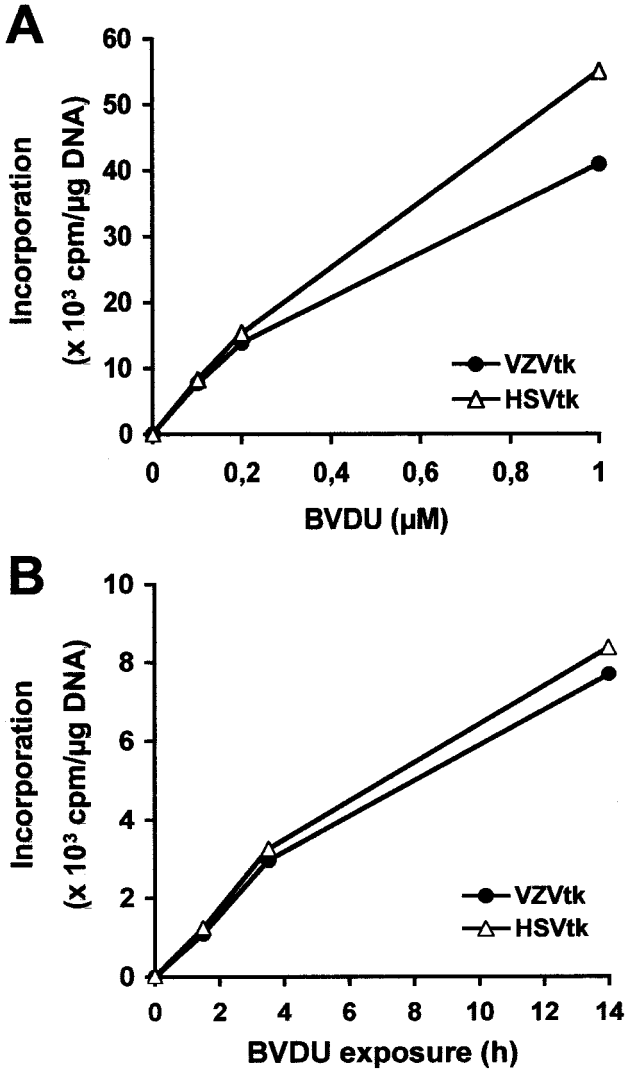

Fig. 3. BVDU incorporation. A, metabolically competent CHO-HSV*tk* and CHO-VZV*tk* cells were exposed to increasing concentrations of radioactively labeled [^{3}H]BVDU for one cell cycle (14 h) and radioactivity (counts per minute per microgram of DNA) in the genomic DNA was determined by scintillation counting. B, incorporation kinetics of 0.1 μM [^{3}H]BVDU into the genomic DNA of CHO-HSV*tk* and CHO-VZV*tk* cells. Isolation of DNA and determination of radioactivity was made as described under *Materials and Methods*.

Induction of AP-1 and Fas Ligand. Inhibition of TS activity was already observed 3 h after addition of BVDU. To see whether this cellular stress causes the activation of immediate early genes that respond to genotoxic insults and are supposed to be involved in the initiation of apoptosis, we analyzed the level of transcription factor AP-1 in the gel retardation assay. As shown in Fig. 7A, in nuclear extracts of CHO-VZV*tk* cells, AP-1 was induced 3 h after exposure to BVDU reaching the maximum 6 h later. After longer BVDU exposure, the AP-1 level slowly decreased. As a positive control, MMS-treated cells were used showing clear AP-1 induction. Competition experiment with nonlabeled AP-1 and an unspecific CREB/ATF oligonucleotide demonstrated the specificity of the AP-1 complex (Fig. 7A, right). Next, we determined the expression of c-Jun protein, which is a constitutive part of AP-1 (Chiu et al., 1988). Indeed, c-Jun was clearly induced 3 to 9 h after BVDU exposure (Fig. 7B).

Previously, it was shown that activation of AP-1 is a crucial component in apoptosis of cells responding by activation of the FasL (Kasibhatla et al., 1998). Therefore, we determined the FasL level in extracts of BVDU-treated CHO-VZV*tk* cells. As shown in Fig. 7C, FasL was enhanced in expression as determined 9 to 28 h after BVDU exposure, which indicates BVDU to activate the Fas-triggered apoptotic pathway.

