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ORIGINAL ARTICLE

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Incorporation of OSI-7836 into DNA of Calu-6 and H460 xenograft tumors

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Abstract OSI-7836 (4′-thio-β-D-arabinofuranosylcytosine) is a novel nucleoside analog in phase I clinical development for the treatment of cancer. As with other nucleoside analogs, the proposed mechanism of action involves phosphorylation to the triphosphate form followed by incorporation into cellular DNA, leading to cell death. This hypothesis has been examined by measuring and comparing the incorporation of ara-C, OSI-7836, and gemcitabine (dFdC) into DNA of cultured cells and by investigating the role of deoxycytidine kinase in OSI-7836 toxicity. We report here additional studies in which the role of cell cycling on OSI-7836 toxicity was investigated and incorporation of OSI-7836 into DNA of xenograft tumors measured. The role of the cell cycle was examined by comparing the toxicity of OSI-7836 in A549 NSCLC cells that were either in log phase growth or had reached confluence. A novel validated LC-MS/MS assay was developed to quantify the concentrations of OSI-7836 in DNA from Calu-6 and H460 human tumor xenografts in mice. Results showed that apoptosis induced by OSI-7836 was markedly greater in cycling cells than in confluent non-cycling cells despite only a modest increase in intracellular OSI-7836 triphosphate concentration. The LC-MS/MS assay developed to measure OSI-7836 incorporation into DNA had an on-column detection limit of 0.25 fmol, a quantification limit of 0.5 fmol, and a sensitivity of approximately 0.1 pmol OSI-7836/μmol dThy. Concentrations of OSI-7836 in splenic DNA (0.4 pmol OSI-7836/μmol dThy) averaged fivefold less than the average concentration in Calu-6 and H460 xenograft DNA (3.0 pmol OSI-7836/μmol dThy) following a 400 mg/kg dose of OSI-7836. Concentrations of OSI-7836 in Calu-6 tumor DNA isolated 24 h following a dose of 400, 1000, or 1600 mg OSI-7836/kg were approximately 1.3, 1 and 1.3 pmol OSI-7836/μmol dThy, respectively. Concentrations of OSI-7836 in DNA from H460 and Calu-6 xenografts did not appear to increase during repeated administration of 400 mg OSI-7836/kg on days 1, 4, 7, and 10. The majority of OSI-7836 in DNA from Calu-6 and H460 tumors of mice dosed with 1600 mg/kg was located at internal nucleotide linkages, similar to dFdC and ara-C. In conclusion, cell cycling studies supported the hypothesis that OSI-7836 cytotoxicity is dependent upon DNA synthesis. A validated LC-MS/MS assay was developed that could quantify OSI-7836 in DNA from tissues. The assay was used to show that OSI-7836 was incorporated in internal linkages in tumor DNA in a manner that was dose-independent at the doses tested and did not appear to accumulate during repeated dosing. The results suggest that if DNA incorporation is a toxic event, the relationships between administered dose, DNA incorporation, and toxicity are complex.

Keywords OSI-7836 · DNA incorporation · Apoptosis · Cell replication · Liquid chromatography/mass spectrometry

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Introduction

Nucleoside analogs have been used as anticancer therapeutics for decades. While these compounds have traditionally been most effective against liquid tumors, the success of gemcitabine (dFdC) has spurred interest in the

development of nucleoside analogues against solid tumors. OSI-7836 (4′-thio-β-D-arabinofuranosylcytosine) [20] is one such nucleoside analog with demonstrated efficacy against human tumor cell lines and solid tumor xenograft models including colon, renal, non-small-cell lung (NSCLC) (including H460, Calu-6 and A549 cells), prostate and pancreatic cancer [4, 21, 23]. OSI-7836 is currently in phase I clinical trials [8, 18].

OSI-7836 is an ara-C analog with a sulfur substitution at the cyclic oxygen (Fig. 1) [24] that arrests cells in the G_2/M phase of the cell cycle [2]. OSI-7836-TP (the triphosphate form of OSI-7836) is a substrate for DNA polymerases α, δ, and γ that inhibits DNA synthesis and is incorporated into DNA primer-templates [2, 16], although the extent of incorporation and the inhibitory effects of the compound are different depending on the polymerase. In CEM cells, OSI-7836 is metabolized to the triphosphate form and incorporated into DNA and is a poorer substrate for cytidine deaminase than ara-C [2, 11]. Cells deficient in deoxycytidine kinase are less susceptible to the toxic effects of OSI-7836 [2]. Together these in vitro data suggest that, like ara-C and dFdC [13], the efficacy of OSI-7836 depends on intracellular phosphorylation and subsequent incorporation into DNA.

To achieve a better understanding of the effects of OSI-7836 in vitro and more importantly in vivo, a series of studies were conducted to examine the effect of cell replication on OSI-7836 toxicity, the extent and nature of incorporation of OSI-7836 into xenograft tumor and mouse splenic DNA, and the effects of OSI-7836 on cell replication and apoptosis within tumor xenografts. Radioactive compounds have traditionally been used to examine DNA incorporation and were used here to compare incorporation of subtherapeutic doses of OSI-7836, ara-C, and dFdC in xenograft tumor DNA. In order to examine DNA incorporation following therapeutic doses, a new LC-MS/MS method was developed that allowed detection and quantification of incorporation following extended treatment with OSI-7836.

