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The Multifunctional Deoxynucleoside Kinase of Insect Cells Is a Target for the Development of New Insecticides

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ABSTRACT

The antiherpetic agent (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) was found to be an efficient substrate for recombinant *Drosophila melanogaster*-deoxyribonucleoside kinase with a K_m of 4.5 μM and a V_{max} of 400 nmol/μg protein/h compared with 1.3 μM and 62.5 nmol/μg protein/h, respectively, for the natural substrate thymidine. Mammalian cytosolic thymidine kinase-1 does not recognize BVDU as a substrate. In sharp contrast to mammalian cells, the insect *D. melanogaster* and *Spodoptera frugiperda* (Sf) embryonic cells proved highly sensitive to the cytostatic action of BVDU. BVDU was efficiently metabolized to its 5′-mono-, 5′-di- and 5′-triphosphate derivatives in the insect cell cultures and abundantly incorporated into the insect cell DNA. BVDU prevented the *D. melanogaster* cells to initiate the S phase of their cell cycle, and exposure of *S. frugiperda* cells to BVDU led to a dose-dependent retardation of the insect cells in the S phase of their cell cycle. Both inhibition of nucleic acid synthesis (through the 5′-triphosphate of BVDU) and inhibition of thymidylate synthase (through the 5′-monophosphate of BVDU) would account for the cytostatic activity of BVDU against the insect cells. Because of the virtual lack of cytotoxicity of BVDU against mammalian cells, the drug should be considered highly selective in its cytostatic action against the insect cells. When added to the food of *S. frugiperda* larvae, BVDU caused a remarkable decrease in the weight gain of the larvae and heavily compromised the transformation of the larvae to the pupae and their subsequent adult (moth) phase. Our data indicate that insect multifunctional deoxyribonucleoside kinase should be considered an entirely novel and attractive target in the development of new nucleoside types of highly selective insecticidal drugs.

Recently, a multifunctional deoxyribonucleoside kinase (dNK) was purified from *Drosophila melanogaster* (Dm) embryonic (*S*-2) cell cultures and its kinetic properties were characterized (Munch-Peterson et al., 1998a,b). The Dm-dNK showed a broad nucleoside substrate specificity with varying efficiency. The K_m values for dThd, dCyd, dAdo, and dGuo were 0.9, 1.0, 109, and 654 μM, respectively, and the V_{max} values for the different deoxynucleosides varied only by <2-fold (Munch-Peterson et al., 1998a,b). The recognition of the four different nucleosides by one and the same enzyme is unprecedented and differs markedly from the situation in mammalian cells. Mammalian cytosolic thymidine kinase (TK) is only able to phosphorylate dThd (and dUrd) as the natural substrate (Kit, 1976), whereas mitochondrial TK-2 can phosphorylate both dThd (and dUrd) and dCyd, albeit with a preference for dThd (Kit, 1976; Johansson and Karlsson, 1997). The mammalian dCyd kinase does not recognize dThd as a substrate, but in addition to dCyd, it can also convert dAdo and dGuo to their 5′-monophosphate derivatives (Johansson and Karlsson, 1995). Finally, mitochondrial dGuo kinase recognizes both dGuo and dAdo as a substrate (Johansson and Karlsson, 1996). The cDNA cloning of Dm-dNK revealed a sequence similarity to mammalian dCK, dGK, and TK-2 as well as to the herpes simplex virus (HSV) TK (Johansson et al., 1999). Munch-Petersen and colleagues revealed that the substrate specificity, size, and other characteristics of Dm-dNK is more related to TK-2 than to any of the other mammalian dNKs (Munch-Petersen et al., 1998a,b) and that the highest level of sequence similarity of Dm-dNK cDNA was with TK-2 (Johansson et al., 1999).

TK-2 shares several kinetic properties with herpetic TKs (including the pronounced substrate specificity for both dCyd and dThd) (Gentry et al., 1983), and tumor cells transfected with the gene encoding for the HSV TK becomes exquisitely sensitive to the cytostatic activity of a number of antiherpetic drugs (Balzarini et al., 1985, 1989). The sequence of Dm-dNK

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ABBREVIATIONS: dNK, deoxyribonucleoside kinase; Dm, *Drosophila melanogaster*; Sf, *Spodoptera frugiperda*; TK, thymidine kinase; BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; BVDU-MP, 5′-monophosphate BVDU; BVDU-MP, 5′-diphosphate BVDU; BVDU-TP, 5′-triphosphate BVDU; dThd, thymidine; dTMP, thymidine-5′-monophosphate; dTTP, thymidine-5′-triphosphate; TS, thymidylate synthase; HSV, herpes simplex virus; VZV, varicella-zoster virus; AZTMP, azidothymidine monophosphate; AZTDB, azidothymidine diphosphate.

also showed that the enzyme is closely related to HSV TKs. Therefore, we decided to evaluate a variety of antiherpetic nucleoside analogues for their affinity for Dm-dNK and wanted to investigate whether Dm-dNK might be a useful target enzyme for the metabolic activation of antiherpetic compounds resulting in a potential cytostatic activity against insect cells. We found that the potent antiherpetic compound (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) (De Clercq et al., 1979) was not only an excellent substrate for Dm-dNK, but that it also seriously retarded or arrested the insect cell cycle progression and insect cell DNA synthesis, resulting in a pronounced selective cytostatic activity against the insect cells compared with mammalian cells. Our data revealed that Dm-dNK is a new, attractive target for the development of insecticidal drugs that are selectively cytostatic to insect, but not mammalian, cells.

Materials and Methods

Cells. Embryonic cells from the insects *D. melanogaster S*-2 and *S. frugiperda* (Sf-9) were cultivated in plastic 75 cm^2 culture bottles (Iwaki, International Medical, Brussels, Belgium) at 26°C. *S*-2 cells were seeded in insect cell medium consisting of Schneider's *D. melanogaster* powder medium (Serva, Boehringer Ingelheim, Heidelberg, Germany) (24.06 g, $CaCl_2$ 0.6 g, $NaHCO_3$ 0.4 g in 100 ml aqua distillata), adjusted to pH 6.5 with NaOH, and containing 10% inactivated (56°C, 30 min) fetal calf serum and 0.15% penicillin/streptomycin solution (final solution). Sf-9 cells were cultured in TC 100 basal medium (Life Technologies, Paisley, Scotland) containing 10% heat-inactivated fetal calf serum, 240 nM $ZnSO_4.7\ H_2O$, 16 μM $AlK(SO_4)_2.12\ H_2O$, L-glutamine (1 mg/ml), and dehydrated tryptose phosphate broth (Life Technologies) at 2.6 g/liter. The cells were subcultured twice weekly by scraping from the bottom of the cell culture flasks and were then resuspended in the insect cell culture medium at an initial density of ~250,000 cells/ml.