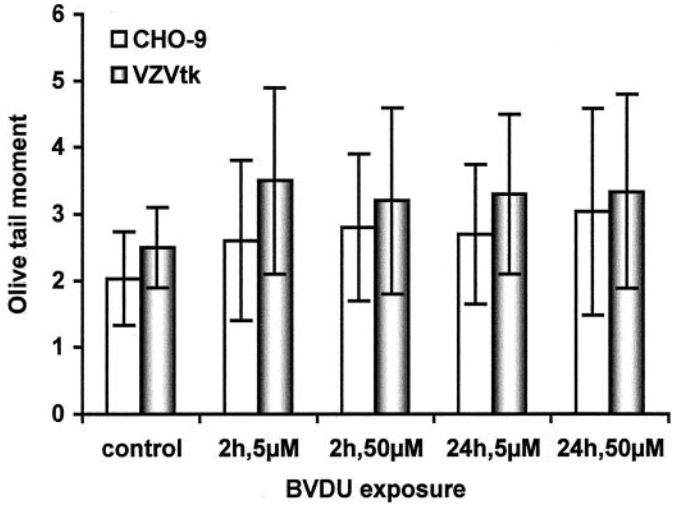

Fig. 4. Induction of DNA strand breaks. DNA strand breaks were determined by alkaline SCGE (comet assay) after exposure of CHO-9 and CHO-VZV*tk* cells to BVDU. Analysis of DNA migration (related to the induction of DNA strand breaks) was performed by image analysis system (Kinetic Imaging Ltd.; Komet 4.0.2.; Optilas, Puchheim, Germany), using Olive tail moment as defined standard. The means of three independent experiments $\pm$ S.D. are shown.

Caspase Activation. Next, we analyzed caspase activity in CHO-VZV*tk* cell extracts after BVDU exposure. As shown in Fig. 8A, the executive caspase-3 was the most activated caspase 24 h after exposure to BVDU, reaching a 4-fold maximum of induction 48 to 72 h after exposure. To examine the involvement of initiator caspases, the activities of caspase-8 and -9 were determined. Caspase-8 was activated in parallel to caspase-3. On the other hand, caspase-9 was not significantly activated during the whole period after exposure.

To elucidate the importance of caspase activation in BVDU-induced apoptosis, we conducted experiments with caspase inhibitors. As shown in Fig. 8B, a broad-spectrum caspase inhibitor (zVAD-fmk) abrogated apoptosis by ~70%. The caspase-8 inhibitor (zIETD-fmk) blocked apoptosis by ~60%, whereas the inhibitor of caspase-3-like caspases (-3 and -7) reduced BVDU-induced apoptosis by ~30%. In control experiments, the inhibitors proved to be active against the corresponding caspases (Tomicic et al., 2002). The data indicate that caspase-3 is indeed a downstream executive caspase of BVDU-triggered apoptosis whose activation is regulated by caspase-8. The caspase inhibitors used had no effect on BVDU-induced necrosis (Fig. 8B).

