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Polyamine Content of *Pneumocystis carinii* and Response to the Ornithine Decarboxylase Inhibitor DL-α-Difluoromethylornithine

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Difluoromethylornithine (DFMO; eflornithine hydrochloride [Ornidyl]), a suicide inhibitor of the key polyamine biosynthesis enzyme ornithine decarboxylase (ODC), is effective in treating *Pneumocystis carinii* pneumonia, a common opportunistic infection associated with AIDS. Despite DFMO's specificity for ODC, the reason for its selective toxicity against *P. carinii* is unknown since both host and parasite are dependent on the same enzyme for polyamine biosynthesis. A new high-performance liquid chromatography method was used with *P. carinii* cells isolated from infected rat lungs to measure polyamine content, to confirm the presence of ODC, and to examine the effect of DFMO on polyamine concentrations. Putrescine, spermidine, and spermine were found to be present at 2.00 ± 0.54, 1.26 ± 0.51, and 1.59 ± 0.91 nmol (mg of protein)$^{-1}$, respectively, neither unusually high nor low values. ODC's specific activity was 79 ± 11 pmol (mg of protein)$^{-1}$ h^{-1}, again not a remarkable value. However, the rates of both DFMO-induced polyamine depletion and subsequent repletion upon DFMO removal were unusually high. A 3-h exposure to 1 mM DFMO in vitro caused the depletion of putrescine, spermidine, and spermine to levels 12, 29, and 16%, respectively, of that of control cells. After DFMO removal and incubation for 1 h in serum-free media, polyamine levels returned to 78, 88, and 64%, respectively, of that of the control cells not exposed to DFMO. Since such depletions and repletions usually occur over periods of days rather than hours, these rapid changes may provide a clue to the selective action of DFMO against *P. carinii* and may guide the development of new compounds and an optimal drug administration schedule for DFMO.

Polyamines are low-molecular-weight, positively charged compounds found in all living cells. The most important of these are putrescine, spermidine, and spermine. Polyamines seem to play important roles in cell growth and differentiation, with the rapid proliferation of many cell types marked by an increase in intracellular polyamine concentrations (27, 38). Inhibition of polyamine biosynthesis has, therefore, been considered as an approach in the development of chemotherapies to treat cancer and infectious diseases (36).

The first step of polyamine biosynthesis is the production of either putrescine from ornithine by ornithine decarboxylase (ODC) or agmatine by arginine decarboxylase, with putrescine being produced by agmatine ureohydrolase. In both cases, aminopropyl groups from decarboxylated *S*-adenosylmethionine are sequentially added to putrescine, forming spermidine and then spermine. The biosynthesis of polyamines can thus be blocked by interrupting the supply of either putrescine or decarboxylated *S*-adenosylmethionine.

Difluoromethylornithine (DFMO), an enzyme-activated, suicide inhibitor of ODC, effectively blocks polyamine biosynthesis in those cells dependent on ODC for the production of putrescine (5) and has been widely used as an investigational drug to treat many cancers. Following the discovery of activity against *Trypanosoma brucei brucei* in an animal model (3), subsequent clinical development led to DFMO being approved by the Food and Drug Administration for the treatment of African sleeping sickness caused by *Trypanosoma brucei gambiense*, and it is highly effective (40).

Pneumocystis carinii is a fungus that causes *P. carinii* pneumonia (PCP) in persons with AIDS, patients treated for cancer, and others with conditions causing severe immunosuppression (19). All current therapies for PCP have significant levels of toxicity, lack complete efficacy, or both (11). DFMO was found to be active against PCP, first with a limited number of AIDS patients treated on a compassionate basis (24) and later in open clinical trials (24, 34). Recent controlled clinical trials demonstrated that, although the rate of successful completion of therapy with DFMO (39%) compared well with that with a combination of trimethoprim and sulfamethoxazole (40%), DFMO was most often discontinued because of a lack of response, whereas the trimethoprim-sulfamethoxazole combination was discontinued because of toxic drug effects; 49% of patients failed to respond to DFMO but only 21% failed to respond to trimethoprim and sulfamethoxazole (37). Initially, DFMO was reported to be inactive in a PCP rat model (18), but it was subsequently found to be active both in vitro (10) and in a rat model in which the drug was constantly available in the drinking water as a 3 or 4% solution (8, 9). ODC, the target of DFMO, was reported to be absent in *P. carinii* (29), but later, partially purified *P. carinii* cells were found to incorporate a radiolabeled precursor into polyamines, suggesting polyamine biosynthesis by *P. carinii* despite the fact that ODC activity remained undetectable (20). Subsequently, ODC was demonstrated in *P. carinii* after the removal of an interfering substance (33).

