Published in final edited form as:

Microbiology (Reading). 2004 May ; 150(Pt 5): 1215–1224. doi:10.1099/mic.0.26889-0.

Polyamine metabolism in a member of the phylum Microspora (*Encephalitozoon cuniculi*): effects of polyamine analogues

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Abstract

The uptake, biosynthesis and catabolism of polyamines in the microsporidian parasite *Encephalitozoon cuniculi* are detailed with reference to the effects of oligoamine and arylamine analogues of polyamines. *Enc. cuniculi*, an intracellular parasite of mammalian cells, has both biosynthetic and catabolic enzymes of polyamine metabolism, as demonstrated in cell-free extracts of mature spores. The uptake of polyamines was measured in immature, pre-emergent spores isolated from host cells by Percoll gradient. Spermine was rapidly taken up and metabolized to spermidine and an unknown, possibly acetamidopropanal, by spermidine/spermine N^1-acetyltransferase (SSAT) and polyamine oxidase (PAO). Most of the spermidine and the unknown product were found in the cell incubation medium, indicating they were released from the cell. bis(Ethyl) oligoamine analogues of polyamines, such as SL-11144 and SL-11158, as well as arylamine analogues [BW-1, a bis(phenylbenzyl) 3-7-3 analogue] blocked uptake and interconversion of spermine at micromolar levels and, in the case of BW-1, acted as substrate for PAO. The *Enc. cuniculi* PAO activity differed from that found in mammalian cells with respect to pH optimum, substrate specificity and sensitivity to known PAO inhibitors. SL-11158 inhibited SSAT activity with a mixed type of inhibition in which the analogue had a 70-fold higher affinity for the enzyme than the natural substrate, spermine. The interest in *Enc. cuniculi* polyamine metabolism and the biochemical effects of these polyamine analogues is warranted since they cure model infections of *Enc. cuniculi* in mice and are potential candidates for human clinical trials.

INTRODUCTION

The microsporidia are a group of obligate intracellular parasites of the phylum Microspora. With over 144 genera and 1200 species, these organisms parasitize a wide range of hosts

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from insects to fish, non-primate mammals and man (Wittner & Weiss, 1999). Microsporidia are true eukaryotes containing a nucleus, nuclear membrane, intracytoplasmic membrane system, a Golgi apparatus, and chromosome separation on mitotic spindles. No mitochondria or centrioles are present. Microsporidia form characteristic unicellular spores which extrude a polar tube through which sporoplasm is passed to inoculate a host cell (Wittner & Weiss, 1999). Little is known concerning the metabolism of the microsporidia, except for a few studies identifying glycolytic enzymes and metabolites which are excystment requirements (Weidner *et al.*, 1999). Molecular studies have recently characterized RNA and the full genome sequence of *Encephalitozoon* (*Enc.*) *cuniculi* is now known (Wittner & Weiss, 1999; Katinka *et al.*, 2001).

The microsporidia have become increasingly important as human pathogens in AIDS. There are approximately eight genera with species proven to be pathogenic in humans. *Enterocytozoon* (*Ent.*) *bienusi*, an enteric parasite which causes intractable diarrhoea in AIDS, is the most common species (Wittner & Weiss, 1999; Weber *et al.*, 1994).

Chemotherapy for microsporidia infections has relied on the benzimidazoles and fumagillin, which are generally effective with exceptions: albendazole is not effective versus *Ent. bienusi* and fumagillin has cytotoxic effects, especially in AIDS patients (Didier, 1997; Costa & Weiss, 2000; Dieterich *et al.*, 1994; Weiss *et al.*, 1994a; Katiyar *et al.*, 1994).

With this as background, our work has focused on polyamine metabolism in the microsporidia using *Enc. cuniculi* as model organism (Bacchi *et al.*, 2001). Polyamines are low-molecular-mass polycations, found universally in cells, which function as cofactors for macromolecule synthesis, in osmotic control, and as structural facilitators for nucleic acids (Cohen, 1998). The major polyamines are putrescine, spermidine and spermine. Polyamines are absolutely required for cell division and differentiation and their internal concentrations are carefully controlled by synthesis, degradation, excretion and uptake from the environment. In mammals and many protozoa, synthesis occurs from the conversion of ornithine to putrescine through ornithine decarboxylase (ODC) (Bacchi & Yarlett, 2002), although the enteric parasite *Cryptosporidium parvum* has a plant-like pathway in which arginine is the starting point for putrescine synthesis through arginine decarboxylase (Keithly *et al.*, 1997). One or two aminopropyl groups, originating from decarboxylated *S*-adenosylmethionine, are added to putrescine to form spermidine and spermine, respectively. The limiting enzymes of polyamine synthesis are ODC and *S*-adenosylmethionine decarboxylase (AdoMetdc), both of which are highly inducible and have short half-lives in the cell (Marton & Pegg, 1995). Mammalian cells, as well as some protozoa, take up spermine and spermidine, interconverting them to spermidine and putrescine, respectively. The key enzymes in interconversion are spermidine/spermine N^1-acetyltransferase (SSAT) and polyamine oxidase (PAO). SSAT is also highly inducible by exogenous polyamines, and the trio of ODC, AdoMetdc and SSAT serves to keep intracellular polyamines at relatively stable levels (Marton & Pegg, 1995).