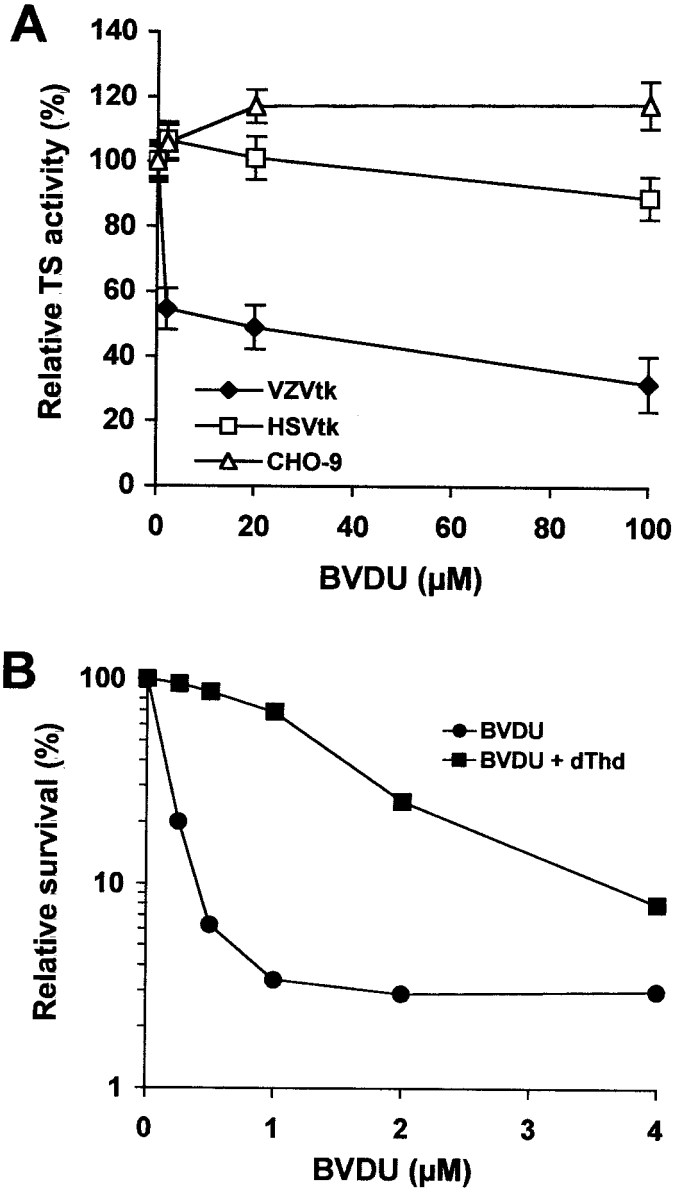

Fig. 5. Inhibition of TS activity and clonogenic survival after coexposure of VZV*tk* cells with thymidine. A, TS inhibition. CHO-9, CHO-HSV*tk*, and CHO-VZV*tk* cells were exposed to increasing BVDU concentrations for 3 h, and thereafter crude extracts were prepared and assayed for TS enzymatic activity as described under *Materials and Methods*. B, clonogenic survival. The VZVtk cells (clone C1E) treated with increasing BVDU concentrations in medium with dialyzed serum in the absence or presence of exogenous 20 μM dThd. The experiment was done in duplicate.

Overexpression of Dominant-Negative FADD. To further substantiate the role of Fas/FasL-triggered pathway in BVDU-induced apoptosis, we transiently overexpressed DN-FADD in CHO-VZV*tk* cells. As shown in Fig. 8C, expression of DN-FADD resulted in a significant reduction of BVDU-induced apoptosis. This indicates that caspase-8 was indeed activated by BVDU-triggered induction of FasL stimulating the Fas receptor.

Expression and Cleavage of Apoptotic Proteins. The intact PARP protein (p113) was cleaved into characteristic fragments, as shown 24 to 72 h after exposure to BVDU. The cleavage occurred in parallel to the activation of caspase-3 (Fig. 9A) that was shown to cleave PARP as one of its downstream targets. The intact Bid protein (p22) was cleaved into the fragments of 15 and 6.5 kDa, of which only the larger fragment (p15) was identified (Fig. 9B). Cleavage occurred 36 h after drug exposure, which followed caspase-8 activation, substantiating the previously published data on caspase-8-mediated cleavage of Bid (Chou et al., 1999).

Recently, we have shown that caspase-9-driven cleavage of Bcl-2 is a hallmark of GCV-induced apoptosis in HSV*tk*-expressing CHO cells (Tomicic et al., 2002). During the course of BVDU-induced apoptosis, however, the Bcl-2 cleavage product (23-kDa fragment) was not detected. In fact, there was no change in expression of either Bcl-2 or Bcl-x_L. On the other hand, a 4-fold induction of the proapoptotic Bax protein was determined at 72 h after treatment with the drug (Fig. 9C). Expression of Fas remained unchanged, whereas FasL (here determined in the total cell extract) was induced at 24 h after exposure to BVDU (shown in Fig. 9D), substantiating the data presented above (Fig. 7C). A very late (96 h after exposure) and significant release of AIF from mitochondria into the cytosol was observed (Fig. 9E). At the same time, however, only an insignificant release of mitochondrial cytochrome *c* was detected, implying that most of the mitochondria were still intact at that stage. This indicates that BVDU-triggered mitochondria-mediated apoptosis in CHO-VZV*tk* cells is only a secondary phenomenon.

Discussion

With regard to chemical structure and the potential mode of action, BVDU is rather uncommon among the nucleoside analog antiviral drugs. Although the strongly modified "sugar" moieties of other antiherpes or anti-HIV drugs either cause severe distortions of DNA structure or chain termination upon incorporation into genomic DNA, the deoxyribose of BVDU is entirely unchanged and, therefore, enables the formation of regular phosphodiester linkages in the DNA backbone. The only structural distinction between thymidine and BVDU is the substitution of the 2-methyl group of thymidine by a bromovinyl substituent in the latter compound.