Since the polyamine biosynthesis target of DFMO is unlike that of any other drug used to treat PCP, the activity of DFMO is a novel lead for improved therapy. The attractiveness of this target is enhanced by the relatively low toxicity of DFMO. However, the further rational development of such a therapeutic lead requires a knowledge of the basis for selective action, which is completely unknown for DFMO and PCP. The work reported here is a study of the effect of DFMO on polyamine biosynthesis by isolated *P. carinii* cells and provides

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data supporting a hypothesis for the selective action of DFMO against *P. carinii.*

MATERIALS AND METHODS

Source of *P. carinii.* The induction of intense PCP in rats was as described previously (21). In brief, animals were maintained in a barrier colony, treated with antibiotics to prevent other opportunistic infections, and immunosuppressed by the inclusion of 1.5 mg of dexamethasone (liter of drinking water)$^{-1}$. One week after immunosuppression was initiated, the rats were inoculated intratracheally by the instillation of homogenized lung tissue taken from a rat heavily infected with *P. carinii*. The donor lungs were prescreened to ensure that no other pathogens were present. A second inoculation followed the first by 4 days. Approximately 3 weeks after the first inoculation, the rats showed typical signs of PCP, including weight loss by approximately 30%, a hunched-back posture, listlessness, rapid shallow breathing, and some instances of cyanosis.

Purification of *P. carinii.* The technique for the isolation and purification of *P. carinii* was a modification of a process previously described (33). Sterility was maintained throughout. A set of lungs weighing 2 to 5 g was sliced into small pieces and mechanically homogenized in 10 ml of homogenizing buffer composed of equal parts of NKPC buffer (2.68 mM KCl, 1.47 mM KH_2PO_4, 51.1 mM Na_2HPO_4, 7.43 mM NaH_2PO_4, 62 mM NaCl, 0.05 mM $CaCl_2$, 0.05 mM $MgCl_2$) and 100 mM dithiothreitol in water. The final volume of the crude homogenate was adjusted to 30 ml of homogenizing buffer per gram of lung tissue. Large debris was removed by centrifugation at 32 × *g* for 5 min, and *P. carinii* cells were pelleted from the supernatant by centrifugation at 5,000 × *g* for 10 min. The erythrocytes in the *P. carinii*-containing pellet were lysed by resuspending the pellet in 10 ml of NKPC, which was made 0.85% with respect to NH_4Cl, and this suspension was incubated for 5 min at 37°C. The *P. carinii* cells were pelleted by centrifugation at 5,000 × *g* for 10 min, and host DNA was removed by resuspending the pellet in 10 ml of homogenizing buffer containing 0.10% DNase I type IV (Sigma Chemical Co., St. Louis, Mo.) and incubating at 37°C for 10 min. The purified *P. carinii* cells were washed three times in NKPC buffer (centrifugation at 5,000 × *g* for 10 min for each wash) to remove the digestion products. The isolated *P. carinii* were examined by light microscopy with Giemsa-stained smears as previously described (33). After the removal of the lungs, the total time for *P. carinii* isolation was approximately 70 min and, with the exception of homogenization at room temperature and the two 37°C incubations totaling 15 min, all steps were carried out at 0 to 4°C.