Chemotherapeutic interest in the polyamine pathway was initially spurred by the development of enzyme-activated inhibitors of ODC [DL-α-difluoromethylornithine hydrochloride (DFMO)] and AdoMetdc (MDL 73811, AbeAdo; Bacchi & Yarlett, 2002).

Although these were not effective clinically as anti-tumour agents, DFMO found use clinically against African sleeping sickness (Schechter & Sjoerdsma, 1989) and AbeAdo in experimental trypanosome infections (Bitonti *et al.*, 1990). More recent attention, however, has focused on polyamine analogues such as bis(ethyl)norspermine (BE 3-3-3) and bis(ethyl)homospermine (BE 4-4-4) as anti-tumour agents. These agents are taken into mammalian cells on polyamine transporters and down-regulate ODC and AdoMetdc, while up-regulating SSAT. The net result is that polyamine synthesis is shut down while remaining intracellular polyamines are acetylated and excreted. The resulting decline in polyamine levels causes apoptosis and cell death, since the analogues do not substitute functionally for natural polyamines (Marton & Pegg, 1995; Casero & Woster, 2001; Frydman & Valasinas, 1999; Bernacki *et al.*, 1995).

Our recent work has demonstrated that several classes of polyamine analogues block *in vitro* growth of *Enc. cuniculi* and sterilize host monolayers at micromolar levels. These agents also cured well-validated laboratory model infections of *Enc. cuniculi* (Zou *et al.*, 2001; Bacchi *et al.*, 2002). In the present study, we examine these analogues for effects on polyamine metabolism in *Enc. cuniculi.*

METHODS

Strain and growth conditions

Enc. cuniculi was obtained from Dr Ann Cali of Rutgers University. It was cultivated in RK-13 (rabbit kidney) or HEK (human embryonic kidney, ATCC CRL-1573) cells. Cells were grown to 80% confluency in Falcon T-75 flasks (Becton-Dickinson) at 37 °C in 5% CO_2/air, and infected with 5×10^5 spores. Infected monolayers were also trypsinized and split into additional flasks. After 1 week of culture, greater than 50% infectivity was obtained. The medium used was Minimal Eagle’s Medium, Earle’s salts, L-glutamine, 7% (w/v) fetal bovine or newborn calf serum (Visvesvara *et al.*, 1991). The medium was changed twice (RK-15 cells) or three times (HEK cells) weekly, and the old medium containing mature spores was pooled and aseptically stored at 4 °C.

Enzyme preparations

Intact mature spores were obtained from supernatant culture media and stored aseptically at 0–4 °C (Bacchi *et al.*, 2001). Spores were washed in 150 mM PBS [0·9% (w/v) NaCl] containing 1% (w/v) SDS and resuspended in PBS prior to breaking. Spores were poured into 2 ml microfuge tubes containing 425–600 nm glass beads (Sigma) and disrupted (6–8 min at 4 °C) using a Mini Bead Beater (Bio Spec Products) or Vortex Mixer and adapter (#13000-50-V1; Mo Bio Laboratories) (Weiss *et al.*, 1994b). Homogenate preparations with greater than 90% breakage (by microscopy) were collected and cleared by centrifugation at 2200 ***g*** for 10 min. The cleared supernatants were used to determine SSAT and PAO activity.

Gradient purification of pre-emergent spores

The large-scale gradient procedure described previously (Bacchi *et al.*, 2001) was used to obtain purified preparations of pre-emergent spores. Usually 10 heavily infected (50–80 %) monolayers from Falcon T75 flasks were trypsinized, harvested and ground in a glass vessel for 6–8 min (3–4 × 2 min grinding) at 0–4 °C. The breakage medium contained W-T medium (described below under ‘Incubation studies using pre-emergent spores’) plus a protease inhibitor cocktail containing 2·7 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 0·15 mM aprotinin, 2·2 mM leupeptin and 0·5 mM DTT in 50 mM KCl/0·15 M Na_3PO_4, pH 7·4. Cell homogenates were passed through 12 μm and 5 μm filters and the final filtrate was centrifuged for 10 min at 15 000 ***g*** on 50% (v/v) stock isotonic Percoll (1 ml/11 ml Beckman 14 × 89 mm tube; Green *et al.*, 1999). Two bands were obtained, a broad one at 1·018–1·035 g ml^{-1} (light band) and a narrower one at 1·102–1·119 g ml^{-1} (heavy band). We had demonstrated from the previous study that the heavy band contained pre-emergent spores and immature spores while the light band contained debris, spore cases and some immature stages (Bacchi *et al.*, 2001). Since the heavy fraction exhibited far greater metabolic activity than the light fraction (Bacchi *et al.*, 2001), we used it for the data presented in the present study. Cell counts of suspensions in several experiments prior to and post-incubation indicated that no lysis of these forms had occurred.

