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Cell Cycle-Dependent Inhibition of the Proliferation of Human Neural Tumor Cell Lines by Iron Chelators

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ABSTRACT. The current studies were designed to examine the conditions under which the ferric iron chelator desferrioxamine (DFO) arrested cell cycle progression and hence the proliferation of neural cell lines *in vitro*. DFO arrested proliferation at different stages of the cell cycle depending on the concentration and duration of drug exposure. Twenty-four-hour treatment with 160 μM DFO arrested glioma cells in G_1, whereas 72-hr treatment with 10 μM DFO acted to slow the passage of glioma cells through the cell cycle, eventually accumulating in G_2/M. Another iron chelator, ADR 529, also inhibited the proliferation of glioma cells by lengthening the period of the cycle and causing the cells to arrest in G_2/M. The effects of 10 and 160 μM DFO were irreversible after 24 and 48 hr, respectively, and 10 μM DFO became cytotoxic after 3 days. These observations demonstrate that DFO has different effects on the proliferation of neural tumor cell lines depending on the concentration and time of exposure, which result in different sites of cell cycle arrest. These different *in vitro* actions of DFO may have ramifications for the successful application of iron chelator therapy *in vivo*. BIOCHEM PHARMACOL 51;11:1553–1561

KEY WORDS. iron chelators; neural tumors; desferrioxamine; cell cycle; DNA synthesis

DFO† is an iron chelator with high affinity for ferric iron and is used extensively in the treatment of iron overload [1]. In addition, DFO is a potent antiproliferative agent *in vitro*, which preferentially arrests the proliferation of particular types of neoplastic cells [2–4]. These observations have stimulated interest in the use of DFO as an antineoplastic drug. However, the efficacy of DFO as an antineoplastic agent *in vivo* has at best been equivocal [5–8]. DFO has a short plasma half-life [9]; therefore, one possible explanation for the lack of success with DFO as an antineoplastic drug is that this agent acts in a cell cycle-dependent manner and so is not present for a sufficiently long enough period to arrest all the cells. In support of this hypothesis, in those studies that reported the successful anti-neoplastic activity of DFO *in vivo*, the drug was administered by continuous infusion [5–7]. Hence, elucidating the cell cycle-dependent action of DFO is important for the successful application of this drug for chemotherapy.

DFO is thought to attenuate proliferation by inhibiting the activity of the iron-requiring enzyme ribonucleotide reductase (ribonucleoside-diphosphate reductase, EC 1.17.4.1), thereby limiting the supply of deoxyribonucleotides available for DNA synthesis [10–13]. One consequence of inhibiting DNA synthesis would be an accumulation of cells at the G_1/S interface of the cell cycle which has been observed in a variety of cell types [12, 14–16], including neural tumor cell lines [17–19]. However, other groups have reported that DFO also arrests cells at the G_2/M phases [12, 20, 21], consistent with the action of other iron chelators, such as ADR 529 [ICRF 159, (±) 1,2-di(3,5-dioxopiperazin-1yl)propane, NSC-120,943, Razoxane] [22–27]. These observations suggest the existence of more than one iron chelator-sensitive site of arrest in the cell cycle that can be accessed by DFO. Determining the basis of these differences in the action of iron chelators may account for the variable responses that neoplastic cells have to these agents and may suggest more efficacious chemotherapeutic regimes. Therefore, the aim of this study was to investigate the sites of cell cycle arrest produced by DFO.

Materials and Methods

Cell Culture

Two glioma (2607 and 2981) cell lines and one neuroblastoma (DAOY) cell line were obtained from Mr. Philip Jacobsen (Department of Neuropathology, Royal Perth Hospital, Western Australia, Australia) [28]. All experiments were performed using cells within 20 passages of the initial passage received. The cells were cultured in modified Eagle's medium supplemented with Earle's salts (Gibco Laboratories, Santa Clara, CA), 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO), 10% heat-inactivated FBS, streptomycin (100 μg/mL) and penicillin (100 μg/mL), all from Commonwealth Serum Laboratories, Melbourne,

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† *Abbreviations:* DFO, desferrioxamine; and FBS, fetal bovine serum.

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Australia. Under these conditions the doubling times of DAOY, 2607, and 2981 cells were 24, 25, and 29 hr, respectively.

Drug Treatments

Cells were seeded from logarithmically growing cultures at 6.5×10^3 cells/mL/cm^2, irrespective of the volume of the culture vessel used. Following seeding, cells were allowed to attach for 6 hr prior to the beginning of any treatments, including serum deprivation. Subsequently, DFO (desferrioxamine mesylate, Ciba Geigy, Sydney, NSW, Australia) was added at 0, 24, and 48 hr after the cell attachment period, and the experiments were terminated at 72 hr. The iron chelators and iron salts were prepared immediately prior to use. DFO was dissolved as a 3.2 or 12.8 mM solution in either PBS (140 mM NaCl, 0.35 mM KH_2PO_4, Na_2HPO_4) or tissue culture medium and diluted as required. Similarly, ADR 529 (donated by Farmitalia Carlo Erba, Milan, Italy) was dissolved as a 50 mM solution in PBS at 37°. Ferric and ferrous iron were prepared from their ammonium sulfate salts (Sigma). In experiments where the medium was replaced, the cells were washed twice with fresh medium, which in the case of the FBS deprivation experiments was devoid of serum.