Preparation of *P. carinii* extracts for polyamine analysis. Isolated *P. carinii* cells were suspended in NKPC buffer and, while kept chilled with an ice jacket, ultrasonicated for 20 min at 40 W and a 70% duty cycle (Heat Systems; Ultrasonics Inc., Plainview, N.Y.). Nuclei and cell ghosts were removed by centrifugation at 2,000 × *g* for 5 min. An aliquot of the supernatant was retained for protein assay, and the balance was deproteinized by heating in a boiling-water bath for 2 min. The boiled sample was clarified by centrifugation at 2,000 × *g* for 5 min, and the final supernatant was defined as the extract. Protein content was determined with the Bio-Rad (Melville, N.Y.) protein dye-binding assay with bovine serum albumin as the standard.

Polyamine assay. High-performance liquid chromatography (HPLC) analysis of polyamines in the extracted samples depended on the use of an AccQ.Fluor kit (designed for amino acid analysis and donated by Waters Corp., Milford, Mass.) for the precolumn derivatization of polyamines, allowing highly sensitive fluorescence detection. The adaptation of this AccQ.Fluor kit for polyamine analysis and the chemicals, conditions, and equipment used were exactly as described previously (22).

ODC assay. Lysed preparations of purified *P. carinii* for ODC assays were prepared as described for the analysis of polyamines except that extraction was in ODC assay buffer (30 mM Tris HCl, 1 mM EDTA, 1 mM dithiothreitol, 50 μM pyridoxal-5′-phosphate [pH 8.0]), and 4 ml of the ODC assay buffer per gram of lung tissue was used in the initial *P. carinii* homogenate. The homogenates were clarified by centrifugation at 30,000 × *g* for 30 min and then dialyzed against ODC assay buffer as previously described (33). Individual ODC assays had a final volume of 0.50 ml and contained 0.40 ml of the dialysate, 50 μM ornithine, 50 μM GTP, 100 U of penicillin ml^{-1}, and 100 μg of streptomycin ml^{-1}. Assurance that no viable microbes were present in the incubation mixture was obtained by streaking a small amount of the incubation mixture onto blood agar plates, which were incubated for 48 h at 37°C. The assay mixtures were incubated for 0, 0.5, 1, or 2 h and then deproteinized by boiling and analyzed for polyamine content.

DFMO. DFMO was supplied by Marion Merrell-Dow, Cincinnati, Ohio.

RESULTS

A newly developed HPLC polyamine assay system which was based on a modification of the Waters AccQ.Fluor amino acid analysis system was used (22). Figure 1A shows a chromatogram of a *P. carinii* extract, and Fig. 1B shows the same extract with added polyamine standards so that the reinforced peaks confirm peak identifications. The validity of measurements depended also on the lack of host contamination of *P. carinii* preparations. Giemsa-stained smears of isolated *P. carinii* revealed that no host cells or host cell organelles were visible by light microscopy. For each preparation at least 100 fields, which included more than 5,000 *P. carinii* trophozoites and 500 cysts, were examined; no host cells or host cell nuclei were found in any of these preparations. A control or "blank isolation" was made from lungs not infected with *P. carinii*, and this control was devoid of both measurable protein and measurable polyamines. A further test for host contamination was by transmission electron microscope examination of isolated *P. carinii*. No intact mitochondria or other host cell organelles could be found, either in scans of sections cut from separate blocks or in subsequent prints of random photographs made of the preparation. Other than *P. carinii*, the only structures found were tubular extensions of *P. carinii* organisms and membranes of unknown origin; no intact host cell organelles were found.

The polyamine contents of eight independent, freshly isolated populations of *P. carinii* cells were analyzed, and the following concentrations were found (in nanomoles of polyamine per milligram of protein ± the standard deviation): putrescine, 2.00 ± 0.71; spermidine, 1.26 ± 0.51; and spermine, 1.59 ± 0.91. This same polyamine assay was used to demonstrate ODC activity by detecting the putrescine produced from ornithine. GTP (50 mM) was included in the assay since GTP has been shown to activate ODC (25). Figure 2 shows the results of this assay. The specific activity of ODC was 79 ± 11 pmol of putrescine (mg of protein)$^{-1}$ h^{-1}. A blank ODC assay was performed with all components present, including ornithine and the coenzyme pyridoxal phosphate, and no putrescine was detected at 2 h. This contrasts with the significant background level observed in previous studies using an assay depending on the capture of $^{14}CO_2$ released from labeled ornithine. Again, to ensure that the ODC measured was exclusively from *P. carinii*, a control preparation was made from lungs not infected with *P. carinii*. This control was devoid of ODC activity.