Polyamine analysis

Polyamine content was quantified by reversed-phase HPLC using a Perkin-Elmer LC-410 pump and a percosphere C18-5 μ 150×4·6 mm column (Perkin Elmer). Heavy gradient fractions (pre-emergent spores) were extracted with 10% (w/v) trichloroacetic acid (TCA) at 0–4 °C. Denatured protein was removed by centrifugation and extracts were pre-column derivatized with 0·8 g *o*-phthalaldehyde l^{-1} (2 : 1) dissolved in 3 ml methanol added to 30·9 g boric acid l^{-1} containing 24 g KOH l^{-1} (pH 10·4). The derivatized samples were quantified with a Perkin-Elmer LC 240 fluorescence detector using an excitation wavelength of 320 nm and an absorption wavelength of 455 nm. Signals were integrated using β-RAM (IN/US Systems) Version 3.1 software. Concentrations of polyamines were determined relative to standards. The HPLC solvent gradient used and other details have been described previously (Yarlett *et al.*, 1994).

Enzyme assays

All incubations were at 37 °C. SSAT was measured according to Libby *et al.* (1989) in a reaction containing 0·06 M Bicine buffer (pH 8·0), 0·15–6 mM spermine or spermidine, 0·1 μCi (0·5 nmol) [1-^{14}C]acetyl-CoA and 50 μl spore enzyme preparation. Reactions were incubated for 30 min, stopped by addition of 20 μl 0·5 M hydroxylamine, and placed on ice. Reaction tubes were heated for 3 min in a boiling water bath and centrifuged to remove protein. Clarified reactions were spotted on P-81 phosphocellulose filters. Filters were washed with distilled water, then methanol, dried and counted. Activity is expressed as nmol (mg protein)$^{-1}$ (30 min)$^{-1}$.

PAO was measured spectrophotometrically (Beckman DU 640) according to the method of Childs & Bardsley (1975) in 0·1 M glycine buffer (pH 7·0) containing 10 units of horseradish peroxidase [Sigma Type VIA: 1 unit will oxidize 1 μmol 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) min^{-1} at 25 °C at pH 5·0], 5 mM ABTS and 22–220 μg *Enc. cuniculi* protein. Final volume was 1 ml. Assays (in duplicate) were started by addition of 1 mMN^1-acetylspermine. Assays were linked to reduction of ABTS by peroxidase and measurement of oxidized product at 420 nm, using a six-compartment cell holder at 37 °C. The extinction coefficient of ABTS at 420 nm is $E=3{\cdot}6 \times 10^4\ M^{-1}\ cm^{-1}$ (Childs & Bardsley, 1975). Studies examining polyamine analogues as substrates of PAO had N^1-acetylspermine left out of the reaction. Blanks containing protein but no N^1-acetylspermine were run with each reaction, and subtracted from substrate runs. Initial velocity plots were obtained using Beckman KINETICS Software (Beckman Instruments).

Incubation studies using pre-emergent spores

The effect of polyamine analogues on polyamine synthesis and interconversion was examined on intact pre-emergent spores. Heavy gradient preparations were incubated in W-T medium based on those employed by Weidner & Trager (1973) and Weidner *et al.* (1999) in their studies of *Nosema* excystation and containing 3 mM ATP, 1 mM GTP, 0·5 mM NAD$^+$, 2 mM glucose 6-phosphate, 0·5 mM acetyl-CoA, 1 mM sodium pyruvate, 2 mM glucose in 8 mM NaCl, 138 mM Na_3PO_4, pH 6·8. We had previously used 150 mM Na_3PO_4/0·8% NaCl (Bacchi *et al.*, 2001), but obtained better uptake and synthesis activity with W-T incubation medium.

Pre-emergent spores from heavy gradient fractions were incubated in the presence of 5 μCi (125 pmol) L-[2,3-^{3}H]ornithine/4 nmol L-methionine for polyamine synthesis and 0·25 μCi (2·1 nmol) [1-^{14}C]spermine for polyamine interconversion in suspensions containing a 250 μl aliquot of cell suspension (100–500 μg protein) in W-T medium. Polyamine analogues and radiolabelled substrates were added at the start of the 1 or 2 h incubation period. After incubation, preparations were centrifuged at 12 000 ***g*** for 10 min, and pellets were washed in incubation medium and lysed with 250 μl of 10% (w/v) TCA. Lysates were kept at 0–4 °C overnight and frozen until analysis. Supernatants from incubations were aspirated from pellets and frozen until analysis. All incubations were done in duplicate.