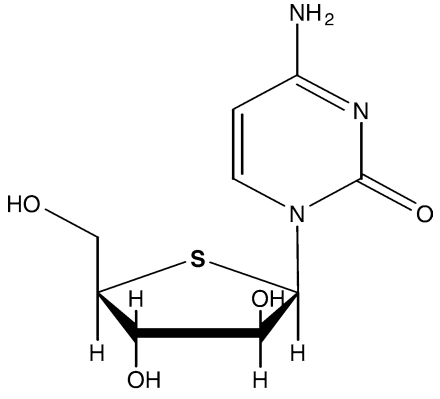

Fig. 1 Structure of OSI-7836

Materials and methods

Cytotoxicity assays

Assays were conducted to determine whether the cytotoxic effects of OSI-7836 were dependent on DNA replication.

Incorporation of thymidine (dThy) into DNA was used to evaluate DNA synthesis and demonstrate that confluent cells were cycling at a significantly reduced rate as compared to those in log-phase cultures. Triplicate cultures of log-phase and confluent A549 NSCLC cells were treated with 5 μCi ^{3}H-dThy (specific activity 20 Ci/mmol; Sigma-Aldrich, St Louis, Mo.) for 15 min, after which time adherent cells were harvested by trypsinization, incubated in 10% trichloroacetic acid for 15 min on ice, washed and analyzed using a Beckman LS6500 scintillation counter (Beckman, Fullerton, Calif.).

Triphosphate formation was determined in duplicate log-phase and confluent cultures of A549 NSCLC cells treated with 10 μ*M* ^{3} H-OSI-7836 (5 μCi of material with specific activity 8.2 Ci/mmol; Moravek Biochemicals, Brea, Calif.) for 72 h. At the end of drug treatment, adherent and non-adherent cells were combined, fixed on ice in 70% ethanol, sedimented at 200 *g* for 10 min, and the supernatant analyzed using a Hewlett Packard 1100 HPLC (Hewlett Packard, Wilmington, De.), a 5-μm Supelcosil SAX1 column (25 cm × 3 mm; Supelco, Bellefonte, Pa.) and a gradient elution method (buffer A, 5 m*M* ammonium phosphate, pH 4.0; buffer B, 700 m*M* ammonium phosphate, pH 4.6). ^{3}H-OSI-7836 and its metabolites, including ^{3}H-OSI-7836-triphosphate, were detected using an in-line Radiomatic 500TR series flow scintillation analyzer and quantified using the Flo-One software package (Packard, Meridian, Ct.).

The rate of apoptosis was measured in duplicate log-phase and confluent cultures of A549 NSCLC cells. Cells were treated with 10 μ*M* OSI-7836 (non-radioactive) for 72 h, after which time adherent and non-adherent cells were collected, sedimented and fixed in 10% buffered formalin for 15 min at room temperature. Samples were then washed with PBS, sedimented onto slides and stained with 1 μg/ml Hoechst 33258 (Molecular Probes, Eugene, Ore.). Apoptotic nuclei were visualized using fluorescence microscopy, and the apoptotic indices were determined from a minimum of 300 nuclei per sample.

Animal dosing for in vivo dose-response and time course studies

A series of studies, using mice bearing human tumor xenografts, were conducted to quantify and compare the incorporation of radioactive OSI-7836, dFdC and ara-C (Sigma-Aldrich) into tumor DNA; to quantify incorporation of OSI-7836 into tumor DNA following

extended administration at therapeutic doses; and to determine the effect of OSI-7836 on cell replication and apoptosis in vivo. Human tumor cell suspensions were implanted subcutaneously into the flank of female CD-1 nu/nu mice (Charles River, Wilmington, Mass.) and tumors were allowed to reach approximately 300 mg prior to administration of compound. All compounds were administered intravenously. Animals were humanely euthanized, following the AVMA guidelines [14], at selected time points or when they were under undue stress as defined in the animal care and use protocol approved by the OSI Animal Care and Use Committee. All animals were maintained in the AAALAC-accredited OSI Animal Facility, and all studies were conducted with the approval of the OSI Animal Care and Use Committee and in accordance with the Guide for the Care and Use of Laboratory Animals [22].

The amount of radioactive OSI-7836, dFdC and ara-C incorporated into Calu-6 xenograft DNA was quantified and compared. Mice bearing Calu-6 xenografts were given 40 μCi/day for 3 days of ^{3}H-dThy (specific activity 20 Ci/mmol; Sigma-Aldrich) plus 40 μCi/day for 3 days of ^{3}H-OSI-7836 (specific activity 7.7 Ci/mmol), ^{3}H-dFdC (specific activity 14 Ci/mmol) or ^{3}H-ara-C (specific activity 21.1 Ci/mmol; Moravek Biochemicals). Tumors were collected 24 h after the last dose. DNA from the entire tumor was isolated and hydrolyzed, and radioactivity was quantified following methods described below (''DNA isolation and enzymatic hydrolysis'', ''Quantification of radioactive incorporation'').

The amounts of OSI-7836 incorporated into H460 or Calu-6 xenograft and splenic DNA were determined following single and repeat doses of OSI-7836. Mice were dosed on days 1, 4, 7 and 10 with 0, 400, 1000, or 1600 mg OSI-7836/kg. Tumors and spleens (400 mg/kg dose only) were collected 24 h after each dose (days 2, 5, 8 and 11). DNA was isolated and hydrolyzed, and DNA incorporation was quantified following methods described below (''DNA isolation and enzymatic hydrolysis'', ''Quantification of OSI-7836 incorporation'').