This group, however, does not participate in the formation of hydrogen bonds within the double helix and thus it remains elusive whether and how BVDU incorporation alters the structure and function of DNA. To our knowledge, no investigations on the physicochemical properties of BVDU-substituted DNA have been published. The BVDU metabolite that most probably causes the cytotoxic effects of this drug in metabolically competent target cells is not BVDU-TP but BVDU-MP, which is a potent thymidylate synthase inhibitor causing nucleotide pool imbalance because of thymidine depletion. In this respect, the mechanism of BVDU action crucially differs from that of other antiviral nucleosides, such as

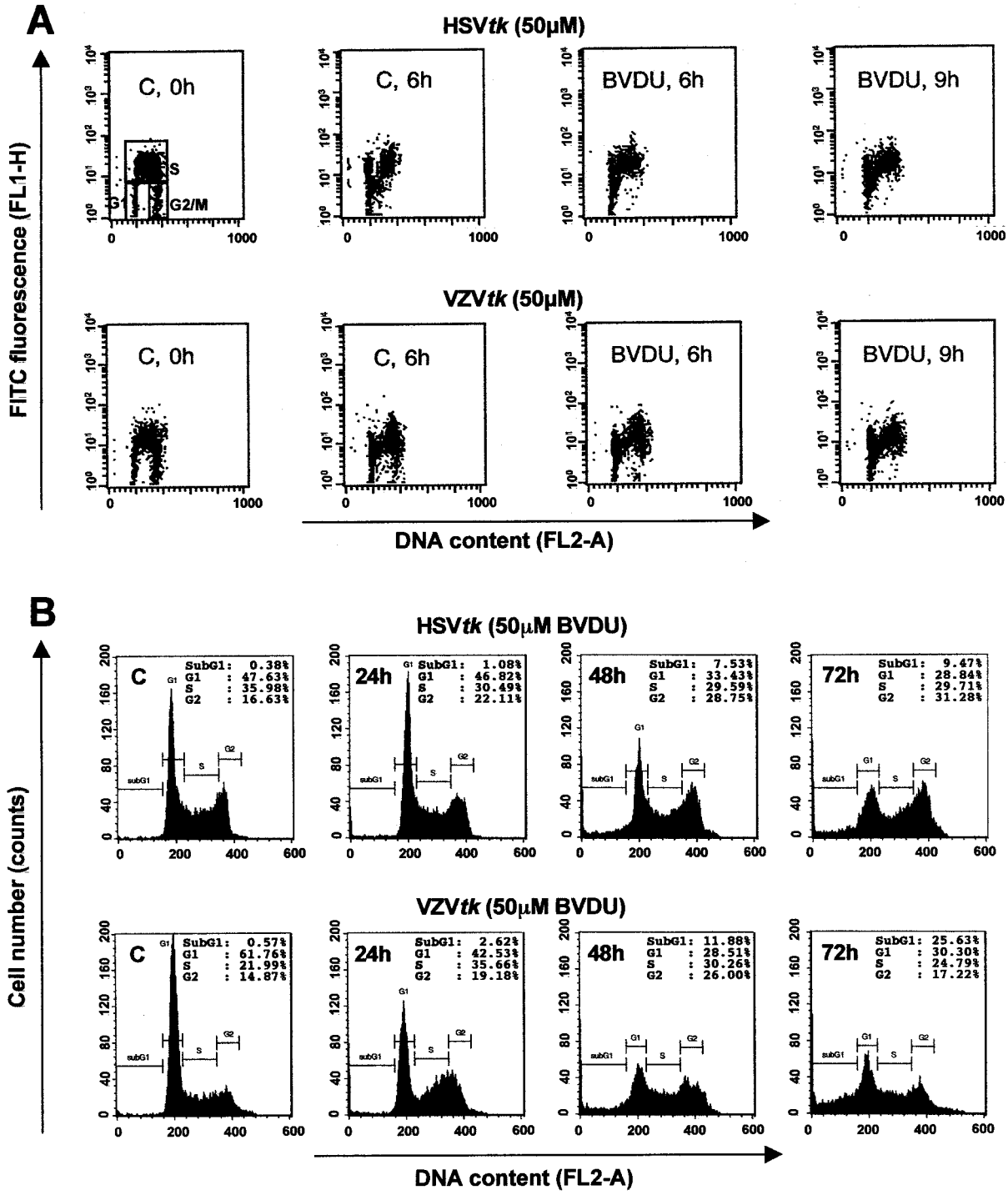

Fig. 6. Cell cycle analysis. A, progression of BrdU-labeled S-phase cells through the cell cycle after BVDU exposure (pulse-chase experiment). Exponentially growing CHO-HSV*tk* and CHO-VZV*tk* cells were pulse-labeled with 10 μM BrdU for 20 min and thereafter immediately treated with 50 μM BVDU. C, 0 h, untreated control, directly prepared after 20-min pulse treatment; C, 6 h, untreated control, prepared 6 h after pulse treatment; BVDU, 6 h/BVDU, 9 h, BVDU-treated cells (for 6 or 9 h) directly after the pulse treatment. Single cell fractions (G1, S, and G2/M) are gated in the control of CHO-HSV*tk* cells. In the two-parameter dot plot diagram, FITC fluorescence (BrdU-positive cells) was plotted against DNA content (PI staining). B, progression of cells through the cell cycle, as determined by flow cytometry. Cells were exposed to 50 μM BVDU for 24 to 72 h. Before analysis, cells were stained with PI. Sub-G_1 fraction, apoptotic cells; C, untreated exponentially growing control cells.