Compounds and Radiochemicals. [CH_3-^{3}H]dThd (radiospecificity 72 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK), and [6-^{3}H]BVDU (radiospecificity 1.0 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Nonradiolabeled BVDU (Fig. 1) was prepared according to a method described previously (Jones et al., 1979). The 5′-monophosphate (BVDU-MP), 5′-diphosphate (BVDU-DP), and 5′-triphosphate (BVDU-TP) of BVDU were synthesized by P. Herdewijn (Rega Institute, Leuven, Belgium). Thymidine (dThd), thymidine-5′-monophosphate (dTMP), and dTTP were obtained from Sigma Chemical Co. (St. Louis, MO).

Expression and Purification of Recombinant Dm-dNK. Dm-dNK was expressed as a fusion protein to glutathione-*S*-transferase. The cDNA sequence encoding Dm-dNK was cloned in the pGEX-5X-1 plasmid (Pharmacia Biotech, Uppsala, Sweden). The protein was expressed in *Escherichia coli* and purified as described (Johansson et al., 1999). The purity of the recombinant protein was determined by SDS-polyacrylamide gel electrophoresis (Phast System, Pharmacia Biotech). The protein concentration was determined with the Bradford Protein Assay (Bio-Rad, Richmond, CA), and BSA was used as the concentration standard.

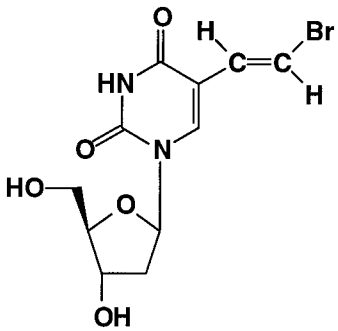

Fig. 1. Structural formula of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU).

dNK and TK-2 Assays. The standard reaction mixture contained 5 mM ATP, 5 mM $MgCl_2.6\ H_2O$, 9 mM KF, 5 mM phosphonoenolpyruvate, 5 μg pyruvate kinase, 10 mM dithiothreitol, 2 μM (0.1 μCi) [CH_3-^{3}H]dThd, 5 μl of the appropriate concentrations of BVDU, BVDU-MP, BVDU-TP, dTMP, or dTTP (i.e., 200, 40, 8, 1.6, 0.32, 0.06, and 0.012 μM), and 10 μl of a purified recombinant Dm-dNK enzyme preparation in a total volume of 40 μl of 0.05 M Tris/HCl, pH 8.0. The reaction mixture was incubated at 37°C for 15 min and the reaction terminated by the addition of 75 μl of ice-cold 0.05 M Tris/HCl buffer, pH 8.0. The reaction proceeded linearly at all substrate concentrations for an incubation period up to >30 min. Then the mixture was applied onto DE81 filter disks to bind phosphorylated [CH_3-^{3}H]dThd, and the remaining nonreacted [CH_3-^{3}H]dThd was removed from the filters by washing with 1 mM ammoniumformate, pH 8.2, ethanol, and ether. The filters were then assayed for radioactivity in a toluene-based scintillation cocktail. The IC_{50} values of BVDU, BVDU-MP, BVDU-TP, dTMP, and dTTP for [CH_3-^{3}H]dThd phosphorylation was defined as the compound concentration that inhibited the conversion of [CH_3-^{3}H]dThd to its 5′-monophosphate by 50%.

The K_i value of Dm-dNK for BVDU using [CH_3-^{3}H]dThd as the natural substrate and the nature of the inhibitory effect of BVDU on Dm-dNK-catalyzed phosphorylation of [CH_3-^{3}H]dThd were determined. BVDU (2 μM and 1 μM) was added to the reaction mixture that contained different concentrations of [CH_3-^{3}H]dThd (i.e., 5, 2, 1.5, 1, 0.75, and 0.4 μM), after which the Dm dNK enzyme (diluted 50,000-fold in potassium phosphate buffer, pH 7.6, containing 20% glycerine, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 0.5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) was added to start the reaction. The additional incubation of the reaction mixture and determination of phosphorylated [CH_3-^{3}H]dThd was performed as described above. Data were analyzed in a Lineweaver-Burk plot to determine the K_i value and kinetics of Dm-dNK inhibition by BVDU. The kinetics of BVDU against purified mitochondrial TK-2 were measured as described for Dm-dNK, but the BVDU concentrations were 0.4 and 0.8 μM, and the enzyme was 100-fold diluted from our TK-2 stock solution (1.7 mg protein/ml).

In the experiments in which the K_m and V_{max} values of BVDU and dThd were determined against Dm-dNK, radiolabeled [6-^{3}H]BVDU (at 20, 10, 5, 2, 1.5, 1, 0.8, and 0.4 μM) and [CH_3-^{3}H]dThd (at 5, 2, 1.5, 1, 0.8, and 0.4 μM) were exposed to the enzyme for 30 min at 37°C. After the reaction was terminated, radiolabeled conversion of [6-^{3}H]BVDU to BVDU-MP and of [CH_3-^{3}H]dThd to [CH_3-^{3}H]dTMP was determined as described above.

Inhibition of Insect Cell Proliferation by BVDU. Approximately 80,000 *S*-2 or Sf-9 cells were suspended in 800 μl of insect cell growth medium and added to the wells of 48-well plates in the presence of varying concentrations of BVDU. The cells were then allowed to proliferate at 26°C and, twice a day, the cell number was determined by use of a Coulter counter type ZM (Coulter Electronics, Harpenden Hertz, UK).

Incorporation of [6-^{3}H]BVDU into Methanol-Soluble and Methanol-Insoluble Material. The metabolism of radiolabeled [6-^{3}H]BVDU was monitored as follows: *S*-2 and Sf-9 cells were seeded at 0.5 to 2 × 10^6 cells/ml in a 5-ml cell culture flask (25 cm^2) and incubated with 1 μM [6-^{3}H]BVDU (5 μCi/5 ml). At 24 h, cells were scraped from the culture flask, centrifuged at 4°C for 10 min at 800*g*, washed 3 times with cold insect cell culture medium (without serum), and extracted with 60% cold methanol. After centrifugation at 2000*g*, the supernatants were filtered and HPLC analysis was performed using a Partisphere SAX radial compression column (Wattman, Clifton, NJ). A linear gradient of 5 mM $(NH_4)H_2PO_4$, pH 5.0, to 300 mM $(NH_4)H_2PO_4$, pH 5.0, was used to separate BVDU and its metabolites. The different fractions of the eluate were assayed for

radioactivity in a toluene-based scintillation cocktail. The radiolabeled 5′-mono-, 5′-di-, and 5′-triphosphorylated derivatives of BVDU were identified with the corresponding synthetically prepared BVDU derivatives, which coeluated on the HPLC chromatograms with the radiolabeled metabolites detected in the cell extracts.