The effect on *P. carinii* polyamine concentrations caused by exposure to DFMO in vitro was measured. *P. carinii* cells were isolated, divided into four aliquots, and suspended in RPMI 1640 tissue culture medium (GIBCO, Grand Island, N.Y.) with no serum added. DFMO (1 mM) was added to three aliquots, and they were incubated at 37°C. One aliquot was extracted for polyamine analysis after 1 h, and another was extracted for analysis after 3 h. DFMO was removed from the third aliquot after 3 h by centrifugation at 5,000 × *g* for 10 min and resuspension in fresh RPMI 1640; these cells were then incubated an additional hour at 37°C and extracted for polyamine analysis. The fourth aliquot was used to provide a control baseline for polyamine concentrations in cells incubated at 37°C; no DFMO was added, and the cells were extracted after 3 h at 37°C. Table 1 presents the results. The data are reported both as the mean polyamine content at each time point and as percentages of the content in control cells. These percentages are the means of the percentages calculated individually for the three independent experiments using the individual controls. There is considerable variation in the polyamine contents of the cells in the three experiments but less variation in the percentages, as was expected since these percentages are based on individual controls. Incubation for 3 h with 1 mM DFMO caused the levels of all three polyamines to decline sharply. Removal of the DFMO allowed substantial restoration of the polyamine content within 1 h.

The mean polyamine concentrations of the 3-h controls were less than the values for freshly isolated *P. carinii*. After a 3-h