GCV and ACV. These agents need to be metabolized to the corresponding triphosphates, which, upon incorporation into DNA, impair the function of the replication complex or activate DNA repair machinery (Thust et al., 2000a,b; Tomicic et al., 2001). TS inhibition, on the other hand, leads to dTTP deprivation and, consequently, accumulation of dUTP that is misincorporated into DNA. Thereafter, uracil in DNA is rapidly excised by uracil-DNA glycosylase leaving an apyrimidinic site that may lead to DNA strand breaks (Curtin et al., 1991; Van Triest et al., 2000). Considering these well known consequences of TS inhibition, it was surprising that both neutral and alkaline SCGE (comet assay), which detects DNA single- and double-strand breaks, was negative after BVDU treatment of CHO-VZV*tk* cells. Similarly, BVDU (in contrast to GCV) was just a weak clastogen and a marginal inducer of sister-chromatid exchanges in CHO-VZV*tk* cells even in the cytotoxic dose range (R. Thust and B. Kaina, unpublished observations).

The findings presented here show that both HSV*tk*- and VZV*tk*-expressing cells incorporate almost equal amounts of BVDU into the genomic DNA, thus reflecting similar BVDU-metabolizing enzyme activities in these clones. However, only CHO-VZV*tk* cells were BVDU hypersensitive, whereas CHO-HSV*tk* cells responded almost in the same way as non-transfected CHO-9 cells. This is in striking contrast to the findings of Balzarini et al. (1985, 1994) reporting a ~3000 times lower BVDU IC_{50} value in HSV*tk*-transfected murine mammary carcinoma FM3A cells compared with the non-transfected cells. One reason for this discrepancy might be that the FM3A cells were deficient for the cytosolic thymidine kinase (TK1) what per se could cause a permanent thymidylate stress, whereas our CHO cells are TK1-proficient. On the other hand, a human breast cancer line and a rat glioblastoma cell line, both of which were TK1-proficient, also became BVDU-hypersensitive upon transfection with the HSV*tk* gene (Grignet-Debrus et al., 2000). So far, we have no pertinent explanation for CHO-HSV*tk* cells to be refractory to the cytotoxic action of BVDU in spite of their capacity to metabolize this nucleoside analog. Nevertheless, from our results it is obvious that BVDU-TP incorporation per se is not responsible for the cytotoxic action of this drug. The cells die from lack of dThd. This was substantiated by experiments with exogenous dThd that protected cells against BVDU-induced loss of colony formation. Together, it seems that the regulation of TS activity is much more complex than hitherto anticipated. As shown in studies with fluoropyrimidines, TS activity can be enhanced by gene amplification. Also, TS shows variable intracellular half-life and its activity depends on the presence of reduced folate cofactors (Pritchard et al., 1997; Kitchens et al., 1999). Although the insensitivity of the HSV*tk*-transfected CHO clones is in line with the poor capacity of BVDU to inhibit TS activity in these cells, TS was strongly inhibited by BVDU in FM3A cells transfected with HSV*tk* (Balzarini et al., 1987). Recently, TS inhibition, drug incorporation and cytotoxicity were studied upon BVDU treatment in a series of HSV*tk*-expressing human and rodent tumor cell lines (Hamel et al., 2001). Although similar TS inhibition and BVDU incorporation rates were observed in all cell lines, some of them were resistant to BVDU. Therefore, it seems to be difficult to explain BVDU-induced cell killing solely on the basis of TS inhibition and/or drug incorporation.