The time course of OSI-7836 incorporation into Calu-6 xenograft DNA was determined following a single dose of OSI-7836. Mice bearing Calu-6 xenografts were given one 400 mg/kg dose of OSI-7836, and tumors were collected at 6, 24, 48 and 72 h after dosing. DNA was isolated and hydrolyzed, and DNA incorporation was quantified following methods described below (''DNA isolation and enzymatic hydrolysis'', ''Quantification of OSI-7836 incorporation'').

Cell replication and apoptosis studies were conducted to elucidate the kinetics of incorporation of OSI-7836 and the short-term effects of the compound on tumor xenografts. Mice bearing Calu-6 xenografts were dosed with a single dose of 100, 400, 1000 or 1600 mg OSI-7836/kg. Tumors were collected at 6, 24, 72 and 96 h after dosing and processed as described below (''Cell replication and apoptosis'').

DNA isolation and enzymatic hydrolysis

DNA was isolated from tissue and cells using Qiagen G-500 DNA isolation columns (Qiagen Sciences, Germantown, Md.) followed by phenol/chloroform extraction. Purified DNA (approximately 100 μl or 1 μg/μl solution) was enzymatically hydrolyzed to the nucleotides and nucleosides using two separate methods. To determine whether OSI-7836 was internally incorporated, DNA was hydrolyzed [20 μg DNA, 2.4 μl 10× MCN buffer, 2.5 μl micrococcal nuclease (nuclease S7, Roche Diagnostics, Indianapolis, Ind.) and H_2O to 24 μl final volume] at 37°C for at least 3 h followed by addition of: 2.6 μl 0.2 *N* HCl and 0.5 μl calf intestinal phosphatase (phosphodiesterase II; Sigma-Aldrich, St Louis, Mo.) and incubation at 37°C overnight. The sample was then divided and a portion (13 μl) treated with bacterial alkaline phosphatase (2 μl of 100 m*M* Tris-HCl, pH 8.0 + 1 μl 0.16 U bacterial alkaline phosphatase; Sigma-Aldrich) + 4.5 μl H_2O for 4 h at 37°C. The difference between the amount of OSI-7836 (determined by LC-MS/MS) in the phosphorylated and dephosphorylated sample was used to determine the extent of internal incorporation. To determine the total amount of OSI-7836 incorporated into xenograft DNA, DNA was hydrolyzed to nucleosides using DNAse I (Sigma-Aldrich), snake venom phosphodiesterase (Worthington, Freehold, N.J.), and bacterial alkaline phosphatase (Sigma-Aldrich) as described previously [15]. The amount of OSI-7836 in each sample was quantified using the LC-MS/MS method described below (''Quantification of OSI-7836 incorporation''). The amount of dThy in each sample was quantified using a Hewlett Packard 1100 HPLC with UV detection (Supelcosil 18-S C-18 25 cm × 4 mm, flow rate 1.5 ml/min, isocratic 10% MeOH in water; 254 nm wavelength UV detection).

Quantification of radioactive incorporation

The amount OSI-7836, dFdC, and ara-C incorporated into DNA was quantified using a Hewlett-Packard 1100 HPLC with diode array detection (Supelcosil 18-S C-18 4 mm × 25 cm) with a flow rate of 1.5 ml/min. For OSI-7836, buffer A was 50 m*M* KH_2PO_4 (pH 6.5) and buffer B was 100% methanol, and for ara-C, buffer A was 25 m*M* KH_2PO_4 (pH 6.5) and buffer B was 100% methanol. The gradient for OSI-7836 was 0–8 min 5/95 B/A and 8.5–11 min 20/80 B/A, and for ara-C was 0–6 min 0/100 B/A, 6.5–8 min 20/80 B/A, and 8–9.5 min 0/100 B/A. Fractions were collected into scintillation vials every 30 s using a RediFrac fraction collector (Pharmacia LKB Biotechnology, Alameda, Calif.), and radioactivity quantified using an LS6500 liquid scintillation counter (Beckman). Ara-C, dFdC, OSI-7836, and dThy eluted at approximately 4, 7, 7, and 11 min, respectively. The amount of modified nucleoside was normalized to the amount of incorpo-

rated dThy and the dose of modified nucleoside administered.

Quantification of OSI-7836 incorporation

The amount of OSI-7836 in DNA was quantified using a newly developed LC-MS/MS method. DNA hydrolysate (50 µl) was spiked with 50 µl internal standard OSI-8530 (2-^{13}C-1,3-$^{15}N_2$-1-(4′-thio-β-D-arabinofuranosyl)cytosine; 4 ng/ml in acetonitrile/water 1:4 v/v) and with 1.425 ml 0.1 *M* ammonium acetate (pH 8.5). Each sample was applied to a C18 SPE cartridge that was equilibrated twice with 2 ml methanol followed by 2 ml 0.1 *M* ammonium acetate (pH 8.5), washed with 1 ml water and 1 ml hexane, eluted with 2 ml methanol, dried, and reconstituted in 50 µl LC mobile phase buffer. A 5-µl aliquot of the sample was injected onto a Micromass Quattro LC-MS/MS (Manchester, UK) operating in a positive ion mode that preseparated nucleosides on a Supelcosil 18-S C-18 reverse-phase column. The flow rate was 0.2 ml/min. Buffer A was 95/5/0.1 water/methanol/1 *M* NH_4 acetate, buffer B was 5/95/0.1 water/methanol/1 *M* NH_4 acetate, and the gradient was A/B 100/0 from 0 to 4 min, A/B from 100/0 to 0/100 from 4 to 7 min, A/B 0/100 maintained until 10 min, return to initial condition from 10 to 11 min, and equilibrate for 9 min. The sample eluted from the column at approximately 6 min (Fig. 2).