HPLC analysis

Lysates and supernatant incubation media from incubations of intact pre-emergent spores with L-[2,3-^{3}H]ornithine or [1-^{14}C]spermine were analysed by HPLC using procedures outlined previously (Bacchi *et al.*, 2001). An IN/US flow-through radio-detector monitored radioactivity and the IN/US β-2 RAM software package (IN/US Systems) was used for data peak integration. Using this system, we obtained the following retention times, based on radiolabelled standards: ornithine, 12 min; putrescine, 24 min; spermidine, 28 min; spermine, 31 min. Acetamidopropionaldehyde had a retention time of 3·5 min, based on UV absorption analysis.

Uptake studies

Pre-emergent spore preparations were incubated for 15 min in duplicate with varying concentrations of [1–4 terminal methylenes-^{14}C]spermine, [terminal methylenes-^{3}H(N)]spermidine [1,4-^{14}C]putrescine or L-[2,3-^{3}H]ornithine. The W-T incubation medium was used, with 0·2 ml reaction mixtures centrifuged through 0·2 ml mineral oil at 12 000 ***g*** for 0·5 min. Aqueous and oil layers were aspirated, and the tips of microfuge tubes were cut off and analysed by liquid scintillation counting in the presence of Beckman Ready Protein scintillation fluor (Beckman Instruments) as in Goldberg *et al.* (1998).

Protein determination

Protein content was estimated by the Bradford method, using BSA as standard.

Chemicals

Radiolabelled chemicals were purchased as follows: [1–4 terminal methylenes-^{14}C]spermine (113 mCi mmol^{-1}; 4·18 GBq mmol^{-1}), Amersham Pharmacia Biotech; L-[2,3-^{3}H]ornithine (40 mCi mmol^{-1}; 1·48 GBq mmol^{-1}), Moravek Biochemicals; [1-^{14}C]acetyl-CoA (59 mCi mmol^{-1}; 2·2 GBq mmol^{-1}); [terminal methylenes-^{3}H(N)]spermidine (25 mCi mol^{-1}; 0·93 GBq mmol^{-1}); [1,4-^{14}C]putrescine (110 mCi mmol^{-1}; 4·07 GBq mmol^{-1}), NEN Life Science Products.

Polyamine analogues were obtained as follows: SL-11061 (BE 4×4), SL-11093, SL-11144, SL-11158, Dr Benjamin Frydman, SLIL Biomedical (synthesis: Valasinas *et al.*, 2001; Bacchi *et al.*, 2002); BW-1, Dr Patrick Woster, Wayne State University (synthesis: Zou *et al.*, 2001). MDL 72521, MDL 72527, MDL 27695, DFMO and difluoromethylarginine were gifts from Marion Merrell Dow. Other chemicals were obtained from Sigma.

RESULTS

Polyamine content of pre-emergent spores

Heavy gradient fractions were analysed by HPLC and fluorescence detection for polyamine content after isolation and washing. Analysis of seven separate preparations yielded a mean of 38·15±27·8 nmol (mg protein)$^{-1}$ for spermine and 23±15·8 nmol (mg protein)$^{-1}$ for spermidine. Neither putrescine nor cadaverine were found using techniques sensitive to 50 pmol per sample (Yarlett *et al.*, 1994). We had earlier reported polyamine content of mature, released spores to include low levels of putrescine as compared to spermidine and spermine (Coyle *et al.*, 1996).

Enzymes of polyamine metabolism

Mature spores were broken using the beadbeater technique (Weiss *et al.*, 1994b), clarified by centrifugation and, used as cell-free extracts, assayed for SSAT and PAO, enzymes of polyamine degradation. Results in Table 1 are compared to previously reported data for the synthetic enzymes ODC, SSAT and AdoMetdc (Bacchi *et al.*, 2001). PAO is four times more active than AdoMetdc and >17-fold more active than ODC. Spermine was the preferred substrate for SSAT, while N^1-acetylspermine was the most active substrate for PAO. Other activity/inhibitor data for SSAT and for PAO are given under ‘Effects of polyamine analogues on polyamine metabolism of pre-emergent spore preparations’. Arginine decarboxylase activity was less than 0·0036 nmol (mg protein)$^{-1}$ (2 h)$^{-1}$ – below the lower limit of detection for the assay. This activity was not inhibitable by difluoromethylarginine (DFMA), a specific inhibitor of the enzyme (Bitonti *et al.*, 1982). Likewise, DFMA was not an inhibitor of ODC, while DFMO at 1 mM was 55% inhibitory (Bacchi *et al.*, 2001).