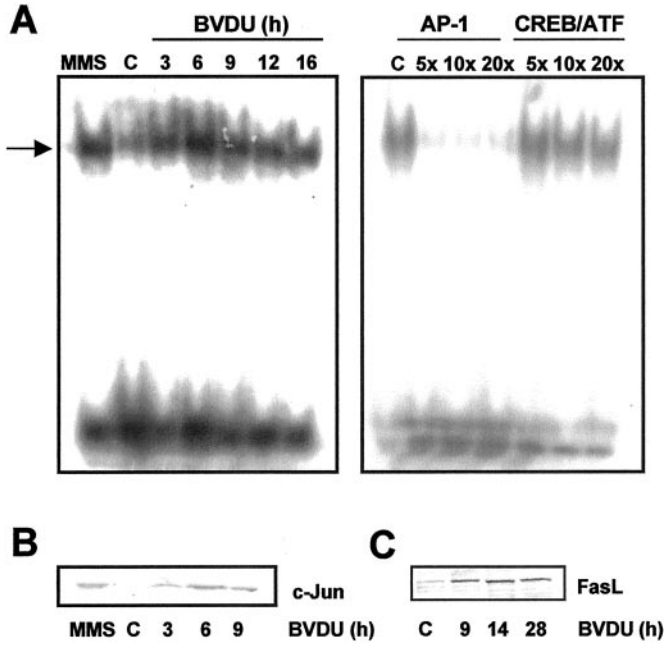

Fig. 7. Induction of AP-1, c-Jun, and FasL. A, For AP-1 bandshift analysis CHO-VZV*tk* cells were treated with 1 μM BVDU, nuclear extracts were isolated and incubated with specific AP-1 oligonucleotide (left). Positive control (designated MMS): mouse fibroblasts prepared 6 h after 1-h treatment with 2 mM MMS. The specific binding complex is indicated with an arrow; the lower band represents nonbound oligonucleotides. Competition experiments of radioactively labeled AP-1 were performed with different molar excess of nonlabeled AP-1 and (unspecific) CREB/ATF oligonucleotides (right). B, expression of c-Jun after exposure of CHO-VZV*tk* cells to 1 μM BVDU, as determined by Western blot analysis with anti-c-Jun pAb. C, induction of FasL in cytoplasmic extracts after exposure to 5 μM BVDU, as determined with anti-FasL monoclonal antibody.

It was recently shown that some VZV*tk*- or HSV*tk*-transfected human cancer cell lines undergo apoptosis after exposure to BVDU (Grignet-Debrus et al., 2000). However, because only the internucleosomal fragmentation assay was applied, necrotic cells remained undetected. Thus, up-to-date evidence as to the predominant way of cell death in VZV*tk*-expressing cells after BVDU exposure has not yet been provided. It was therefore interesting to find out that CHO-VZV*tk* cells undergo both apoptosis and necrosis (with similar frequencies) upon exposure to BVDU. In this respect BVDU-triggered cytotoxicity in CHO-VZV*tk* cells strikingly differs from that induced by the purine analogs ACV, GCV, and penciclovir in CHO-HSV*tk* cells. These agents induce mostly apoptosis, whereas the frequency of necrosis remains rather low over a wide range of drug concentrations and postexposure periods tested (Thust et al., 2000a; Tomicic et al., 2002).

The molecular mechanism of BVDU-induced cell killing has not been elucidated before. Our results demonstrate that BVDU-induced apoptosis is characterized by early and long-lasting activation of c-Jun and AP-1. Also, BVDU triggers in the same time period up-regulation of FasL, whereas Fas remains unchanged in expression. FasL expression is regulated via AP-1 (Kasibhatla et al., 1998), which provides an explanation for their concomitant induction. A possible explanation for the lack of Fas induction could lie in the fact that CHO cells harbor mutated p53 protein (Hu et al., 1999) that cannot fulfill its role as a transcription factor in activat-