The peak areas of the *m/z* 260–112 product ion of OSI-7836 and *m/z* 263–115 product ion of OSI-8350 were determined. Quantification was performed using a weighted ($1/x^2$) linear least squares regression analysis generated from a standard curve of spiked salmon testes DNA (see below) or spiked Calu-6 DNA hydrolysate. All analytical runs were evaluated on the following acceptance criteria: a minimum of five calibration standards within ±25% absolute deviation with no more than two standards excluded from the curve and at least two-thirds of quality control samples and 50% of each level within ±25% of the nominal values, if quality control samples were analyzed. The number of picomoles of OSI-7836 in each sample was then normalized to the micromoles of dThy per sample.

Cell replication and apoptosis

Tumors and tissues from OSI-7836-treated mice were fixed in 10% neutral buffered formalin for 24 h then placed in 70% ethanol until tissues could be processed and embedded in paraffin. Blocked tissues were sectioned at approximately 4 µm and slides were stained with hematoxylin and eosin. The number of apoptotic and mitotic tumor cells (Fig. 3) in approximately 500 neoplastic cells was quantified by histological examination. Results are expressed as percent of neoplastic cells that were undergoing mitosis (an approximation of cell replication) or apoptosis.

Results

In vitro cytotoxicity assays

Results from the cytotoxicity assays are presented in Fig. 4. The amount of OSI-7836-induced apoptosis, as determined by Hoechst staining, was significantly less in

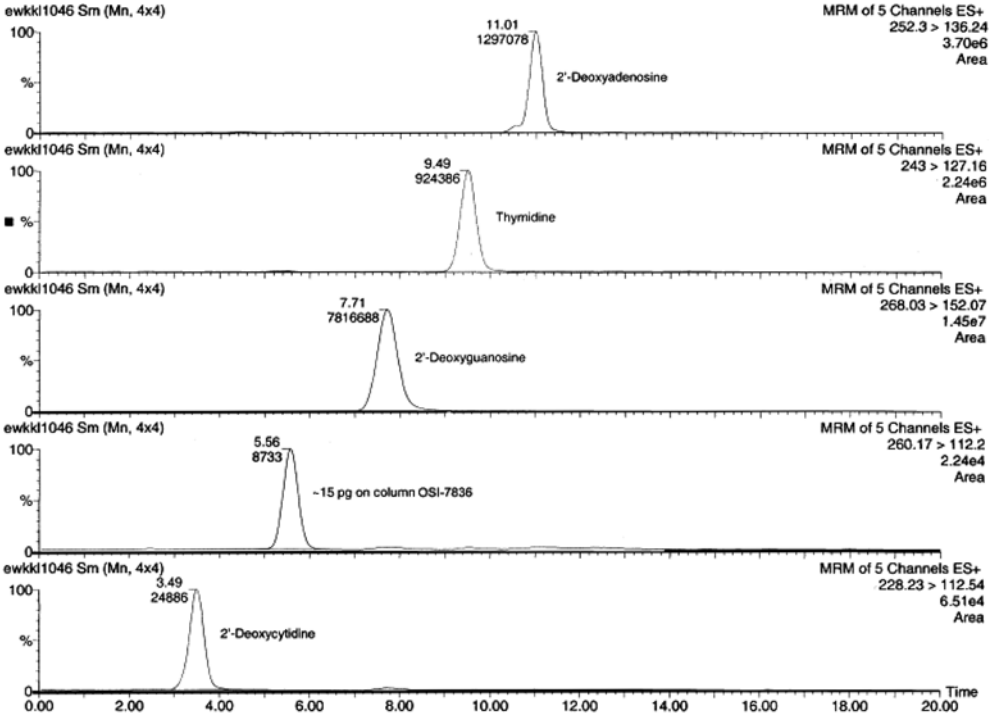

Fig. 2 Sample chromatogram of enzymatically hydrolyzed salmon testes DNA spiked with OSI-7836 sufficient for a 15 pg on-column injection

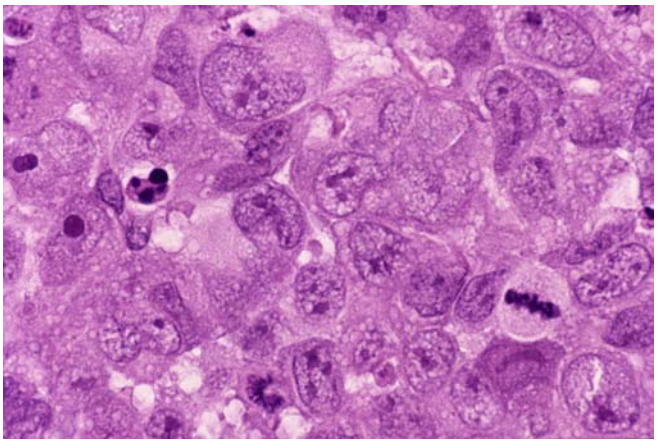

Fig. 3 Example of a mitotic figure and an apoptotic body in a Calu-6 xenograft tumor. These histological morphologies were used to measure the effect of OSI-7836 on mitosis and apoptosis in Calu-6 tumors

confluent non-cycling A549 cells than in log-phase growth cells. In order to determine whether this difference was due to suppression of basal levels of drug activation (phosphorylation) in non-cycling cells, OSI-7836-TP levels were measured. While there was a slight decrease in the amount of OSI-7836-TP formed in the confluent, non-cycling cells compared to that in the log-phase cells, the difference was not enough to account for the observed decrease in apoptosis. Collectively, these results support the hypothesis that DNA synthesis/cell replication is a necessary process in the cytotoxic mechanism of OSI-7836.