Measures of Proliferation and Viability

Cellular proliferation was measured in terms of either DNA synthesis or cell enumeration. DNA synthesis was measured by the incorporation of [*methyl*-^{3}H]thymidine (ICN Flow Inc., Irvine, CA). For these experiments cells were seeded in a volume of 200 μL/well and at a density of 1.25×10^4 cells/mL in 96-well flat-bottomed plates (ICN). [^{3}H]Thymidine at 1 μCi/well was applied for the final 4 hr of the culture period indicated in the text. Labeled DNA was precipitated onto glass fiber discs using a PHD harvester (Cambridge Technology Inc., MA), and the discs were suspended in 2 mL of Optifluor-O liquid scintillant (Canberra Packard, Sydney, NSW, Australia) to allow counting of the β-emissions.

For the enumeration experiments, the cells were detached with 0.05% trypsin, 0.02% EDTA, 100 mM PBS, at 37°, following a PBS wash after which the trypsin and EDTA were inactivated with spent tissue culture medium. Cells were collected by centrifugation at 200 *g* for 5 min at room temperature and counted on a Neubauer hemocytometer. Viability was determined by the exclusion of eosin, present at a final concentration of 0.1% (v/v). At least 100 viable cells were counted per treatment in each experiment.

Cell Cycle Analysis

The frequency of cells in the various cell cycle phases was determined by enumerating the distribution of nuclei containing double-stranded DNA, using a Becton-Dickinson FACScan flow cytometer (San Jose, CA). For these experiments, cells grown in either 25 or 75 cm^2 flasks were harvested and resuspended in 2 mL of tissue culture medium at a concentration of 10^6 cells/mL. Diploid nuclei were then stained with propidium iodide (Sigma), in the presence of Triton X-100 (Sigma) at final concentrations of 50 μg/mL and 0.2%, respectively. Staining due to RNA was obviated by the addition of Ribonuclease A (Type I, Sigma) to a final concentration of 0.2 mg/mL. The reactants were left on ice for at least 5 min prior to filtration through an 80 μm mesh. All of the above harvesting and staining procedures were performed on ice.

It should be noted that the cell cycle distribution of DFO-treated cells was compared with that of untreated cells; cells were harvested within one cell doubling of drug application because (i) this was the period during which 10 or 160 μM DFO acted to arrest proliferation, and (ii) the proportion of untreated cells in G_0/G_1 increased relative to those in G_2/M or S with increasing time in culture. Analysis of the frequency of cells in the cell cycle phases was performed using the Cellfit software (Becton-Dickinson). During acquisition, the data were gated to exclude doublets, using the manufacturer's method.

Statistics

The differences between treated and untreated groups were examined by Analysis of Variance, while any estimates of Goodness of Fit were made using the Chi-Squared test.

RESULTS

Inhibition of the Growth of 2607 Glioma Cells by DFO

DFO inhibited DNA synthesis in 2607 cells in a manner that depended on both the concentration and duration of treatment (Fig. 1a). The DFO concentrations required to inhibit proliferation by 50% (IC_{50}) decreased geometrically as a function of increasing drug exposure (Fig. 1b), with mean values of 2.55, 4.45, and 19.4 μM for incubations of 72, 48, and 24 hr, respectively. The IC_{50} values fitted the expression $IC_{50} = 19.40^{25\ hr/2^{x}\ hr}$ ($P < 0.001$), where 19.40 is the mean IC_{50} after a 24-hr incubation with DFO, 25 hr is the doubling time of 2607 cells, and x the incubation period with DFO. This observation indicates that the amount of DFO required to arrest the cells is functionally related to the number of cell doublings and, therefore, the cell cycle. One implication of this result is that DFO is able to arrest the growth of 2607 cells within the first cell-doubling period of its application. This was confirmed by the demonstration that a 24-hr exposure of the cells to DFO attenuated the expected doubling in cell density (Fig. 2a). Similarly, the density of 10 μM DFO-treated cells did not increase over a 72-hr incubation period (Fig. 2b). As had been shown with the thymidine incorporation studies (Fig. 1), the effects of DFO on cell density depended on both the concentration and period of incubation (Fig. 2). Furthermore, the maintenance of cell viability, at 24 or 72 hr (Fig. 2), indicated that inhibition of proliferation measured by