Thymidylate Synthase (TS) Activity Measurements by Assaying the Tritium Release from [5-^{3}H]dCyd in Intact *S*-2 and Sf-9 Cells in the Presence of BVDU. Activity of TS in the intact insect *S*-2 and Sf-9 cells was measured by estimation of tritium release from [5-^{3}H]deoxyuridylate that had been formed in the intact cells from 20 μM [5-^{3}H]dCyd, in the reaction catalyzed by TS. The procedure for the determination of tritium release has been described previously (Balzarini and De Clercq, 1984). Briefly, 6×10^6 insect cells were collected by centrifugation at 200*g* for 8 min and resuspended in 1 ml fresh insect medium. Then 240 μl of this cell suspension (1.5×10^6 cells) was added to 300 μl of medium (control) or an appropriate amount of inhibitor together with 30 μl of medium, containing nonradiolabeled substrate (final concentration, 20 μM dCyd). After a 30-min preincubation period at 37°C, 15 μl (15 μCi) of radiolabeled substrate [5-^{3}H]dCyd was added, and at 0, 30, and 60 min, 100 μl of the reaction mixture was withdrawn and mixed with 250 μl of a cold suspension of carbon black (160 mg/ml) in 5% trichloroacetic acid. After centrifugation at 1100*g* for 10 min, 200-μl samples of the supernatant (containing tritiated water derived from the tritium release during the TS-catalyzed conversion of [5-^{3}H]dUMP to dTMP) were analyzed for radioactivity.

Flow Cytometric Insect Cell Cycle Analysis. Exponentially growing *S*-2 and Sf-9 cells were exposed to BVDU at 4.0, 0.8, 0.16, and 0.032 μM. At 24, 48, and 72 h, the DNA of the cells was stained with propidium iodide using the CycleTEST PLUS DNA Reagent Kit (Becton Dickinson, Le pont de Claix, France). The DNA content of the stained insect *S*-2 and Sf-9 cell cultures was assessed by flow cytometry on a FACScan equipped with CellQuest software (Becton Dickinson). Cell debris and cell clumps were excluded from the analysis using conventional dot plot gating.

In Vivo Assay on Insecticidal Activity. BVDU was examined for its growth-inhibitory and lethal activities against larvae of *S. frugiperda* in an artificial diet feeding assay. Various amounts of the drug were added to the solid and liquid ingredients of the agar-based diet to result in final drug concentrations of 100, 200, and 500 μM BVDU. Six-day-old larvae of *S. frugiperda* were placed individually on portions of the diet in plastic containers (diameter = 4 cm, height = 3 cm). Twenty larvae were used for each BVDU concentration and for the (drug-free) control. The weight of each larva was determined every other day until prepupation or premature death of the larvae. Mortality was registered during larval and pupal development, and the hatch of the adults was carefully observed. Additional details on the preparation of the diet and the implementation of the *S. frugiperda* bioassay for testing insecticidal compounds were described previously (Breuer and Schmidt, 1996).

Results

Affinity of BVDU for Dm-dNK and Mitochondrial TK-2. The inhibitory activity of BVDU against the recombinant purified Dm-dNK and TK-2 was evaluated in the presence of 2 μM [CH_3-^{3}H]dThd as the natural substrate. BVDU dose dependently inhibited dThd phosphorylation by Dm-dNK and TK-2 with IC_{50} values of 2.6 μM and 0.34 μM, respectively. When different concentrations of BVDU (2 μM and 1 μM) were evaluated in the presence of different [CH_3-^{3}H]dThd concentrations between 5 μM and 0.4 μM, Lineweaver-Burk plots of the data revealed competitive inhibition of BVDU against [CH_3-^{3}H]dThd with a K_i value of 0.66 μM and a K_i/K_m value of 0.44, indicating a pronounced affinity of the drug for the Dm-dNK (Fig. 2). Because the choice of Tris as the buffer system may lower the association constant for the Mg^{2+}-ATP complex compared with other buffers, we also have replaced Tris by 50 mM HEPES, pH 8.0, and performed similar kinetic experiments in the presence of 1 and 5 mM both $MgCl_2$ and ATP. Virtually identical K_i/K_m values were obtained under these experimental conditions (K_i/K_m = 0.44 and 0.46, respectively). When the kinetics of BVDU were examined against mitochondrial TK-2, competitive inhibition with respect of dThd as the natural substrate was recorded (Fig. 2). The K_i/K_m ratio was 0.15, which is 3-fold lower than that found for Dm-dNK. Our data strongly suggest that BVDU may act as an alternative substrate for Dm-dNK (and TK-2). Indeed, when examined directly for substrate activity against Dm-dNK using [^{3}H]BVDU as the radiolabeled substrate, a K_m value of 4.5 μM and a V_{max} value of 400 nmol/μg protein/h were found, compared with a K_m value of 1.3 μM and a V_{max} value of 62.5 nmol/μg protein/h for the natural substrate [CH_3-^{3}H]dThd. Thus, the phosphorylating capacity (V_{max}/K_m) of Dm-dNK for BVDU was almost twice as high as for dThd. HPLC analysis of the reaction products revealed the formation of BVDU-MP from BVDU at approximately the same extent as the formation of dTMP from dThd (data not shown). The identity of the BVDU-MP formed was attested by verifying its retention time on the HPLC chromatograms with synthetically prepared BVDU-MP. No other phosphorylated BVDU derivatives (i.e., the 5′-diphosphate of BVDU) were detected in the HPLC chromatograms. Interestingly, Dm-dNK was subject to pronounced feedback inhibition by