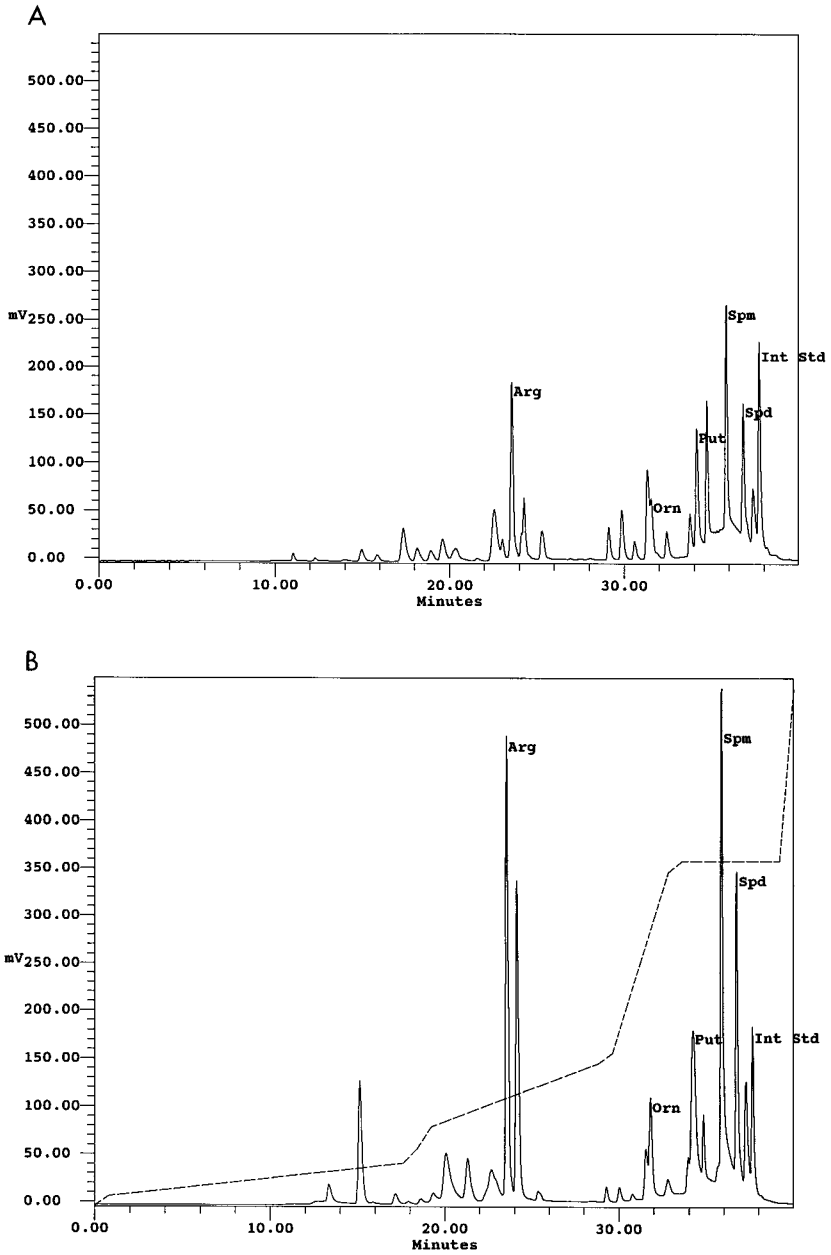

FIG. 1. HPLC tracings. (A) *P. carinii* extract. (B) *P. carinii* extract to which polyamine standards were added, thus reinforcing and confirming the identities of the peaks in panel A. Arg, arginine; Orn, ornithine; Put, putrescine; Spm, spermine; Spd, spermidine; Int Std, internal standard (1,7-diaminoheptane). The peak in panel B eluting just after arginine is the result of a combination of the contributions of an unidentified contaminant present in the ornithine, putrescine, spermine, and spermidine standards together with a peak present in the *P. carinii* extract seen in panel A. The dashed line in panel B represents the elution gradient.

incubation at 37°C, the mean putrescine, spermidine, and spermine concentrations were 34, 29, and 29%, respectively, of the means of freshly isolated cells from the same preparations. Since the isolation method used was relatively quick (just over 1 h) and most of the steps were at 0 to 4°C, the polyamine content of freshly isolated cells may more closely represent the in situ state.

DISCUSSION

The effect of DFMO on *P. carinii* polyamine content (Table 1) indicates that ODC is critical to polyamine production, but this is true for the host as well. The nature of *P. carinii* ODC does not account for the selective action of DFMO, since an earlier study demonstrated that *P. carinii* ODC is less sensitive to DFMO than mammalian ODC (33). If *P. carinii* maintained a high polyamine content, it might be particularly sensitive to interruption of polyamine biosynthesis, but the values do not indicate high polyamine concentrations. In reports on mammalian cells, putrescine concentrations have ranged from 0.04 to 4 nmol (mg of protein)$^{-1}$, spermidine concentrations have ranged from 2.1 to 35 nmol (mg of protein)$^{-1}$, and spermine concentrations have ranged from 1.5 to 22 nmol (mg of protein)$^{-1}$ (1, 7, 16, 17, 23, 26, 39). An earlier paper on *P. carinii*

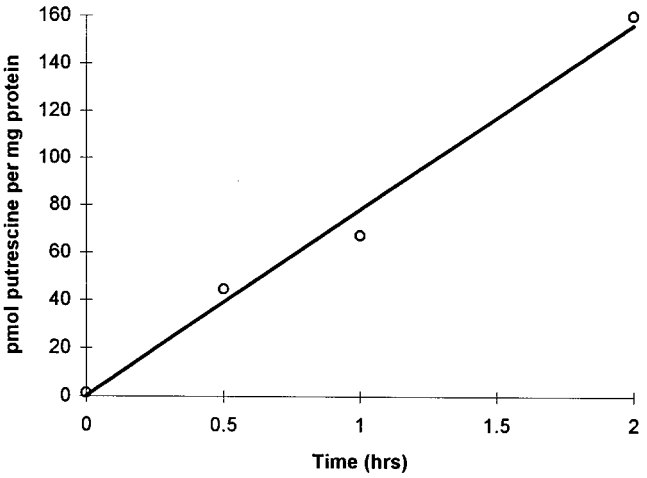

FIG. 2. Ornithine decarboxylase activity levels as determined by the detection of putrescine production.