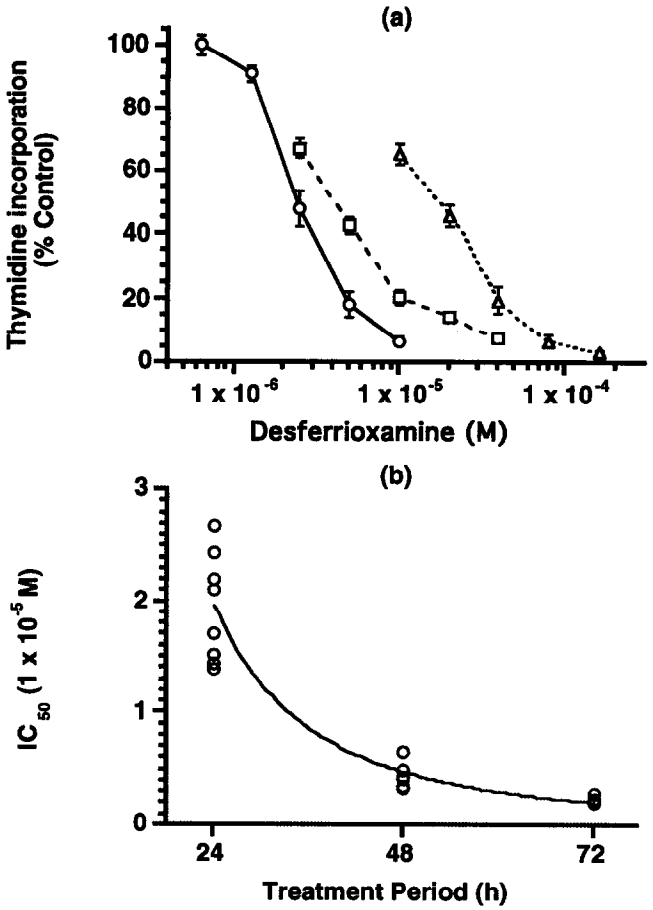

FIG. 1. Inhibition of the proliferation of 2607 cells by DFO as a function of time and drug exposure. Cells were exposed to DFO for 24 (△), 48 (□), and 72 (○) hr, and [^{3}H]thymidine incorporation was measured 72–76 hr post-seeding. The percent control mean ± SEM values for 8 (24 hr), 7 (48 hr) and 5 (72 hr) experiments are shown in (a) and were calculated from the following thymidine incorporation values: 38,100 ± 9,700 (24 hr), 38,090 ± 8,940 (48 hr) and 37,323 ± 9,860 (72 hr) dpm. The IC_{50} values were determined from the individual experiments used for part (a) and are shown as the open symbols in (b). The curve represents the predicted IC_{50} values according to the function $IC_{50} = 19.40^{\frac{25\ hr/2x\ hr}{}}$, where 19.40 is the mean IC_{50} after 24-hr incubation with DFO, 25 hr the doubling time of 2607 cells, and x the incubation period.

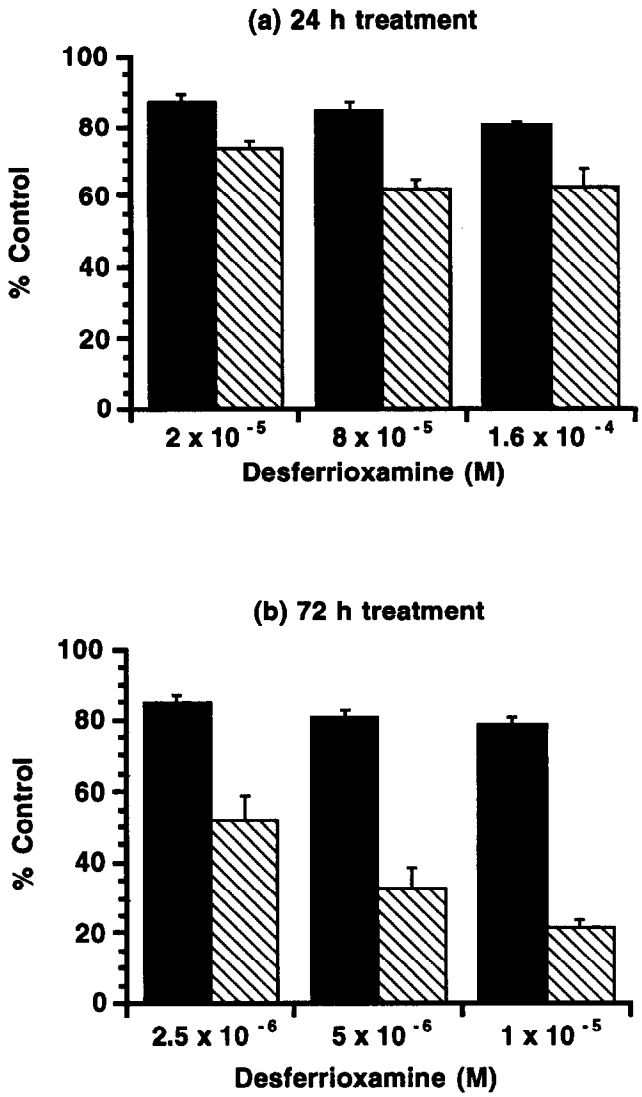

FIG. 2. Effect of DFO on the density and viability of 2607 cells. The effects of DFO on glioma cell density ▨ and viability ■ were measured following either 24 (a) or 72 (b) hr of treatment. The changes in cell density and viability are expressed relative to untreated cultures as percent control calculated using 467,280 ± 72,000 (24 hr) and 517,188 ± 36,557 (72 hr) viable cells as the control values. Shown are the mean and SEM values of 6 experiments for the 24- and 72-hr drug exposure periods.

either thymidine incorporation (Fig. 1) or cell enumeration (Fig. 2) was due to cytostasis rather than cytotoxicity.