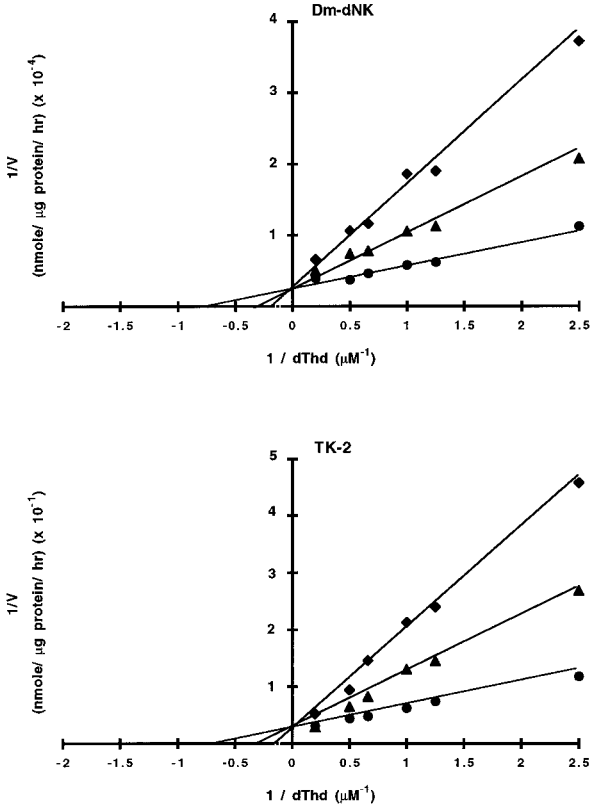

Fig. 2. Double-reciprocal (Lineweaver-Burk) plots for inhibition of Dm-dNK by BVDU at 2 (♦), 1 (▲), or 0 μM (●), and for inhibition of mitochondrial TK-2 by BVDU at 0.8 (♦), 0.4 (▲), or 0 μM (●).

dTTP, BVDU-TP, and BVDU-MP, but not by dTMP. The IC_{50} values of the phosphorylated dThd and BVDU metabolites ranged between 3.1 and 12 μM, respectively, in the presence of 2 μM [CH_3-^{3}H]dThd (Table 1). It should be noted that the kinetic experiments for Dm-dNK were performed with the fusion dNK protein to glutathione *S*-transferase. However, we have shown previously that the affinity values for nucleoside substrates obtained for this recombinant enzyme preparation were comparable with those obtained from purified Dm-dNK derived from the *D. melanogaster* insect cells (Munch-Peterson et al., 1998a,b; Johansson et al., 1999).

Inhibition of the Growth of *S*-2 and Sf Cells and a Variety of Mammalian Cells in Cell Culture. The *S*-2 and Sf-9 insect cells were seeded at ~80,000 cells per 1-cm^2 well of a 48-well microplate and grown in the presence or absence of different concentrations of BVDU. Nontreated control cells grew exponentially as a function of the incubation time and reached the stationary growth phase within 80 (*S*-2) and 104 (S9) h after seeding (Fig. 3). By that time, the insect cells proceeded through 2.5 to 3 complete cell cycles. At concentrations of 0.16 and 4 μM, BVDU virtually completely blocked *S*-2 and Sf-9 insect cell proliferation at all time points (up to 104 h), respectively. BVDU, administered at a concentration as low as 0.032 μM (for *S*-2 cells) and 0.16 μM (for Sf-9 cells) still inhibited cell proliferation. The IC_{50} values, or inhibitory concentration of BVDU that inhibit insect cell proliferation by 50%, were 0.11 and 0.86 μM for *S*-2 and Sf-9 cells, respectively (Fig. 3). Thus, BVDU was markedly cytostatic against the insect cells, being at least 5-fold more potent an inhibitor of *S*-2 cell proliferation than of Sf-9 cell proliferation. In contrast, BVDU was only marginally cytostatic ($IC_{50} \geq 100$ μM) to mammalian cells (including murine leukemia L1210 and breast carcinoma FM3A cells, human T-lymphocyte wild-type CEM/0, Molt/4F, and TK-deficient CEM/TK$^-$cells, human B-lymphoblast Raji and TK-deficient Raji/TK$^-$ cells, human wild-type osteosarcoma and TK-deficient osteosarcoma cells, human embryonic lung cells, and human cervix carcinoma HeLa cells), attesting to the highly selective susceptibility of insect cells to the growth-inhibitory effects of BVDU. Thus, whereas the insect cell proliferation was inhibited at BVDU concentrations <1 μM, none of the human cell lines investigated were markedly inhibited in their growth by BVDU at 100 μM (Table 2).

Metabolism of [6-^{3}H]BVDU in *S*-2 and Sf-9 Cell Cultures. Both *S*-2 and Sf-9 cell cultures efficiently metabolized [6-^{3}H]BVDU (Table 3). On exposure of the insect cell cultures to 1 μM BVDU for 24 h, the majority of BVDU-derived radiolabel was recovered in the methanol-insoluble fraction in *S*-2 cells but in the methanol-soluble fraction in Sf-9 cells. Whereas a total of 0.36 pmol radiolabel/10^6 cells was recovered in the 60% methanol-soluble BVDU-MP + BVDU-DP +

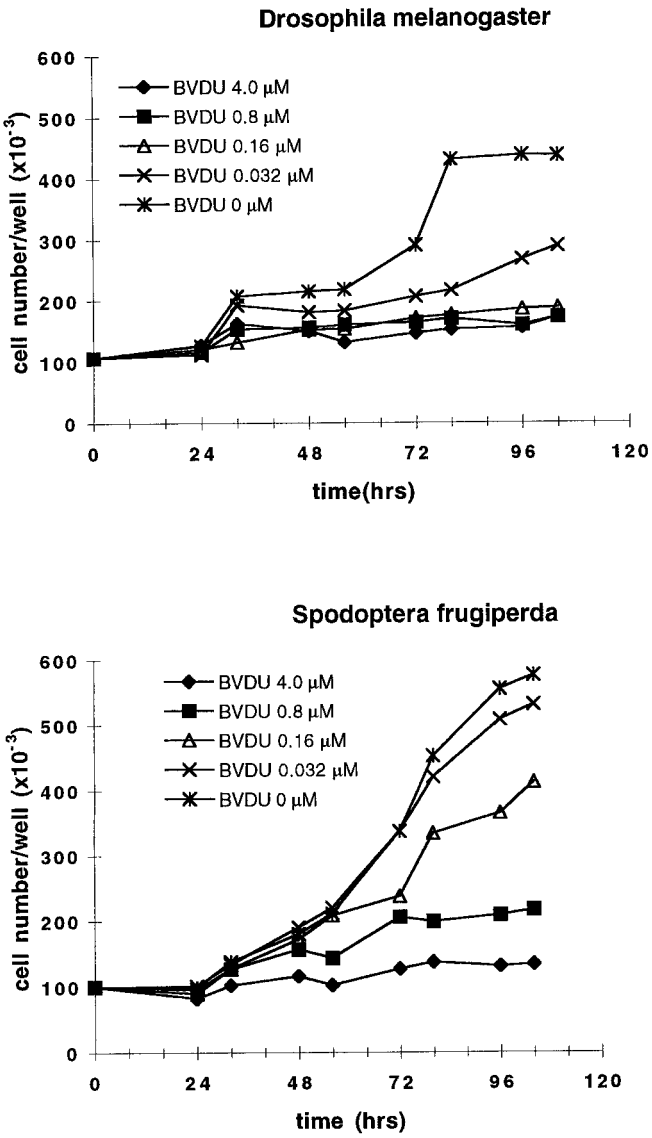

Fig. 3. Growth of *S*-2 and Sf-9 cells in the presence of 4.0, 0.8, 0.16, and 0.032 μM BVDU as a function of time.