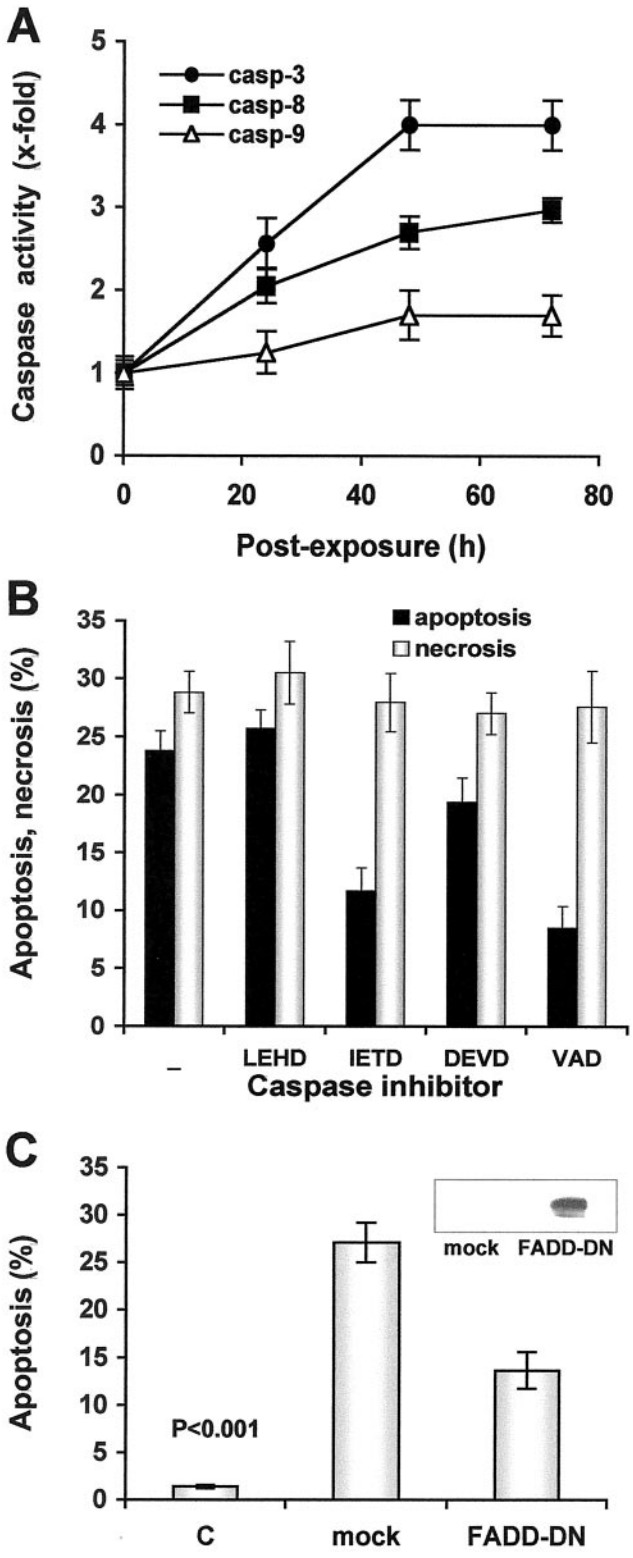

Fig. 8. Caspase activation and overexpression of DN-FADD. A, relative caspase activity in CHO-VZV*tk* cells exposed for 14 h to 5 μM BVDU, as determined at different postexposure times by caspase activity assay. The means of three independent experiments ± S.D. are shown. B, modulation of apoptosis by caspase inhibitors. CHO-VZV*tk* cells were preincubated for 10 h with caspase inhibitors (80 μM) and then coincubated with 5 μM BVDU for additional 60 h. The inhibitor concentration was adjusted in preliminary experiments, specifically depleting caspase activities by ~90% and having no undesired cross-inhibitory effects on other caspases. LEHD, IETD, DEVD, and VAD are specific caspase-9, caspase-8, caspase-3/-7, and a broad-spectrum caspase inhibitor, respectively. Apoptosis was determined using annexin V-FITC flow cytometry. The means of three independent experiments ± S.D. are shown. C, modulation of BVDU-induced apoptosis by overexpression of DN-FADD. CHO-VZV*tk* cells were either mock (pcDNA3)- or DN-FADD-transfected, as shown by Western blot analysis in the upper right of the figure. Apoptosis was determined by annexin V-FITC flow cytometry. The mean of three experiments ± S.D. ($P < 0.001$) is shown.