Effects of DFO on the Cell Cycle Phase Distribution of 2607 Glioma Cells

DFO has previously been shown to arrest a leukemia cell line at different phases of the cell cycle as a function of DFO concentration [12]. The possibility that the concentration-dependent effects of DFO shown in Figs. 1 and 2 were explicable in terms of different sites of cell cycle arrest was examined by flow cytometry. To assess the magnitude of cell cycle arrest produced by DFO, its effects were compared with FBS deprivation, which is thought to cause a maximal accumulation of cells in the G_1 phase of the cell cycle. Lowering the FBS concentration in the tissue culture medium to 0.06% (v/v) completely arrested the cells within the first doubling period of 2607 cells without compromising the viability of the cells over 72 hr (data not shown). In addition, cells deprived of FBS for 24 hr accumulated in G_1 at the expense of cells in S or G_2/M (Fig. 3a). The application of 160 μM DFO for 24 hr also blocked the passage of 2607 cells into the S and G_2/M phases of cycle to approximately the same extent as FBS deprivation (Fig. 3a). In contrast, 10 μM DFO caused a significant proportion of cells to accumulate in G_2/M relative to untreated cells (Fig. 3b). Interestingly, the cell cycle distribution of FBS-

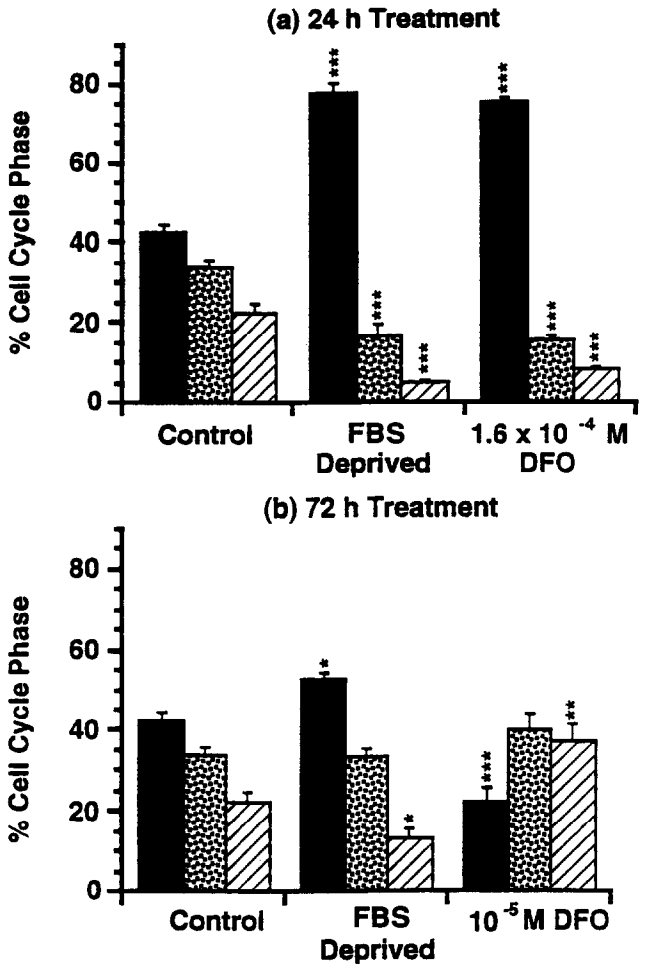

FIG. 3. Effect of DFO and FBS-deprivation on the cell cycle distribution of 2607 cells. 2607 Glioma cells were treated with 160 μM DFO for 24 (a) and 10 μM DFO for 72 (b) hr, and the resultant changes in cell cycle phase distribution were determined by flow cytometry. Similarly, the cells were FBS-deprived for the indicated periods. The cell cycle phases are shown as G_1 (■), S (▨), and G_2/M (▨) and as the mean and SEM of at least 7 experiments, with significant differences indicated by (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$.

deprived cells also accumulated in the later parts of the cycle with increasing time of serum starvation (Fig. 3). The FBS-deprived cells did not proliferate over 72 hr (data not shown).

The effects of DFO on the cell cycle of 2607 cells (Fig. 3) were similar using another glioma cell line, 2981, and a neuroblastoma cell line, DAOY (data not shown). DAOY cells were more sensitive to inhibition by DFO, and so an accumulation of G_2/M cells could be obtained with 72 hr of treatment with 1.25 μM DFO (data not shown).