TABLE 1
Inhibitory effects of dTTP, dTMP, BVDU-TP, BVDU-MP, and BVDU on Dm-dNK using 2 μM [CH_3-^{3}H]dThd as the natural substrate

Compound	IC_{50}[a]
	μM
dTTP	12 ± 1.7
dTMP	> 500
BVDU-TP	6.9 ± 0.55
BVDU-MP	2.6 ± 0.60
BVDU	2.6 ± 0.45

[a] IC_{50}, or compound concentration required to inhibit Dm-dNK-catalyzed [CH_3-^{3}H]dThd phosphorylation by 50%. The data represent the mean ± S.D. and were derived from two to three independent experiments.

TABLE 2
Inhibitory activity of BVDU against the proliferation of a variety of murine, human, and insect cells

Cell Type	IC_{50}[a]
	μM
L1210/0	23 ± 9.6
FM3A/0	29 ± 13
CEM/0	302 ± 32
CEM/TK$^-$	210 ± 166
Molt/4F	> 100
Raji/0	89 ± 5.2
Raji/TK$^-$	79 ± 20
HOS	> 250
OST/TK$^-$	548 ± 107
HEL	> 200
Spodoptera Sf-9	0.11 ± 0.04
Drosophila S-2	0.86 ± 0.47

[a] IC_{50}, or compound concentration required to inhibit cell proliferation by 50%.

BVDU-TP fraction of *S*-2 cells, 30-fold higher amounts of BVDU metabolites (i.e., 11 pmol radiolabel/10^6 cells) had accumulated into the methanol-soluble fraction of Sf-9 cells. In the Sf-9 cell cultures, a slightly lower amount of radiolabel was recovered in the 60% methanol-insoluble fraction (i.e., nucleic acids) than in *S*-2 cells (Table 3). The ratios for accumulation of mono- versus di- and triphosphates of BVDU markedly differed between both insect cell lines. Whereas 73% of the total phosphorylated BVDU metabolites represented BVDU-TP in *S*-2 cells, the predominant BVDU metabolite in Sf-9 cells was BVDU-MP (77%). Thus, *S*-2 cells showed a markedly better conversion of BVDU to BVDU-TP and subsequent incorporation into nucleic acid than did Sf-9 cells, which predominantly tend to accumulate BVDU-MP and allow less incorporation of BVDU into their nucleic acids.

Inhibition of Tritium Release from [5-^{3}H]dCyd by dThd and BVDU in Intact *S*-2 and Sf-9 Cell Cultures as a Tool to Measure In Situ TS Activity. The inhibitory effect of different concentrations of dThd and BVDU on the tritium release from 20 μM [5-^{3}H]dCyd was evaluated in intact *S*-2 and Sf-9 cells. Tritium is released from [5-^{3}H]dCyd after formation of [5-^{3}H]dCyd-derived [5-^{3}H]dUMP and subsequent conversion of [5-^{3}H]dUMP to unlabeled dTMP in the TS-catalyzed reaction. dThd inhibited tritium release from [5-^{3}H]dCyd by 50% at ~5 μM and 49 μM in *S*-2 and Sf-9 cells, respectively. BVDU, however, effected a 50% inhibition of tritium release from [5-^{3}H]dCyd at an IC_{50} value of 0.6 and 1.0 μM in *S*-2 and Sf-9 cells, respectively (Fig. 4), thus at a 10- to 50-fold lower concentration than dThd.

Effect of BVDU on Cell Cycle Progression in Drug-Exposed *S*-2 and Sf-9 Cell Cultures. Fig. 5 shows the cell cycle distribution of *S*-2 (A) and Sf-9 (B) cell cultures exposed to different BVDU concentrations for 24, 48, and 72 h. At the lowest BVDU concentration (i.e., 0.05 μM), the *S*-2 cells accumulated in the S phase of the cell cycle after 24 h and slowly progressed to mitosis (G2/M phase) at longer incubation times. At higher drug concentrations (i.e., 0.5 and 5 μM), the cells were unable to enter the S phase and were arrested at the G1/S boundary. In contrast, the cell cycle distribution of Sf-9 cells was not markedly affected at 0.05 μM BVDU. In the presence of 0.5 μM, a moderate accumulation of Sf-9 cells in the S phase was noted throughout the entire incubation period. Treatment with 5 μM BVDU caused pronounced synchronization of the Sf-9 cell cultures, leading to predominant accumulation of the cells at the G1/S boundary of the cell cycle at 24 h, in the S phase at 48 h, and at the G1/S phase at 72 h.

Insecticidal Activity of BVDU. BVDU was added to an artificial diet of *S. frugiperda* larvae at 500, 100, and 20 μM, and the weight of the larvae during growth was determined (Fig. 6A). Retardation of larval growth was most striking at the highest BVDU concentration (500 μM); after 9 days, the mean weight of the larvae was only 17% of that of control animals, and all larvae subsequently died. At 100 μM BVDU, a significant (25–30%) growth retardation of the larvae was observed, which resulted in ~20% larval death (prepupal mortality), but also in ~50% pupal mortality. Virtually all adults that survived the pupal period were seriously affected (moulting defects, deformed wings, etc.). Only ~1 of 20 larvae eventually developed to an intact moth. Although 20 μM BVDU did not result in a statistically significant growth retardation of the larvae, 5% of the larvae died in the prepupal stage. Those larvae that survived the prepupal state further developed to apparently normal adults (Fig. 6B).

Discussion

In *D. melanogaster* embryonic *S*-2 cells, only a single dNK (Dm-dNK) was found being able to phosphorylate all four deoxynucleosides (Munch-Petersen et al., 1998a,b). So far, this property is unique and clearly differs from the more specialized mammalian dNKs that show a more stringent substrate specificity. However, because HSV type 1, HSV-2, and varicella-zoster virus (VZV) also encode a dNK enzyme that efficiently recognizes both dThd and dCyd as a substrate and proved able to phosphorylate a variety of synthetic guanosine analogues (i.e., acyclovir, ganciclovir, penciclovir, etc.) (Balzarini et al., 1993 and references therein), antiherpetic compounds also might be recognized as substrates by Dm-dNK. From an initial search of a variety of antiherpetic compounds, BVDU emerged as the most potent inhibitor of dThd phosphorylation by Dm-dNK. BVDU is an excellent substrate for HSV-1 and VZV TK and is also recognized as an efficient substrate by mitochondrial TK-2 (Cheng et al., 1981b), but not by mammalian TK-1 (Cheng et al., 1981a; Balzarini et al., 1982) or by mammalian dCK (unpublished observations). As a matter of fact, BVDU is a highly selective antiherpetic agent because of its virtual lack of metabolism (and associated toxicity) in mammalian cells. Therefore, our findings that BVDU is a very efficient substrate for Dm-dNK may be of great interest in view of the development of novel Dm-dNK substrates as cytostatic agents with selectivity for insect cells. Indeed, BVDU was found to have a pronounced cytostatic activity against both the *S*-2 and Sf-9 cells. These cell lines are derived from two insect species that belong to two different families within the arthropod class of insects. The observations strongly suggest that insect dNK can be regarded as an attractive novel target for the development of potent and selective insecticides. This premise was attested further by our findings that BVDU caused a detrimental effect on the normal developmental process of *S. frugiperda* larvae to the adult stage.