Polyamine synthesis and uptake by heavy gradient fractions

We had initially examined polyamine metabolism in mature spores freshly isolated from supernatant culture media, but found this activity to be very low (Bacchi *et al.*, 2001). Early studies on polyamine metabolism in pre-emergent spores used both heavy and light gradient fractions incubated in 150 mM Na_3PO_4-buffered 0·8% (w/v) NaCl, yielding wide-ranging results (Bacchi *et al.*, 2001). Nevertheless, these data indicated that uptake and conversion of L-[2,3-^{3}H]ornithine + 500 μM L-methionine to putrescine, spermidine and spermine occurred at about 12% of the rate of interconversion of [1-^{14}C]spermine to spermidine and putrescine [55·4 nmol (mg protein)$^{-1}$ (2 h)$^{-1}$, 12 preparations, versus 444·8 nmol (mg protein)$^{-1}$ (2 h)$^{-1}$, 9 preparations, respectively; Bacchi *et al.*, 2001].

We also examined uptake of polyamines in pre-emergent spores to compare rates of uptake. Table 2 lists K_m and V_{max} values of an experiment in which pre-emergent spores were examined in a rapid uptake study. Incubation mixtures (0·2 ml) containing increasing concentrations of radiolabelled ornithine, putrescine, spermidine and spermine were centrifuged through 0·2 ml mineral oil at 12 000 ***g***. Incubations of 15 min were used in a procedure we have used for uptake studies with African trypanosomes and obtained negligible carry-over of exogenous label (Goldberg *et al.*, 1998). Table 2 shows that, of the four molecules studied, spermine had the lowest K_m value (12·5 nmol) and the highest V_{max} [10 000 nmol (15 min)$^{-1}$]. One gauge of the efficacy of transport is the V_{max}/K_m ratio. For spermine, this ratio was 800, which was >100-fold higher than ratios obtained with data from other substrates. Other preparations yielded similar results.

In a series of experiments, we also examined the effects of various ionophores and ion channel reagents on spermine uptake. Used at 10–100 μM versus [1-^{14}C]spermine uptake, the following agents had little (<10 %) or no effect over 15 min uptake (two preparations, duplicate determinations): gramicidin S, amiloride, nifedipine, nitrendipine, monensin. At 100 μM, valinomycin was 57% inhibitory (two determinations: range 10–100 μM) while KCN was 31% inhibitory and sodium azide was not inhibitory.

Effects of polyamine analogues on polyamine metabolism of pre-emergent spore preparations

In recent work, we had demonstrated that polyamine analogues having a backbone of repeating *N*-butyl subunits, such as pentamines, oligoamines or bis(aryl)-substituted 3-7-3 analogues (Fig. 1), sterilized *Enc. cuniculi*-infected monolayer cells and cured two murine model infections (Bacchi *et al.*, 2002). We began mechanistic studies with some of the compounds that proved to be active *in vivo*, incubating pre-emergent spore preparations with 8 μM (0·25 μCi) [1-^{14}C]spermine for 1 or 2 h, and examining the resulting 10% TCA extracts by HPLC and radiodetection for reduction in uptake and interference with spermine interconversion. In an initial series of three experiments, we found that 10 μM SL-11144 and SL-11158 inhibited spermine uptake by 66% (59–77 %) and 63% (32–89 %), respectively. With 0·25 μCi [terminal methylenes-^{3}H(N)]-spermidine as substrate, SL-11144 also inhibited uptake 88·5% (single preparation) and SL-11158 58·8% (48·6–68·9%; two preparations) (data not shown).

In the course of these studies, we found a peak appearing at 3–5 min, which did not correspond to known polyamine standards or N^1-acetylspermine. The peak did, however, correspond to acetamidopropionaldehyde, a byproduct of polyamine interconversion which is excreted from cells (Marton & Pegg, 1995). The confirmation of the identity of this peak, however, awaits MS analysis.

In several experiments, we examined the effects of polyamine analogues on overall spermine uptake, and production of spermidine and acetamidopropionaldehyde. Table 3 gives results of a typical experiment (duplicate incubations) using the analogues SL-11061, SL-11158 and BW-1. In this experiment, we monitored internal (pellet) as well as external (supernatant medium) concentrations of spermine and products. All three analogues caused significant (70–80%) reduction in total spermine uptake, and higher inhibition of spermidine (82–95 %) and acetamidopropionaldehyde (76–92 %) production. SL-11061 had somewhat lower inhibition of total uptake (69·3%) as well as lower inhibition of spermidine (82·6%) and acetamidopropionaldehyde (76·3%) production. BW-1, although having a median effect on total uptake (73·4 %), inhibited production of both metabolites ≥90 %. Although little spermidine was found as excretion product, acetamidopropionaldehyde was present in control incubations, and was reduced in SL-11158 (20 %) and BW-1 (27 %) supernatants. If incubations continued for 2 h, supernatant media of analogue-treated cells had 3·2- to 11·5-fold more spermine remaining in the medium than controls incubated without analogues (not shown), indicating that less uptake was occurring in these cells.