The observation that 160 μM DFO prevented entry of cells into S phase and that 10 μM DFO-treated cells accumulated in G_2/M (Fig. 3) suggested that the low concentrations of DFO attenuated the passage of cells through S phase. When cells treated with 10 μM DFO were harvested at 24 hr post-drug addition, a significant number had accumulated in G_2/M within 24 hr, at the expense of cells in G_1 (Fig. 4). The proportion of DFO-arrested cells in G_2/M remained relatively constant between 24 and 72 hr post-DFO application, while the cells in G_1 declined by 72 hr, as compared with the untreated cells (Fig. 4). In contrast, the proportion of S phase cells remained constant over 72 hr of DFO administration. Since the cell density also remained constant, the latter result indicates that the rate of DNA synthesis was retarded and thus slowed the progress of cells through S phase. This conclusion is also supported by the studies shown in Fig. 1 where 10 μM DFO inhibited thymidine uptake at 24 and 48 hr by approximately 35 and 80%, respectively.

The observation that 10 μM DFO arrested the progression of 2607 cells through the cell cycle in G_2/M, after slowing their passage through S phase, suggested that other iron chelators that cause cells to accumulate in the latter phases of the cycle might have similar effects during S phase. ADR 529 arrests the proliferation of lymphocytes by arresting cells at G_2/M [27]. When applied to 2607 cells at 2.24 mM, ADR 529 inhibited the growth of 2607 cells without a significant loss of viability over 72 hr (data not shown). Over the same time period ADR also slowed the passage of cells through S phase causing them to accumulate in G_2/M by 72 hr (Fig. 5).

Recovery of 2607 Glioma Cells from DFO Treatment

Cells treated with DFO (10–160 μM) for 24 hr resumed thymidine incorporation within 4 hr of washing the cells free of DFO-containing medium (Fig. 6a). However, this resumption of DNA synthesis does not reflect complete recovery of the cells from DFO treatment because thymidine incorporation in cells treated with 160 μM DFO, washed, and cultured for a further 72 hr was 20% of the

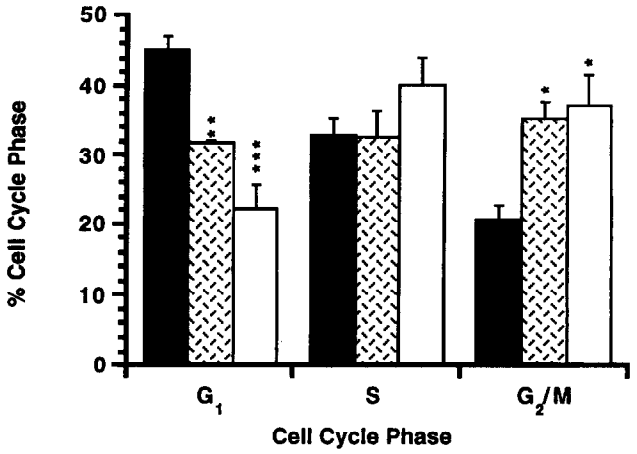

FIG. 4. Effect of 10 μM DFO on the cell cycle distribution of 2607 cells. 2607 Glioma cells were analyzed for the percent of cells in G_1, S, and G_2/M, in untreated cultures (■) and those treated with 10 μM DFO for 24 (▨) and 72 (□) hr by flow cytometry. The mean and SEM values of 3 experiments are shown, with significant differences indicated by (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$.