DNA incorporation

DNA incorporation was measured using traditional radioactive methods and a newly developed LC-MS/MS method. The radioactive method was useful to compare the amount of DNA incorporation across the three compounds since non-radioactive methods were not available for dFdC and ara-C; however, none of the nucleosides could be administered at therapeutically relevant doses because of a significant dilution of the specific activity.

Following three consecutive days of subtherapeutic intravenous doses, the incorporation of OSI-7836 was significantly less than that of dFdC and ara-C (Fig. 5). Based on work in CEM cells, the lower amount of incorporation compared to that of ara-C was most likely due to the lower amount of OSI-7836-TP formed. It is also possible that the lower incorporation of OSI-7836 was caused by its greater ability to inhibit DNA synthesis/cell replication, but this seems unlikely at these low doses which were in the range 0.47–1.28 μg/day (the therapeutic dose in these models was in the range 10–30 mg/day depending on the schedule of administration).

A new method for quantifying the incorporation of OSI-7836 into DNA was developed in order to allow quantification of incorporated drug following dosing

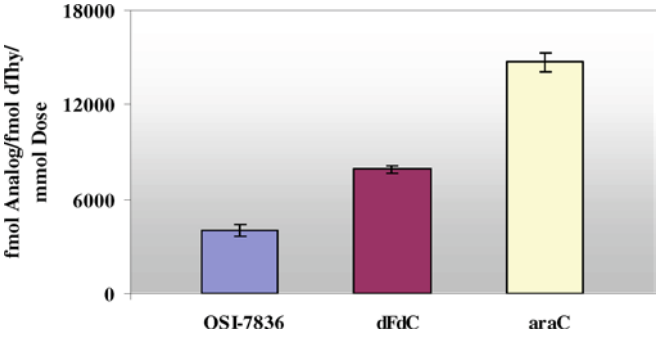

Fig. 5 Incorporation of ^{3}H-OSI-7836, ^{3}H-dFdC or ^{3}H-ara-C into DNA of Calu-6 xenografts following treatment for 3 days with 40 μCi/mouse per day ($1.5–5.2\times10^{-6}$ mmol/day) and normalized to incorporation of ^{3}H-dThy and dose of nucleoside analog (the data are presented as means ± SD of three animals; see "Animal dosing" and "Quantification of radioactive incorporation" for details)

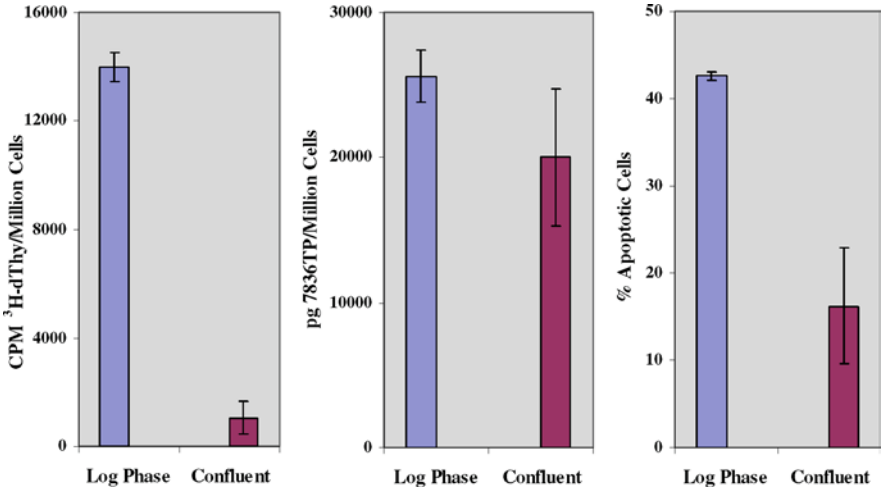

Fig. 4 **a** The amount of ^{3}H-dThy in TCA-precipitable material from either log-phase or confluent A549 cell cultures treated with 10 μ*M* OSI-7836 for 72 h. **b** The amount of OSI-7836 triphosphate present in either log-phase or confluent A549 cell cultures treated with 10 μ*M* OSI-7836 for 72 h (see "Cytotoxicity assays" for details). **c** The percent of apoptotic per normal cells in log-phase or confluent A549 cell cultures treated with 10 μ*M* OSI-7836 for 72 h

Table 1 Calibration curve for LC-MS/MS detection and quantification of OSI-7836 using hydrolyzed salmon testes DNA spiked with OSI-7836

	Theoretical amount (pg) of OSI-7836 applied to the column							
	0.25	0.5	1.0	2.0	5.0	10.0	25.0	60.0
Actual amount on-column (pg)	0.242	0.548	1.16	1.98	4.93	10.1	22.5	61.3
	0.229	0.549	0.94	2.10	5.23	9.84	25.1	58.8
	0.262	0.456	4.47[a]	1.87	4.65	10.2	24.1	63.3
Mean amount on-column (pg)	0.244	0.518	1.05	1.98	4.94	10.0	23.9	61.1
SD ($n-1$)	0.017	0.053	–[b]	0.12	0.29	0.2	1.3	2.3
CV (%)	6.8	10.3	–[b]	5.8	5.9	1.8	5.5	3.7