Effects of SL-11158 on *Enc. cuniculi* SSAT

The key enzyme involved in polyamine interconversion is SSAT which in *Enc. cuniculi* has two to three times the activity with spermine rather than spermidine as substrate. In mammalian cells, SSAT has a short (15 min) $t_{1/2}$ and is highly inducible (to 1000-fold, or 1% of total cell protein; Casero & Pegg, 1993).

To determine the effect of SL-11158 on enzyme kinetics of *Enc. cuniculi* SSAT, we measured enzyme activity in the presence of none, 0·54 and 1·08 mM SL-11158 with increasing concentrations of spermine (2·7, 5·4 and 10·8 mM). Incubations contained 100 μM Bicine buffer, pH 8·0, 17 μM (60 μCi mmol^{-1}) [1-^{14}C]acetyl-CoA, inhibitor and spermine and 114 μg *Enc. cuniculi* spore protein preparation. After 30 min, reactions (in triplicate) were spotted on filter paper discs, washed and counted in Ominfluor. Blank reactions without spermine and protein were also run and subtracted. Results were analysed using a Hanes–Woolf primary plot (V/[S] versus substrate concentration). A K_m value of 17·3 mM was obtained for spermine and a K_i value of 0·24 mM was obtained for SL-11158 from a secondary Hanes–Woolf plot of K_m versus inhibitor concentration (Fig. 2). This is a mixed type of inhibition in which the inhibitor has an affinity for the enzyme which is much higher than that of the substrate.

Inhibitor/substrate studies on *Enc. cuniculi* PAO

We examined a number of compounds as substrates or inhibitors of *Enc. cuniculi* PAO, and compared them to findings in the literature for mammalian PAO (Table 4). None of the compounds, used at the concentrations indicated, affected the commercial horseradish peroxidase reaction in control reactions with H_2O_2 as substrate, without N^1-acetylspermine and homogenate.

Enc. cuniculi PAO utilized N^1-acetylspermine and N^8-acetylspermidine as substrate. It did not utilize spermine, spermidine or bis(benzyl)putrescine. Unfortunately, neither N^1- or N^8-acetylspermidine nor N^1,N^{12}-diacetylspermine were available for study with the *Enc. cuniculi* PAO, but both are substrates for mammalian PAO. In contrast, mammalian PAO utilizes spermine N^1- but not N^8-acetylspermidine, and uses bis(benzyl)putrescine. MDL 27695 is a bis(benzyl) 3-7-3 polyamine analogue which is debenzylated by mammalian PAO (Bitonti *et al.*, 1990), and which is also used as substrate by the *Enc. cuniculi* PAO. Aminoguanidine was not a substrate for mammalian enzyme but, at concentrations below 100 μM, was a substrate for *Enc. cuniculi* enzyme.

The pH optima of the enzymes also differed: pH 10·0 for mammalian PAO and pH 7·0–8·0 for the protozoan enzyme (Table 4). FAD superadded to control reaction mixtures did not stimulate the rate of the *Enc. cuniculi* enzyme. Two enzyme-activated irreversible inhibitors of mammalian PAO were developed by Merrell Dow Research Laboratories, MDL 72521 and MDL 72527 (Bey *et al.*, 1985). These exhibited time- and dose-dependent kinetics and had K_i values of 0·1–0·3 μM for the mammalian enzyme (Seiler, 1987). These analogues, used at up to 100 μM, stimulated the *Enc. cuniculi* enzyme by up to 70% (Table 5).

Table 5 lists activities of polyamine analogues used in this study as substrates or as inhibitors of *Enc. cuniculi* PAO. Substrate studies were done without N^1-acetylspermine in the reaction, while inhibitor studies had 1 mM N^1-acetylspermine as substrate. Activities were determined in duplicate and corrected with blanks run with extract without N^1-acetylspermine. None of the compounds affected rates with H_2O_2 as substrate for horseradish peroxidase.

None of the SLIL amine analogues studied (SL-11061, SL-11093, SL-11144, SL-11158) served as substrate for the *Enc. cuniculi* PAO at 100 μM, and only SL-11144 and SL-11158 were significant inhibitors of the reaction at 100 μM(75 and 57 %, respectively). MDL 27695 (1 mM) was a substrate, having 34% of the activity with the natural substrate, while BW-1 (1 mM), a bis(phenylbenzyl) 3-7-3 analogue of MDL 27695, had 55% of control activity, and stimulated the control reaction (with N^1-acetylspermine as substrate) by 40 %. Pargyline is an inhibitor of monoamine oxidases, but inhibited the full reaction by 80% and did not affect the mammalian enzyme (Table 5). These analogues did not affect the horseradish peroxidase reaction with H_2O_2 as substrate.