We have demonstrated that in insect cells, BVDU is markedly converted (presumably by the multifunctional dNK) to its phosphorylated derivatives and subsequently incorporated into insect cell DNA. Moreover, the insect cells accu-

TABLE 3
Distribution of [6-^{3}H]BVDU metabolites in insect S-2 and Sf-9 cells[a]

Cell Line	Percentage of Total MeOH-Soluble Radiolabel				Total [6-^{3}H]BVDU Label (pmol/10^6 cells)	
	BVDU	BVDU-MP	BVDU-DP	BVDU-TP	MeOH-Soluble Fraction	MeOH-Insoluble Fraction
Sf-9	14.5	77	2.0	6.6	11	5.3
S-2	19.1	7.7	0.3	73	0.36	8.3

[a] [6-^{3}H]BVDU was exposed to the cells at 1 μMfor 24 h. The data represent the mean values of two independent experiments,

mulated in the S phase of the cell cycle or were even prohibited to enter the S phase on BVDU exposure. It is thus far unclear which metabolic event prevented or retarded DNA synthesis caused in the insect cells. Because TS represents the sole de novo enzyme providing cells with thymine nucleotide building blocks for DNA synthesis and because BVDU-MP has been shown earlier to be a potent inhibitor of this enzyme (K_i/K_m ~0.5) (Balzarini et al., 1982), inhibition of TS by BVDU-MP may contribute to the cytostatic activity of BVDU against the insect cells.

We have shown earlier that tumor cells transfected with the HSV-1 or HSV-2 TK gene and subsequently exposed to BVDU are strongly inhibited in their proliferative capacity, primarily because of TS inhibition on intracellular conversion of BVDU to BVDU-MP by the HSV-1 TK or HSV-2 TK (Balzarini et al., 1987; Balzarini and De Clercq, 1989). We also have demonstrated previously that BVDU-MP is a potent inhibitor of partially purified TS (K_i/K_m = 0.5) (Balzarini et al., 1982). We also now found a TS-specific suppression in the intact insect cells by BVDU. The pronounced inhibition of tritium release from [5-^{3}H]dCyd by BVDU cannot just be ascribed to an inhibitory effect of BVDU on [5-^{3}H]dCyd conversion to [5-^{3}H]dCMP by dNK before its additional deamination to [5-^{3}H]dUMP and TS-catalyzed tritium release. Indeed, we found that dThd is much less inhibitory to tritium release from [5-^{3}H]dCyd in the intact insect cells than is BVDU, whereas both dThd and BVDU have a comparable affinity for dNK. The TS inhibition we observed in the intact *S*-2 and Sf-9 insect cells exposed to BVDU may lower the endogenous dTTP pools, resulting in a transient or sustained decreased availability of dTTP for DNA synthesis. Lack of this and other dNTP substrates for DNA synthesis may in turn cause accumulation of the insect cells at the G1/S boundary or in the S phase of the cell cycle, as observed in our experiments. The inhibition of TS in intact BVDU-treated insect cells (as measured by the inhibition of tritium release from [5-^{3}H]dCyd) occurred at drug concentrations that were (approximately) 1 order of magnitude higher than the inhibitory concentrations of BVDU for insect cell proliferation. This may point to yet additional mechanisms to explain the cytostatic action of BVDU. Indeed, inhibition of DNA polymerase by BVDU-TP and incorporation of BVDU into the TCA-insoluble cell fraction (presumably DNA) may also contribute to the accumulation of the drug-treated insect cells in the S phase of their cell cycle. Potent inhibition of DNA polymerase may even prevent the cells to enter the S phase. It should be noted that Sf-9 cells accumulated much more BVDU-MP (300-fold) and BVDU-TP (3-fold) than did *S*-2 cells, although Sf-9 cells showed lesser BVDU-derived radiolabel in their nucleic acids than did *S*-2 cells. This may partly explain the ~5-fold higher sensitivity of *S*-2 cells to the cytostatic activity of BVDU compared with Sf-9 cells. These observations may point to the importance of the conversion of BVDU to its triphosphate, and subsequent incorporation into DNA, in the eventual cytostatic activity.

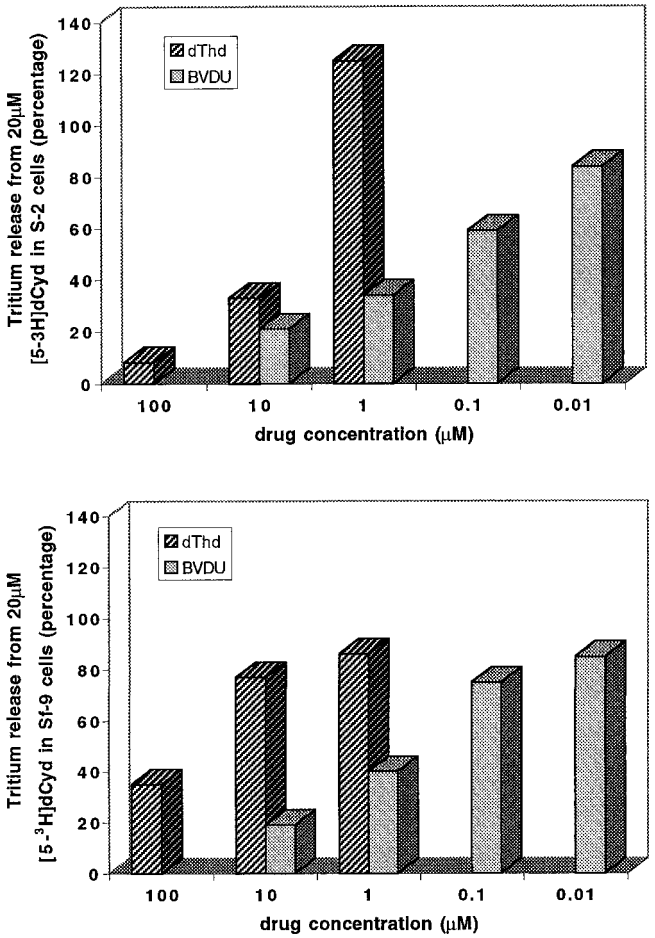

Fig. 4. Effect of BVDU on the release of tritium from [5-^{3}H]dCyd in intact *S*-2 and Sf-9 cell cultures.