[a]Data not included in calculations following SOP guidelines
[b]Insufficient data to calculate

with therapeutically relevant doses. The method, based on LC-MS/MS, had an on-column limit of detection of OSI-7836 in hydrolyzed DNA samples of 0.25 pg or approximately 1 fmol and an on-column linear range of up to 60 pg (Table 1). Based on a 5-µl injection of 1 µg/µl DNA hydrolysate, the limit of sensitivity was estimated to be approximately 60 fmol OSI-7836/µmol dThy. Additional work (data not shown) was done to determine the effect of injection volume on the assay. At least 20 µl (data not shown, 20 µl was the maximum volume tested) was injected without untoward effects on quantification. There have been considerable efforts to use LC-MS/MS to quantify the formation of DNA adducts caused by chemical and radiation exposure with limits of sensitivity of the order of a femtomole to a fraction of a femtomole depending on the adduct [9]. In contrast there has been comparatively little work reported that has used LC-MS/MS to detect and quantify the incorporation of nucleoside analogs into DNA. Previous work with 2′-fluorouridine was able to achieve an on-column limit of detection of 2′-fluorouridine in the low femtomole range but only about 2–6 pmol/µmol dThy could be detected when it was in the DNA hydrolysate [15]. The method described here was comparable in on-column sensitivity to the LC-MS/MS method developed for 2′-fluorocytidine and 2′-fluorouridine. However, we consider this method superior for quantification in DNA hydrolysate, primarily because there was less background interference from the hydrolysate.

OSI-7836 was detected in DNA of tumors of mice following treatment with therapeutic doses. Using micrococcal nuclease and calf intestinal phosphodiesterase, OSI-7836 located on the terminal ends of DNA was released as OSI-7836 while internal OSI-7836 was released as OSI-7836-MP, which was not quantified using the LC-MS/MS method. Subsequent treatment with alkaline phosphatase converts all OSI-7836 MP to OSI-7836. Therefore, the extent of internucleotide incorporation of OSI-7836 was determined by comparing the amount of OSI-7836 in samples with and without alkaline phosphate treatment. Figure 6 shows a considerably greater amount (from approximately threefold to tenfold) of OSI-7836 in alkaline phosphatase-treated samples, indicating that 60–90% of the OSI-7836 present in DNA 24 h following treatment was located at internucleotide linkages.

OSI-7836 was present at greater concentrations in tumor DNA than in splenic DNA 24 h following 1, 4, 7, or 10 days of dosing (Fig. 7). Interestingly the amount of OSI-7836 did not appear to increase over the duration of repeated dosing in either spleen or tumor DNA. In addition, increasing a single dose of OSI-7836 from 400 to 1000 mg/kg did not appear to increase the amount of OSI-7836 present in DNA 24 h later (Fig. 8).

Based on these findings it was hypothesized that OSI-7836 concentrations in DNA did not increase over the 3 days because the OSI-7836 in the DNA was either removed or diluted by cell replication. To test this hypothesis mice bearing Calu-6 tumors received a single dose of OSI-7836 and tumors were collected at various time points thereafter. Surprisingly, the concentration of OSI-7836 in tumor DNA increased with time but remained dose-independent (Fig. 9).

Since the incorporation of OSI-7836 into DNA depends on DNA synthesis, we were curious to know whether this lack of accumulation with repeat administration and lack of dose-response may somehow have been related to the effect of the compound on DNA synthesis. Therefore cell replication and apoptosis were measured in Calu-6 tumors at various times after OSI-7836 administration (Fig. 10). Within 24 h of dosing,

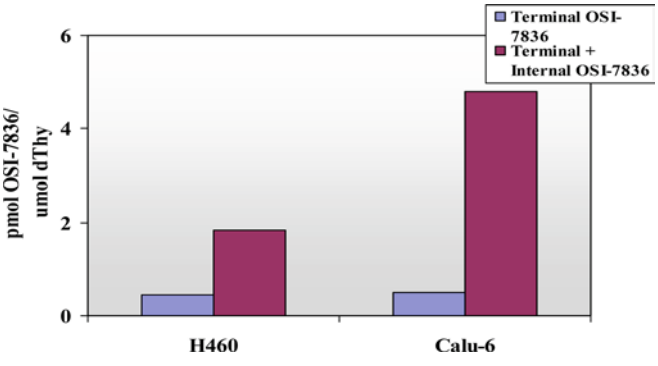

Fig. 6 Concentrations of terminally located and total OSI-7836 in DNA from tumors of mice dosed with 1600 mg OSI-7836/kg. DNA was enzymatically hydrolyzed with micrococcal nuclease and calf intestinal phosphodiesterase to release terminal OSI-7836 and then with bacterial alkaline phosphatase to convert OSI-7836-TP from internal linkages to OSI-7836 to generate terminal + internal OSI-7836 (the data shown are the means of two animals; see "DNA isolation and enzymatic hydrolysis" and "Quantification of OSI-7836 incorporation" for details)