DISCUSSION

Although the microsporidia have been studied as unusual eukaryotic intracellular parasites for many years, little is known regarding their metabolism. Weidner *et al.* (1999) summarized the metabolic capabilities of *Ameson michaelis*, a parasite of the blue crab, indicating glycolysis proceeded in isolated sporoplasms when exposed to exogenous glucose and ATP. Related studies (Dolgikh *et al.*, 1997) indicated at least eight glycolytic enzymes (but not hexokinase) were present in spores of *Nosema grylli*, and concluded that it is likely that glycolytically produced NADH is recycled through a glycerol-3-phosphate dehydrogenase, since other glycolytic dehydrogenases were not detectable. Since hexokinase was also not detectable in several studies, it was postulated that the microsporidia may utilize hexose phosphates directly from the host (summarized in Weidner *et al.*, 1999).

Similarly, the ability of *Enc. cuniculi* to take up spermine and convert it to spermidine and putrescine, coupled with the high efficacy of spermine uptake as opposed to other polyamines or precursors, makes it likely that this microsporidian utilizes polyamine salvaged from the host cell as a major mechanism of acquiring polyamines. The kinetics of transport indicate it is used by *Enc. cuniculi* to scavenge any available polyamines from the host cytoplasm. Other protozoan parasites such as *Trypanosoma cruzi* and *Trichomonas vaginalis* are also believed to obtain most of their polyamines via exogenous sources (Le Quesne & Fairlamb, 1996; Ariyanayagam & Fairlamb, 1997; Yarlett *et al.*, 1994). Since spermine uptake was not blocked by monensin, gramicidin S, ouabain or amiloride, but was inhibited by valinomycin, it appears that Na^+/K^+ ATPase was not involved in uptake, but a universal cation transporter may be responsible. Relatively low inhibition (31 %) of transport obtained with KCN reinforces the finding that the microsporidia have neither mitochondria nor a functional cytochrome system (Undeen, 1990).

The high activity of exogenous spermine uptake in pre-emergent spores, and the increasing focus on polyamine analogues as anti-tumour agents (Casero & Woster, 2001; Frydman & Valasinas, 1999), made it imperative that we examine analogues as anti-microsporidial agents (Bacchi *et al.*, 2002). In mammalian cells, these are taken up through polyamine transporters and down-regulate polyamine synthesis while inducing SSAT and promoting excretion of acetylated polyamines. As polyamine levels fall in treated cells, the concentrations of analogues increase. Since they do not function in cell metabolism as do polyamines, division stops and apoptosis begins (Casero & Woster, 2001; Frydman & Valasinas, 1999; Ha *et al.*, 1998).

In the present study, we worked with pre-emergent spore fractions, which, although relatively homogeneous, were not actively dividing cells. Thus, effects of analogues seen on dividing mammalian cells such as SSAT induction, reduction in ODC and AdoMetdc activities, could not be readily observed. However, these analogues had several definitive effects on polyamine metabolism of *Enc. cuniculi*, including reduction of spermine uptake and interconversion (SL-11144, SL-11158) and mixed inhibition of SSAT (SL-11158). BW-1 was a substrate for PAO and *in vivo* is thus a likely competitive inhibitor of the enzyme with N^1-acetylspermine as substrate. The related analogue MDL 27695 [a bis(benzyl) 3-7-3 analogue; Fig. 1] is debenzylated by mammalian PAO (Bitonti *et al.*, 1990). MDL 27695 at 1 mM had modest (33 %) substrate activity in the *Enc. cuniculi* PAO assay. BW-1, a bis(phenylbenzyl) 3-7-3 analogue, had higher substrate activity (54·9%) in this system, and, used in the presence of the natural substrate, stimulated enzyme activity, by 2·6-fold (Table 5).

These studies indicate that *Enc. cuniculi* has significant polyamine scavenging potential with spermine the preferred substrate and it is likely that interconversion of polyamines readily occurs in this intracellular parasite. SL-11144, SL-11158 and BW-1 interfere with polyamine uptake and interconversion in the intact pre-emergent spores, while SL-11158 gives a mixed type of inhibition of parasite SSAT. BW-1 is a substrate for parasite PAO and

NMR studies in progress indicate two products are formed from [^{13}C]BW-1, a carboxylic acid and an aldehyde, which is consistent with an oxidation pathway (P. Woster, personal communication). All these effects occur at similar analogue concentrations, indicating that the mechanism of action of these agents *in vivo* targets this pathway.