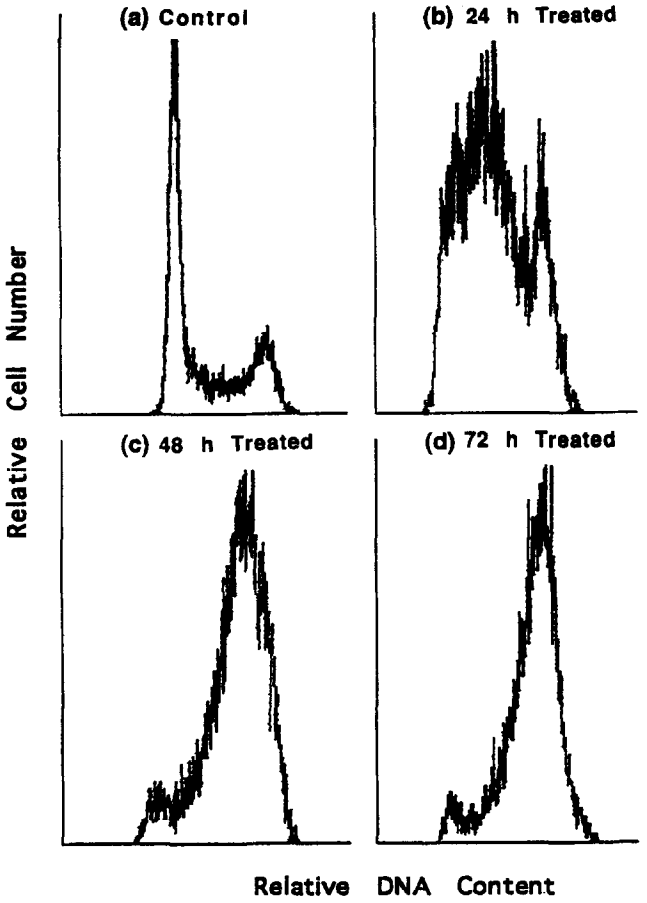

FIG. 5. Cell cycle phase distribution of ADR 529-treated cells as a function of time. Control cells (a) and cells treated with 2.24 mM ADR 529 (b–d) were analyzed by flow cytometry, for the proportion of cells in G_1, S, and G_2/M after 24 (b), 48 (c), and 72 (d) hr of exposure. In the control cells, the left peak represents G_1 DNA, the right peak G_2/M stain, and the region between the peaks the S phase stained DNA. Shown is a representative experiment of which 3 were performed.

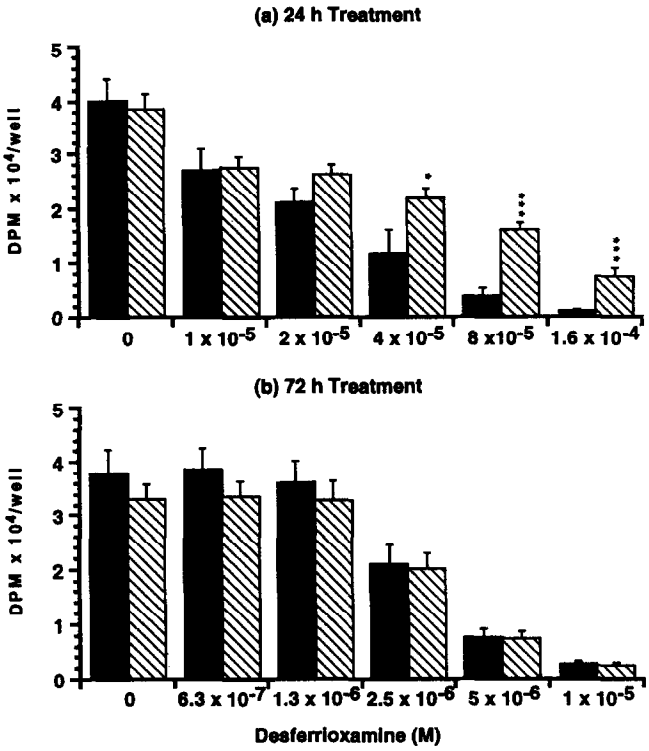

FIG. 6. Effect of cell washing on DFO-inhibited glioma cells. Glioma cells were incubated with graded concentrations of DFO for either 24 (a) or 72 (b) hr, and [^{3}H]thymidine incorporation was measured in cells washed of DFO-containing medium (▨) or not (■). Shown are the mean and SEM values of 6 experiments with significant differences indicated by (*) $P < 0.05$ and (***) $P < 0.001$.

counts of the untreated control (Table 1). Washing of 160 μM DFO-treated cells at 48 and 72 hr also had little effect on the inhibition of thymidine incorporation by DFO (Table 1). Similarly, the addition of either equimolar Fe^{2+} or Fe^{3+}, on any day of drug treatment, was not able to affect a recovery of cells treated with 160 μM DFO (Table 1).

In contrast to the effect of washing observed in Fig. 6a, the removal of 0.6 to 10 μM DFO-containing medium did not cause thymidine to be incorporated within the 4 hr of cell washing (Fig. 6b). However, cells treated with 10 μM DFO were able to resume DNA synthesis sometime after 4 hr of washing, as shown in Table 1. Equimolar ferrous but not ferric iron was also able to reverse the inhibitory effect of 10 μM DFO if added to the cells within 24 hr, later times of addition being ineffective (Table 1). Saturation of DFO with iron (10 and 160 μM) prior to the addition of the complex to the cells completely abrogated the ability of DFO to arrest cellular proliferation.

The observation that 24-hr treatment with 10 μM DFO arrested the proliferation of 2607 cells in an irreversible manner suggested the possibility that this drug regimen may compromise the viability of 2607 cells after 72 hr, a time at which the treated cells are still viable (Fig. 2). Prolonging the exposure of cells to 10 μM DFO for periods longer than 72 hr resulted in a loss of viability as measured in terms of the number of viable cells remaining attached to the culture vessel (Fig. 7). All cultures were denuded of cells completely by 7 days of 10 μM DFO treatment.