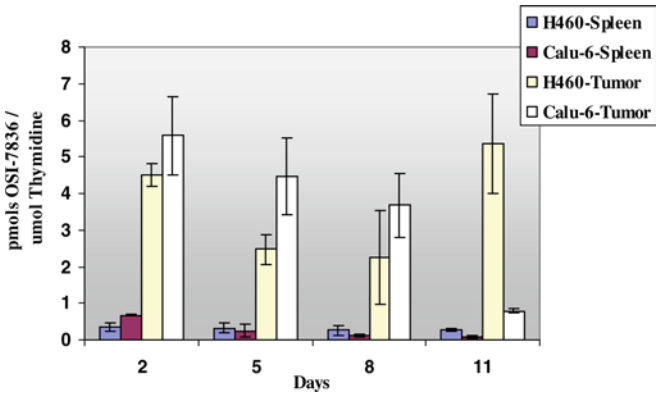

Fig. 7 Concentrations of OSI-7836 in Calu-6 and H460 tumor DNA and corresponding splenic DNA collected from mice dosed with 400 mg/kg on days 1, 4, 7, and 10 (mean of three animals). Incorporation was quantified using LC-MS/MS (see "DNA isolation and enzymatic hydrolysis" and "Quantification of OSI-7836 incorporation" for details)

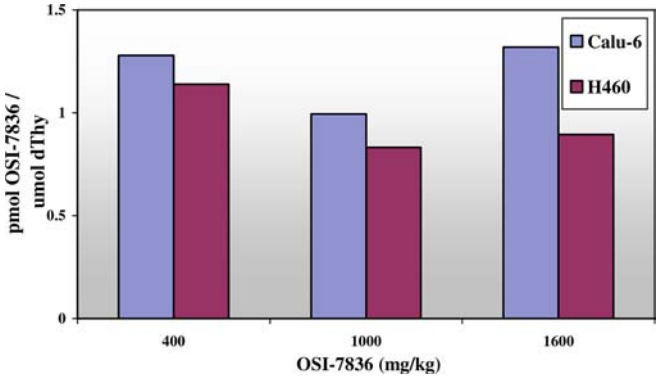

Fig. 8 Concentrations of OSI-7836 in Calu-6 and H460 tumor DNA collected from mice 24 h after doses of 400, 1000, or 1600 mg OSI-7836/kg (mean of two animals). Incorporation was quantified using LC-MS/MS (see "Materials and methods" for details)

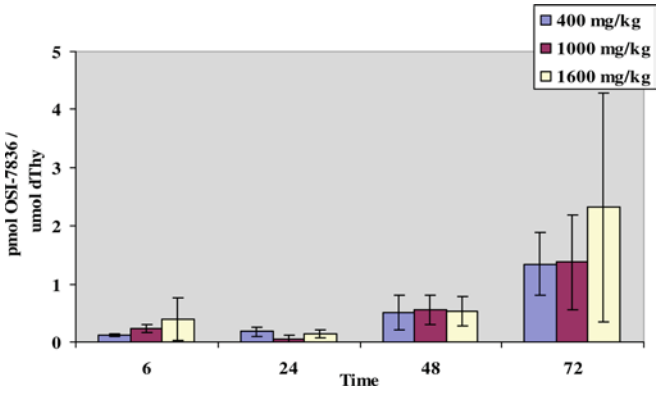

Fig. 9 Concentrations of OSI-7836 in tumor Calu-6 DNA of mice 6, 24 and 72 h after a single dose of 400, 1000 or 1600 mg OSI-7836/kg (mean and SD of three animals). Incorporation was quantified using LC-MS/MS (see "DNA isolation and enzymatic hydrolysis" and "Quantification of OSI-7836 incorporation" for details)

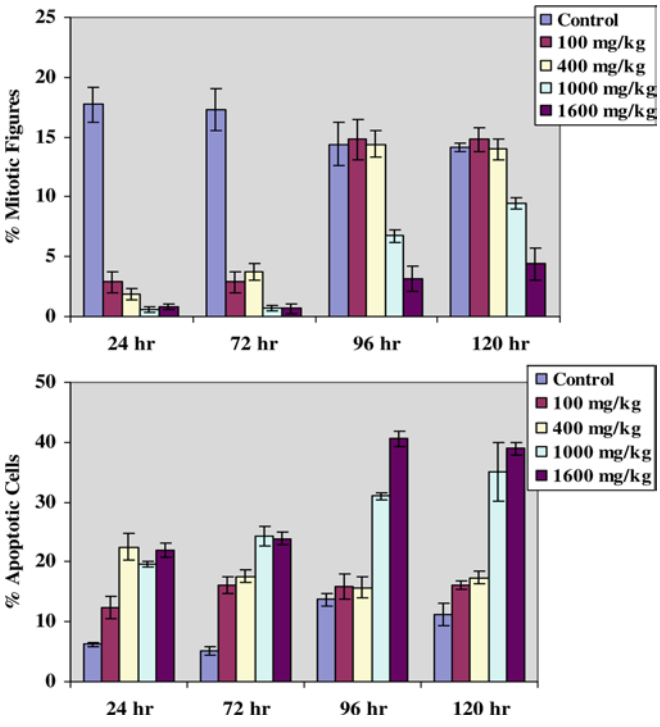

Fig. 10 Effects of OSI-7836 on cell replication and apoptosis in Calu-6 tumors treated with 100, 400, 1000 or 1600 mg OSI-7836/kg (mean and SD of three animals; severe necrosis was present at 400, 1000 and 1600 after 72 h)

the mitotic indices were dramatically reduced at all doses. The effect was still apparent at 72 h and a clear dose-response started to emerge at 96 h. Apoptosis was also dose-dependent but plateaued by 24 h when adjusted for increases in control samples. In most cases the rate of apoptosis was greater than mitosis following treatment. This was not unexpected since the apoptotic process generally takes considerably longer than mitosis.