Munch-Petersen and collaborators have reported that Dm-dNK purified from *D. melanogaster* is not endowed with thymidylate (dTMP) kinase activity (Munch-Petersen et al., 1998a,b), an observation that we could confirm with our recombinant purified Dm-dNK preparation. Therefore, it is intriguing that *S*-2 cells easily convert BVDU-MP into BVDU-TP, whereas in Sf-9 cells, this conversion seems to be limited (as attested by the accumulation of BVDU-MP in the methanol-soluble fraction). There may be two explanations for this phenomenon. Unlike Sf-9 cells, the *S*-2 cells may contain a slightly different dNK that has, like HSV-1 TK, and VZV TK, associated dTMP kinase activity, thus efficiently recognizing and converting BVDU-MP as substrate to BVDU-DP. Hereafter, BVDU-DP is converted further to BVDU-TP by nucleoside diphosphate kinase. Alternatively, Dm-dNK and Sf-dNK may have no associated dTMP kinase activity, as it is the case for HSV-2 TK and mitochondrial TK-2, but the *S*-2 cells, unlike the Sf-9 cells, may contain a cellular dTMP kinase that is able to efficiently convert BVDU-MP to BVDU-DP. The latter metabolite is then readily converted to BVDU-TP by the nucleoside diphosphate kinase. Cellular dTMP kinases can differ markedly to one another depending the nature of the cell line. For example, whereas the anti-HIV drug azidothymidine monophosphate (AZTMP) is easily converted to azidothymidine diphosphate (AZTDP) by dTMP kinase in murine L1210 cells (Balzarini et al., 1987), AZTMP is poorly converted to AZTDP by dTMP kinase of human cells (Furman et al., 1986) because of an unusual low V_{max} value of human dTMP kinase for AZTMP. Therefore, it would not be unlikely that the different processing of BVDU-MP to BVDU-TP in *S*-2 and Sf-9 cells may be attributable to kinetic differences at the level of dTMP kinase. We found that metabolism of [^{3}H]dThd by *S*-2 and Sf-9

cells was very similar with regard to dTMP, dTDP, and dTTP formation (data not shown), suggesting that the kinetic properties of the dTMP kinase in both cell lines for dTMP are not strikingly different. Therefore, additional studies are required to elucidate the molecular basis of the different kinetic behavior of BVDU in intact *S*-2 and Sf-9 cells, and this may become an important issue with regard to the development of novel drugs with selective insecticidal potential.

Our observations that BVDU-MP and BVDU-TP act as efficient product or feed-back inhibitors of Dm-dNK imply that the conversion of BVDU to its 5′-mono- and 5′-triphosphates may become self-regulatory. On the other hand, the inhibition of both TS (by BVDU-MP) and Dm-dNK (by BVDU-MP and BVDU-TP) may efficiently prevent the formation of dTTP. This should cause a sustained shortage of dTTP for DNA synthesis and a concomitantly increased competitive advantage of BVDU-TP over dTTP during its incorporation into DNA.

BVDU is highly selective in its cytostatic action for the insect cells investigated. Whereas the Sf-9 and *S*-2 insect cells were inhibited at IC_{50} values of 0.11 and 0.87 μM, respectively, the mammalian murine cells were inhibited at an IC_{50} value of 23 to 29 μM, whereas human cell proliferation was inhibited at BVDU concentrations of $\geq$100 μM. Also, the TK-deficient cells that virtually lack cytosolic TK and only express mitochondrial TK-2 were poorly sensitive to the cytostatic action of BVDU. Presumably, BVDU cannot efficiently reach the mitochondrial TK-2 to become converted to its phosphorylated derivative or, alternatively, BVDU is phosphorylated in the mitochondria by TK-2, but the phosphorylated product(s) are not harmful for this cellular compartment. These data are in agreement with observations published previously that BVDU very marginally inhibits growth of normal lung fibroblasts at 450 μM (Machida et al., 1982), DNA synthesis of normal primary rabbit kidney cells or Vero cells at 300 μM (De Clercq and Descamps, 1981; Larsson and Öberg, 1981), the proliferation of bone marrow granulocyte-monocyte progenitor cells at 120 to 600 μM (Wingard et al., 1983), and various lymphocyte responses at 150 to 300 μM (Marmer et al., 1982; Wingard et al., 1983). In fact, BVDU has been used in the treatment of herpetic keratitis, herpetic gingivostomatitis, herpes labialis, herpetic encephalitis, and VZV infections (i.e., chickenpox, shingles) with immune-competent and immune-compromised patients. BVDU has been given as eye drops of 0.1 or 0.5%, as a cream of 1.3 or 5%, or orally or parentally (i.v.) at 7.5 to 15 mg/kg/day for 5 subsequent days. At the latter drug doses, no remarkable side effects were observed (De Clercq et al., 1980; Wildiers and De Clercq, 1984; Maudgal et al., 1985a,b). There also are no experimental indications of mutagenicity, carcinogenicity, or teratogenicity at drug doses that are antivirally active in humans (Marquardt et al., 1985; De Clercq, 1986; Jelinek and De Clercq, 1987). Although we found BVDU highly cytostatic against the *D. melanogaster* and *Spodoptera frugiperda* cells, there is at least one report in the literature (Mazzacano and Fallon, 1995) that shows that