DISCUSSION

It has been demonstrated that micromolar concentrations of DFO are able to inhibit the proliferation of neural tumors in both the G_1/S and G_2/M phases of the cell cycle depending on the concentration and duration of drug exposure. Hoyes *et al.* [12] have demonstrated similar concentration-dependent effects of DFO on the cell cycle distribution of leukemia cell lines. However, while DFO induced an increase in the proportion of cells in G_2/M, this was not associated with a complete inhibition of proliferation [12]. Identification of the drug-induced sites in cell cycle arrest is important for (i) determining the duration of drug application, and (ii) gaining insight into the actions of the agent in question. The results of this study and others indicate

TABLE 1. Effect of washing and iron addition on the inhibition of thymidine incorporation in 2607 cells by DFO

	Thymidine incorporation (% of control)			
	Control	10 μM DFO	Control	160 μM DFO
No addition	100	1 ± 0	100	1 ± 0
Fe^{2+} at 24 hr	138 ± 10	101 ± 7	130 ± 6	11 ± 2
Fe^{2+} at 48 hr	133 ± 11	16 ± 1	123 ± 9	5 ± 1
Fe^{2+} at 72 hr	120 ± 11	16 ± 1	107 ± 14	1 ± 0
No addition	100	1 ± 0	100	1 ± 0
Fe^{3+} at 24 hr	121 ± 10	7 ± 2	144 ± 22	4 ± 0
Fe^{3+} at 48 hr	109 ± 16	8 ± 2	134 ± 25	5 ± 0
Fe^{3+} at 72 hr	94 ± 9	3 ± 1	92 ± 23	2 ± 1
No addition	100	1 ± 0	100	1 ± 0
Washed at 24 hr	125 ± 12	93 ± 8	123 ± 8	20 ± 2
Washed at 48 hr	135 ± 10	14 ± 1	121 ± 5	4 ± 0
Washed at 72 hr	132 ± 18	18 ± 2	111 ± 9	1 ± 0

Cells were treated with DFO or its vehicle for either 24, 48, 72, or 96 hr and cultured for a total of 96 hr. At 24, 48, and 72 hr, treatment with DFO or its vehicle was terminated by either washing the cells with fresh medium or addition of Fe^{2+} or Fe^{3+}. The iron salts were added to the medium at the same concentration as the initial DFO concentration. Thymidine incorporation was measured during the final 4 hr of the 96-hr incubation period and expressed as percent of control using 99,700 ± 14,400 dpm as the control value. The means ± SEM of 3 separate experiments are shown.

that DFO acts to arrest cells at the G_1/S border (Fig. 3a) [12, 14–19] and in G_2/M (Fig. 3b) [12, 21]. Thus, the complete inhibition of proliferation by DFO requires that this drug be applied at anti-proliferative concentrations for a period of at least one cell cycle.

Iron is an essential requirement for DNA synthesis because it is necessary for the assembly of active ribonucleotide reductase. Ferric iron is incorporated into the R2 subunit of ribonucleotide reductase by way of an Fe(III)-O-Fe(III) bond [29, 30]. Nyholm *et al.* [13] have suggested that the ferric iron is released during catalysis of deoxyribonucleotides and that this pool is chelated by DFO and so preventing the reassembly of the active enzyme. Thus, DFO-treated cells are thought to arrest at G_1/S because of a paucity of deoxyribonucleotides available for DNA synthesis. This hypothesis is supported by the observations that DFO depletes cellular deoxyribonucleotide pools [10], inhibits cellular ribonucleotide reductase activity [29, 31], and causes cells to arrest at the G_1/S interface [12, 14–19]. In addition, the addition of exogenous iron can reverse the anti-proliferative effects of DFO ([2, 3, 11, 13, 17, 18, 32] (Table 1)) and can restore ribonucleotide reductase activity in extracts of DFO-treated cells [11, 13]. Thus, the observation that 24-hr treatment with 160 μM DFO caused cells to arrest at the interface of G_1/S (Fig. 3a) is consistent with previous studies [12, 14–19] and the hypothesis that this is due to an inhibition of ribonucleotide reductase.

The effect of 10 μM DFO on the cell cycle of 2607 cells is also likely to be due to an inhibition of ribonucleotide reductase activity, albeit a partial inhibition. This hypothesis is supported by the observations that proliferation can be restored by the addition of exogenous iron, that thymidine incorporation is decreased by 10 μM DFO (Fig. 1a), and that ribonucleotide reductase activity is partially inhibited at concentrations that cause leukemic cells to arrest in G_2/M [12]. The fact that cells finally accumulate in G_2/M may be due to the effect of DFO on some other factor necessary for the transition beyond G_2/M, possibly $p34^{cdc2}$, a cyclin-dependent protein kinase required for cell cycle progression through both G_1/S and G_2/M [33, 34]. Interestingly, both the synthesis and kinase activity of $p34^{cdc2}$ is attenuated by DFO at concentrations as low as 10 μM [19, 33, 35]. ADR 529 is commonly reported to arrest cells in G_2/M [24–27] even though stathmokinetic analysis has revealed that it slows the progression of cells in G_1 and S before finally causing the cells to accumulate in G_2 [36]. The results presented here are consistent with ADR 529 having effects on the cycle prior to their accumulation at G_2/M (Fig. 5).