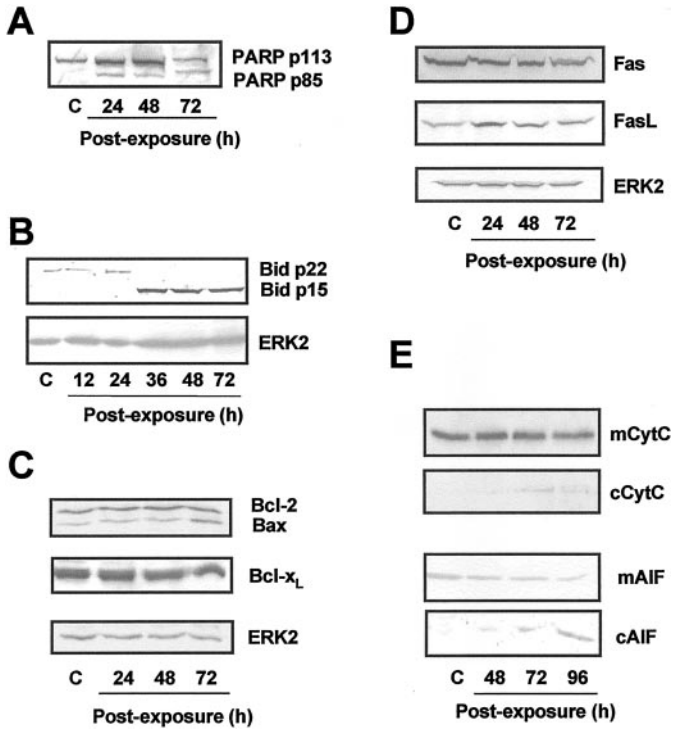

Fig. 9. Expression of apoptotic proteins. A, time course of PARP cleavage in total cell extracts after exposure of CHO-VZV*tk* cells to 5 μM BVDU, as revealed by Western blot analysis. The membrane was stained with Ponceau's as loading control. B, cleavage of Bid, as determined by Western blot analysis with anti-Bid pAb. C, expression of Bax, Bcl-2, and Bcl-x_L, as determined by Western blot analysis. ERK2 shows equal quantities of the proteins on the membrane. D, expression of Fas and FasL in total extracts of CHO-VZV*tk* cells after exposure to 5 μM BVDU, as determined by Western blot analysis. Membranes were incubated with anti-ERK2 pAb as internal loading control. E, release of cytochrome *c* and AIF from mitochondria (mCytC, mAIF) into the cytosol (cCytC, cAIF) at different postexposure times after treatment with 5 μM BVDU, as revealed by incubation of mitochondrial and cytosolic extracts with anti-cytochrome *c* and anti-AIF pAb.

ing the *fas* gene (Muller et al., 1998). Obviously, CHO-VZV*tk* cells undergo apoptosis upon BVDU via Fas in a p53-independent way. We would like to indicate that BVDU parallels to a certain degree the effect of 5-fluorouracil, another TS inhibitor. With this anticancer drug induction of Fas was shown to be involved in cytotoxicity, although, in a p53-dependent manner (Petak et al., 2000; Eichhorst et al., 2001). As already mentioned, FasL is induced and its interaction with Fas is responsible for activation of caspase-8. Fas/caspase-8 activation as a main route of BVDU-induced apoptosis was proven by overexpression of DN-FADD. FADD is a death domain-harboring adaptor molecule that docks to Fas, thus triggering activation of caspase-8 (Chinnaiyan et al., 1995). As expected, transfection with DN-FADD significantly prevented cells from undergoing apoptosis. Involvement of Fas/caspase-8 was further confirmed by use of a specific caspase-8 inhibitor (zIETD-fmk) that abrogated ap-

optosis by ~60%. Therefore, caspase-8 seems to be an initiator caspase that activates procaspase-3 after BVDU exposure. In fact, caspase-3 activation occurred concomitantly with activation of caspase-8. The upstream caspase-9, generally involved in the mitochondrial damage pathway, was only marginally activated. This agrees with the finding that cytochrome *c*, required for activation of caspase-9 (Li et al., 1997), was hardly released from mitochondria upon BVDU treatment. Taken together, the data indicate that BVDU does not induce severe damage to mitochondria, thus not leading to significant cytochrome *c* release and subsequent caspase-9 activation. However, a small quantity of AIF was released from mitochondria 96 h after BVDU exposure.

We also observed cleavage of Bid protein (p22). The activated truncated form of Bid (p15) was shown to recruit cytosolically localized Bax to the outer mitochondrial membrane where it can form pores or channels (Gross et al., 1998). In this way, release of proapoptotic mitochondrial proteins such as cytochrome *c* (Li et al., 1997) and AIF (Susin et al., 1999) is facilitated. Interestingly, the proapoptotic Bax protein was significantly increased in expression 72 h after exposure to the drug, probably because of the stabilization of the protein. The antiapoptotic proteins Bcl-2 and Bcl-x_L remained unchanged in expression after exposure to BVDU. Thus, we assume that changes in the Bcl-2/Bax or Bcl-x_L/Bax ratio are involved in the very late step of apoptosis in a p53-independent manner.

Previously, we reported that GCV induces apoptosis in HSV*tk*-transfected cells by activating the mitochondrial damage pathway, with caspase-mediated cleavage of Bcl-2 to be a hallmark of the response (Tomicic et al., 2002). A significant activation of the Fas/FasL pathway upon GCV treatment was not observed, although the cellular background was essentially the same (CHO-9 cells). Apoptosis was related to incorporation of GCV into DNA causing DNA breaks that were supposed to act as the ultimate trigger of Bcl-2 cleavage (Tomicic et al., 2002). A similar conclusion was gained on the basis of studies with DNA-damaging agents such as UV-C light and alkylating drugs (Ochs and Kaina, 2000; Dunkern and Kaina, 2001). BVDU does not induce significant DNA breakage and replication blockage. Obviously, the agent acts in a different and unique way by activating the AP-1/FasL/caspase-8 pathway. It will be most interesting to find out why some agents have the ability to activate Fas, whereas others preferentially activate the mitochondrial damage pathway without the involvement of Fas. Further studies on the molecular mechanism of BVDU-induced cytotoxicity will clarify this question and give additional answers regarding its suitability as a prodrug in anticancer suicide gene therapy.

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