polyamines reported a putrescine content 9-fold and a spermidine content 47-fold higher than those reported here and a nonreproducible trace of spermine (20). However, those *P. carinii* cells were acknowledged to be contaminated with host cells and they were incubated for 12 to 24 h in media containing 20% fetal calf serum prior to measuring polyamines; both the contamination and the incubation could have affected the polyamine content of the samples.

P. carinii does not have high-level ODC activity. A previous measurement of *P. carinii* ODC which depended on the detection of $^{14}CO_2$ released from carboxyl-carbon-labeled ornithine yielded a specific activity of 11.7 ± 0.2 pmol of CO_2 released (mg of protein)$^{-1}$ h^{-1} (33). The data reported here depended on HPLC detection of putrescine and yielded an activity of 79 ± 11 pmol of putrescine released (mg of protein)$^{-1}$ h^{-1}. The higher specific activity may have been due to the inclusion of GTP in the assay medium, which has been shown to activate *E. coli* and mouse papilloma ODC (25), and/or the greater sensitivity and lower background level of the HPLC assay system. Specific activities of ODC (in picomoles per milligram of protein per hour) in nondividing and unstimulated rat cells range from 6 in liver cells to 94 in kidney cells to 250 in small intestine cells to 11,750 in prostate cells (35).

The data reported here do reveal an unusual feature of *P. carinii* in that DFMO causes very quick polyamine depletion. A 3-h exposure to 1 mM DFMO caused mean reductions of 88% in putrescine, 71% in spermidine, and 84% in spermine (Table 1). In other cells, the rate of DFMO-induced polyamine depletion is not as high and spermine reduction is not as extensive. For example, after a 4-day exposure of a human colon cancer cell line to 1 mM DFMO, there was complete depletion of putrescine and spermidine but only a 49% reduction in spermine content (2). After a 4-day exposure of KB cells to 5 mM DFMO, putrescine and spermidine were 99 and 85% depleted, respectively, but spermine was only 3% depleted (23). Similarly, CHO cells exposed to 5 mM DFMO become depleted of putrescine and spermidine within 3 days; spermine was 10% depleted at 3 days and 43% depleted at 6 days (7). MCF-7 breast cancer cells exposed to 1 mM DFMO for 24 h became 59% depleted of putrescine and 26% depleted of spermidine, but there was a 2.3-fold increase in spermine (39). Since mammalian cells can interconvert polyamines using spermine to maintain minimal concentrations of putrescine (35), the inability of DFMO to cause complete spermine depletion in mammalian tumor cells has been suggested to underlie the limitation of the anticancer activity of this compound (27). The yeasts *Candida tropicalis* and *Candida albicans* become fully depleted of polyamines, including spermine, but only after 20 h of exposure to 6 mM DFMO (30). *Cryptococcus neoformans* exposed to 6 mM DFMO for 48 h was 48% depleted of putrescine and 54% depleted of spermidine; spermine was not detected before or after exposure (31).

An even more striking feature of *P. carinii* polyamine metabolism is the rate at which polyamine concentrations are restored after the removal of DFMO. Within 1 h, the physiologically most important polyamine, spermidine, is restored to 88% of the pretreatment concentration and putrescine and spermine are 78 and 64% restored, respectively (Table 1). Rat HTC hepatoma cells exposed to 1 mM DFMO for 24 h were fully depleted of putrescine, 80% depleted of spermidine, and 20% depleted of spermine. There was essentially no recovery 1 day after the removal of the DFMO. At 2 days, putrescine and spermidine were approximately 55 and 17% restored, respectively, and spermine was fully restored. At 3 days all concentrations were above the pretreatment concentrations (12).