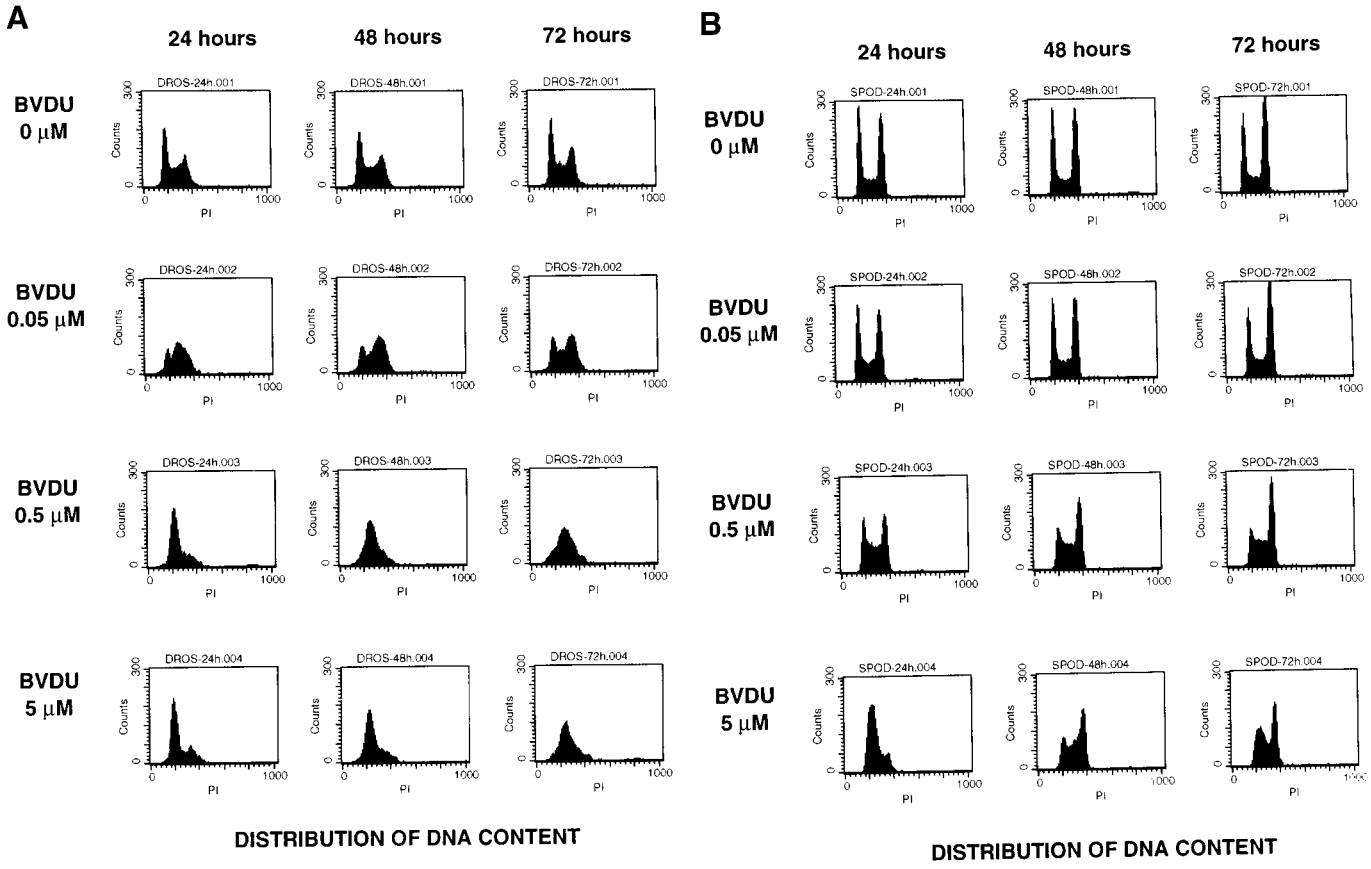

Fig. 5. Cell cycle distribution of BVDU-treated *S*-2 (A) and Sf-9 (B) cell cultures. BVDU was administered to *S*-2 and Sf-9 cell cultures at a final concentration of 0, 0.05, 0.5, or 5 μM. After 24, 48, and 72 h of exposure, the DNA content of the drug-treated cells was analyzed by propidium iodide (PI) staining and flow cytometry. Cell debris and doublets were excluded from the DNA histograms using conventional dot plot gating.

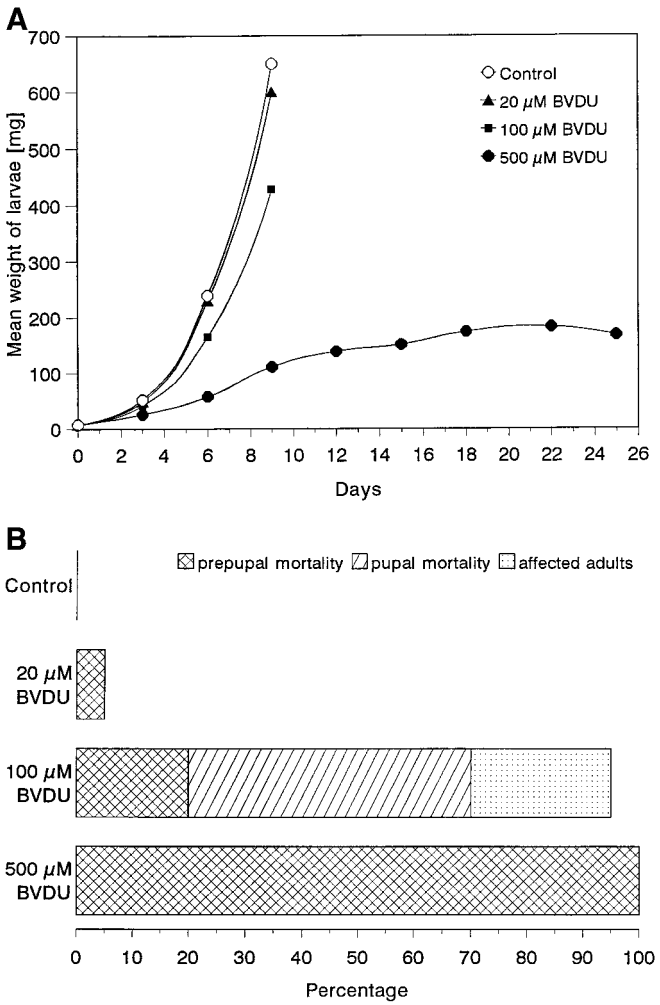

Fig. 6. A, growth curves of *S. frugiperda* larvae in the presence of 500, 100, and 20 μM BVDU. Data represent the mean weight of 20 larvae. B, prepupal (larval) mortality, pupal mortality, and affected adults (moths) in the presence of 500, 100, and 20 μM BVDU.

BVDU is not markedly cytostatic to mosquito cells, but becomes more inhibitory when the mosquito cells were transfected with the TK gene of HSV-1. All observations together point to a selective cytostatic activity of BVDU against several, but not all, types of insect cells.

In conclusion, we have shown that insect cells belonging to different families within the arthropod class of insects are highly sensitive to the cytostatic action of (antiherpes) drugs that are recognized as substrates for phosphorylation by the multifunctional insect dNK ($IC_{50} \leq 0.1$ μM). Because of the similarities between Dm-dNK and herpetic TKs, it would be worth evaluating additional HSV TK-dependent antiherpetic drugs for their (substrate) affinity for Dm-dNK and their potential cytostatic/insecticidal properties. The insect multifunctional dNK may be regarded as a suitable target in the development of an entirely novel type of selective insecticides.

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