Discussion

OSI-7836 is an effective antitumor agent against human xenografts including Calu 6 and H460. Previous work with CEM cells has indicated that the triphosphate of OSI-7836 is required for activity and that accumulation of OSI-7836-TP is much slower and much less extensive than that for ara-C [11], although the difference in the rate of phosphorylation by isolated deoxycytidine kinase is small [19]. Data presented here using A549 NSCLC cells indicate not only that DNA synthesis and cell replication are required for OSI-7836 toxicity (supporting the role of DNA incorporation and/or DNA polymerase inhibition in the antitumor activity) but also that apoptosis is a primary mechanism of cell death. These results are supported by the in vivo observations that mitosis was rapidly reduced following OSI-7836 administration and apoptosis was increased in a dose-dependent manner.

Ara-C is considered a strong chain terminator in vitro but is efficiently incorporated into internucleotide linkages using DNA synthesomes [25] and in intact cells, suggesting that ara-C is not an efficient chain terminator in vivo where the complete replication machinery is present. Similarly, in vitro polymerase studies have shown that following incorporation into DNA, dFdC allows one additional nucleotide to be inserted before significantly reducing synthesis [12], while the DNA synthesome and cell culture studies have demonstrated that dFdC is incorporated internally [6, 17] and is more effective than ara-C at inhibiting DNA synthesis. Similarly, OSI-7836 is also a strong chain terminator in DNA polymerase assays [2, 16] and is predominantly incorporated into internal positions when administered to CEM cells. In the experiments reported here, we demonstrated that when administered to animals, OSI-7836 was also incorporated into tumor DNA primarily at internucleotide linkages. We also showed that at subtherapeutic doses ara-C was incorporated into Calu-6 tumors to a greater extent than dFdC and OSI-7836. The percent of internal vs terminal incorporation was not determined for dFdC and ara-C in these xenografts; however, in H460 and Calu-6 cells, the ratio of internal to terminal incorporation is approximately 12 for dFdC and 3–6 for ara-C and OSI-7836 (unpublished data). Whether this relationship in overall incorporation observed at low doses remains intact at therapeutic doses is not known and will require the development of sensitive non-radioactive detection methods for ara-C and dFdC, for which LC-MS/MS would seem a likely candidate.

Using this newly developed LC-MS/MS method, the incorporation of OSI-7836 into tumor DNA was found to be dose- and time-independent during repeat dosing and dose-independent following a single dose. This was somewhat surprising since there was a dose-response for cell replication, apoptosis, and tumor inhibition (data not shown). We propose that this lack of dose-dependence was caused by a dose-dependent decrease in cell replication coupled to an assumed dose-dependent increase in intracellular OSI-7836-TP concentrations. The continued accumulation of OSI-7836 in DNA after a single dose, and after many plasma half-lives, suggests that OSI-7836, most likely in the triphosphate form, remains in the tumor cells long after blood levels have been reduced and reflects the upswing in mitosis over 24–72 h. The persistence of OSI-7836-TP in an intact solid tumor cell line has been described and proposed as a possible mechanism for the efficacy of OSI-7836 in solid tumor xenografts [11].

DNA incorporation correlates with and has been proposed as the primary mechanism involved in the toxicity of many nucleoside analogs. These studies have provided an in-depth examination of this relationship for OSI-7836 and have yielded very complex results. The cell culture studies described here indicate that DNA synthesis is required for toxicity, implying that incorporation may be a key event. In vivo results, however, show that incorporation is not dose-dependent and is delayed, with maximum incorporation occurring after cell replication is restored and apoptotic rates have reached a plateau. If there was a one-to-one correspondence between the extent of incorporation and toxicity, then one would expect cell replication to remain depressed and apoptosis to increase with increasing concentrations of OSI-7836 in the DNA. What then is the relationship between DNA incorporation of OSI-7836 and toxicity as observed in these studies? There are several possibilities. First, incorporation itself is not required and the phosphorylated form of OSI-7836 is directly responsible for toxicity through another mechanism such as inhibition of ribonucleotide reductase (RR), already proposed as a secondary mechanism of dFdC toxicity [5]. Preliminary results indicate, however, that the effect of OSI-7836 on RR is not greater than that of ara-C and is much less than that of dFdC (personal communication, William Parker, Southern Research Institute, Birmingham, Ala.). Second, as already proposed, OSI-7836-TP interferes with DNA polymerase very shortly after incorporation, interfering with S-phase. This pausing in DNA synthesis could activate cellular processes that trigger apoptosis in some cells. Other cells, however, could continue to replicate entering apoptosis at a rate that results in a plateau in apoptosis. Third, the presence of OSI-7836 in internucleotide linkages (the majority of incorporation measured) triggers cellular responses that lead to apoptosis and/or toxicity. NMR studies on the structural analog 4′-thio-T have demonstrated little perturbation of the DNA structure [3]. However, effects of OSI-7836 and thio-analogs on methyltransferases [1, 10], nuclease sensitivity [7] and OSI-7836 induction of G_2/M rather than S-phase arrest [2] suggest that OSI-7836 is not inert when present in internucleotide linkages.

In summary, these studies demonstrate that OSI-7836 is incorporated into human tumor xenograft DNA following treatment with therapeutic doses of the compound, supporting the qualitative concept that incorporation may play a role in efficacy and/or toxicity. However, quantitative comparisons based on time and dose do not show a straightforward relationship between toxicity/efficacy and concentrations of OSI-7836 in DNA. Therefore while the qualitative relationship between incorporation and toxicity/efficacy may exist, it is overly simplistic to state that there is a quantitative relationship between whole genome DNA incorporation and these endpoints.

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