West African trypanosomiasis caused by *T. brucei gambiense* is the only disease for which the Food and Drug Administration has approved DFMO as a treatment (40). The mechanism of selective toxicity against these trypanosomes does not rest on the sensitivity of parasite ODC to inhibition by DFMO (6). Neither does it rest on polyamine depletion, since constant exposure to DFMO fully depletes putrescine within 24 h but causes only about a 50% reduction in spermidine. DFMO actually induces a low level of spermine accumulation, which does not occur in untreated cells (13). The long half-life of trypanosome ODC causes the effect of DFMO to be long lasting, and this likely contributes to its selective toxicity. In addition, there is a marked increase in the intracellular con-

TABLE 1. Effects on the polyamine content of *P. carinii* caused by exposure to 1 mM DFMO in vitro and recovery after removal of the DFMO

Assay condition	Amt (pmol [mg of protein]$^{-1}$ ± SEM[a]) of indicated polyamine (mean % of paired 3-h control ± SEM[b])		
	Putrescine	Spermidine	Spermine
3-h incubation with no DFMO	771 ± 166	576 ± 727	302 ± 196
1-h incubation with 1 mM DFMO	190 ± 171 (22 ± 16)	447 ± 570 (76 ± 8)	113 ± 119 (35 ± 19)
3-h incubation with 1 mM DFMO	101 ± 90 (12 ± 8)	109 ± 122 (29 ± 14)	45 ± 38 (16 ± 7)
3-h incubation with 1 mM DFMO plus 1 h after DFMO removal	598 ± 191 (78 ± 16)	534 ± 672 (88 ± 36)	148 ± 67 (64 ± 28)

[a] The values are means from three separate experiments using three separate *P. carinii* preparations.
[b] Percentages from individual experiments were calculated on the basis of the 3-h control for that experiment, and means and standard errors of the mean of these percentages are presented.

centration of *S*-adenosylmethionine, which leads to abnormally high-level transmethylation activity (4). In the case of *P. carinii*, rapid recovery of polyamine concentrations after the removal of DFMO indicates that the effect of DFMO is not long-lasting. Nothing is known about possible perturbations in *S*-adenosylmethionine metabolism caused by DFMO, but this is an area of investigation that should be pursued.

The mechanisms for DFMO-induced rapid depletion of polyamines and subsequent restoration upon DFMO removal are completely unknown. No measurements have been made of polyamine degradative enzymes or of the possible loss of polyamines by transport out of the cells. There is no information on the rate of ODC induction. However, the data here do indicate that polyamine repletion is dependent on de novo polyamine biosynthesis and not on the uptake of exogenous polyamines, since polyamines are not included in RPMI 1640 and no serum was added. HPLC analysis of the media directly demonstrated that polyamines were not present as contaminants to a limit of detection of 26 nM (data not shown). Additionally, if uptake of preformed polyamines from the media allowed *P. carinii* to restore intracellular polyamines under the conditions used, DFMO could not have caused the depletion observed.

The rapid recovery of polyamines by *P. carinii* after the removal of DFMO may indicate a particular need for these cells to maintain polyamine concentration control, and the rapid polyamine depletion following exposure to DFMO may be the cause for the selective toxicity of DFMO against this pathogen. These results suggest that relatively low but constant concentrations of DFMO may be optimal for enhancing the selective action of DFMO against this parasite, but common administration protocols do not achieve this. The most frequent mode of DFMO administration in an animal model is by way of the drinking water. Since the half-life of DFMO in rodents is short and animals do not drink at constant rates during the day and night, this protocol does not provide a constant presence of the drug (32). Similarly, the recommended clinical protocol is infusion of 100 mg of DFMO (kg of body weight)$^{-1}$ over 20 min at 6-h intervals for 10 to 14 days and then of 75 mg kg^{-1} administered orally every 6 h for 4 to 6 weeks (41); this protocol also will not maintain a constant plasma DFMO concentration (15).

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