A number of the effects seen at low DFO concentrations differed from those observed with higher concentrations of this iron chelator. For example, the inhibition due to 24-hr incubation with 10 μM DFO could be reversed by the

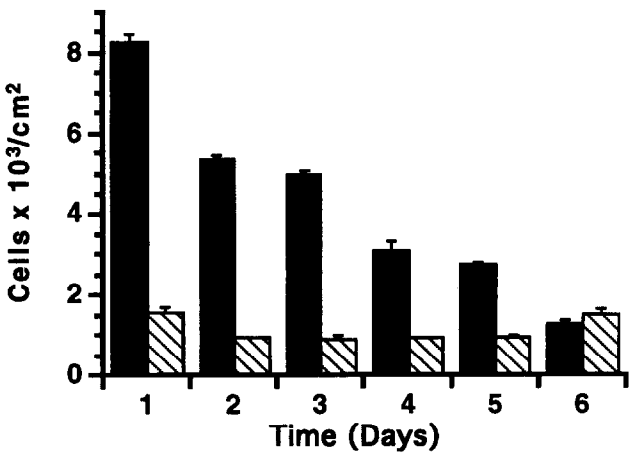

FIG. 7. DFO-induced cell death *in vitro*. Cells were treated with 10 μM DFO for 72 hr after which remaining attached viable (■) and non-viable (▨) cells were counted. Shown are the mean ± SEM values of 3 experiments.

addition of equimolar ferrous iron or the removal of the DFO-containing medium, whereas neither of these procedures had any effect on the inhibition due to 160 μM DFO (Table 1). This observation is explicable in terms of the observation that DFO induces irreversible effects on the proliferation of cells in a manner that depends on the concentration and length of drug exposure, such that short exposures with high concentrations of DFO and longer exposures with low DFO concentrations abolish the capacity of cells to return to the cycle (Table 1). Other groups have also noted that the inhibition of proliferation due to DFO becomes insensitive to iron rescue with increasing drug exposure [3, 32, 37, 38]. These irreversible effects of DFO may simply be due to protracted cell cycle arrest or depletion from the heme or ferritin iron pools [19, 39, 40].

The inhibition due to 10 μM DFO could be reversed by equimolar ferrous but not ferric iron (Table 1). Previous studies have used both forms of iron to reverse the inhibitory effects of DFO [2, 3, 11, 13, 17, 18, 32]. The inability of Fe^{3+} to restore proliferation suggests that the added iron was bound to sites other than DFO. Protracted DFO treatment leads to the depletion of a variety of iron pools [19, 39, 40] and so any exogenously added Fe^{3+} may bind these sites rather than DFO. Since DFO preferentially targets ferric iron, the ferrous iron pools would not be expected to be affected to the same extent. However, DFO binds Fe^{2+}, which in turn can be oxidized to Fe^{3+} [41, 42] and so occupy the iron binding site of this chelator. In support of this hypothesis, the addition of Fe^{2+} changed the color of the medium from red to brown, shortly after its addition, indicative of the oxidation to Fe^{3+}.

It has been argued above, that DFO preferentially chelates the iron supplied via transferrin and not that delivered by other means [43]. Thus, Voest *et al.* [44] have suggested that anti-proliferative concentrations of DFO used in this and many other *in vitro* studies [2, 12, 14–21, 27, 29, 32, 37, 45–47] underestimate the amount of DFO that would be required to arrest proliferation *in vivo*, because bovine transferrin is poorly recognized by the human transferrin receptor [48]. Voest and his colleagues [44] estimated that DFO concentrations in excess of 100 μM are required to produce cytotoxicity in the presence of iron-saturated human transferrin *in vitro*. Given these considerations, DFO would need to be administered either at an effective plasma concentration of least 100 μM for at least one cell cycle length or with agents that block transferrin-mediated iron transport, in order to arrest cellular proliferation. Recent studies have shown that the efficacy of DFO is enhanced when given in combination with antibodies against the transferrin receptor *in vitro* [49, 50]. Since DFO is a very efficient anti-neoplastic agent under conditions where the transferrin-dependent iron transport is restricted, and can be administered for sustained periods using plasma concentrations of approximately 25 μM [9, 51], the combination of DFO and anti-transferrin antibodies may be a viable option for the chemotherapy of neoplasia. Alternatively, DFO could be given at sufficiently high doses to achieve 100 μM plasma concentrations. Visual and auditory neurotoxicity can result from high dose DFO regimens; however, these symptoms usually present after weeks of treatment and usually disappear with the cessation of DFO administation [52–54]. The results of this study suggest that the high doses of DFO need only be applied for the period of one cell cycle to arrest neoplastic growth. The mean cell cycle of human neoplasms has been estimated as between 1 and 2 days [55], a period during which DFO could be administered at high doses with relatively little risk.

Taken together, the above observations also suggest that previous investigations of DFO as an anti-neoplastic agent *in vivo* were hampered by the administration of insufficient quantities of DFO for too short a time. Even so, the reported anti-neoplastic activity of DFO *in vivo* [5–7] suggests that this agent may be of some use in chemotherapy, given careful consideration of its pharmacology and the cell cycle-dependent nature of its anti-proliferative activity.

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