Since existing chemotherapeutic agents for the microsporidia are not universally curative in human infections (Wittner & Weiss, 1999), more effective agents are needed. Continuing studies are aimed at developing more active agents by examining structure–activity relationships and clarifying mechanism(s) of action.

ACKNOWLEDGEMENTS

This work has been supported by grants from the National Institutes of Health (#2RO1-041398-06 to M. W. and A44-AI43094-03 to B. F.), and a Pace University Scholarly Research Award (C. J. B.). The authors thank Jenny Gallardo, Amy Kirner and Maryann Soliman for technical help.

Abbreviations

AdoMetdc	*S*-adenosylmethionine decarboxylase
DFMO	DL-α-difluoromethylornithine hydrochloride
ODC	ornithine decarboxylase
PAO	polyamine oxidase
SSAT	spermidine/spermine N^1-acetyl-transferase

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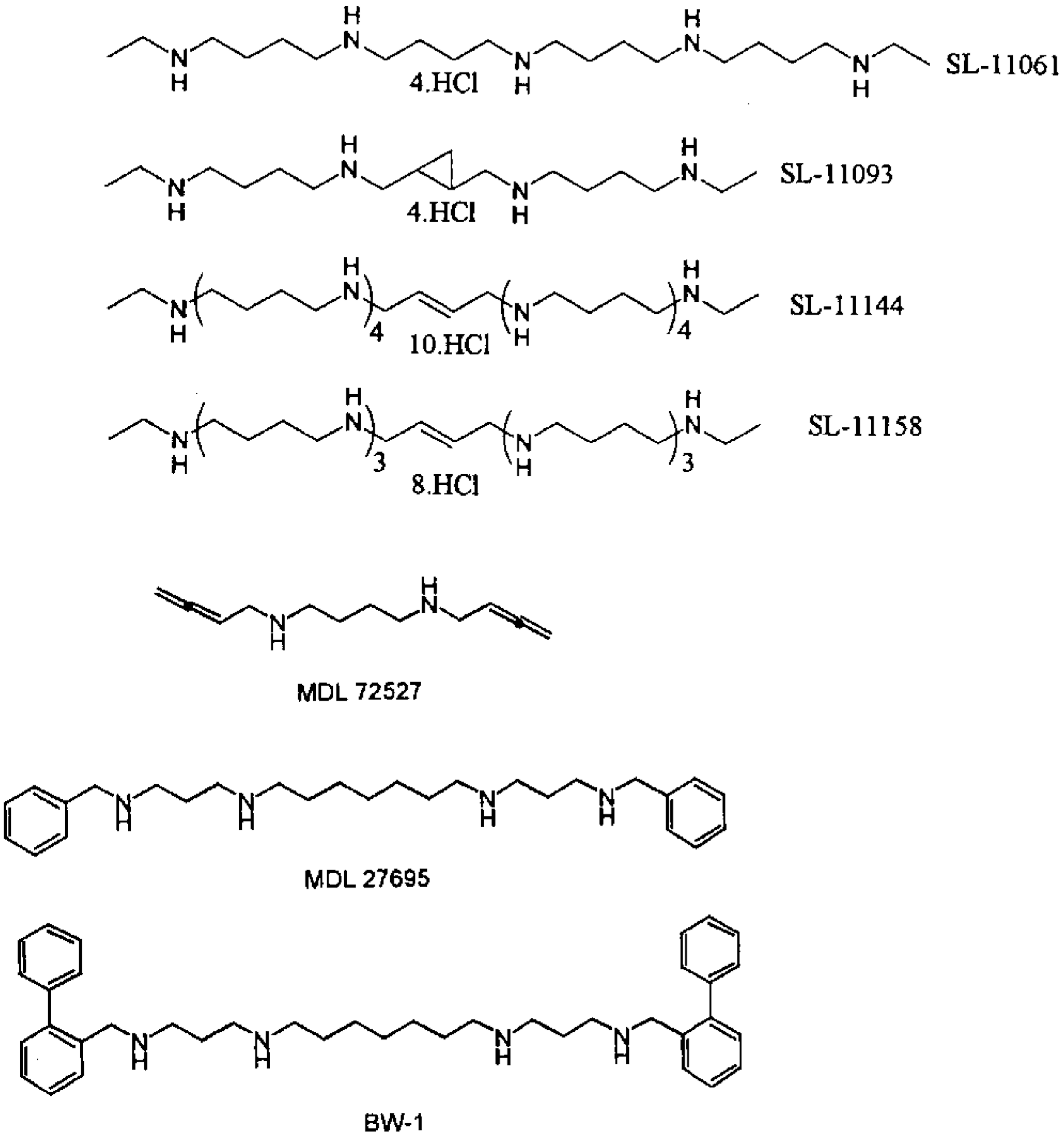

Fig. 1.

Structures of polyamine analogues used in this study.

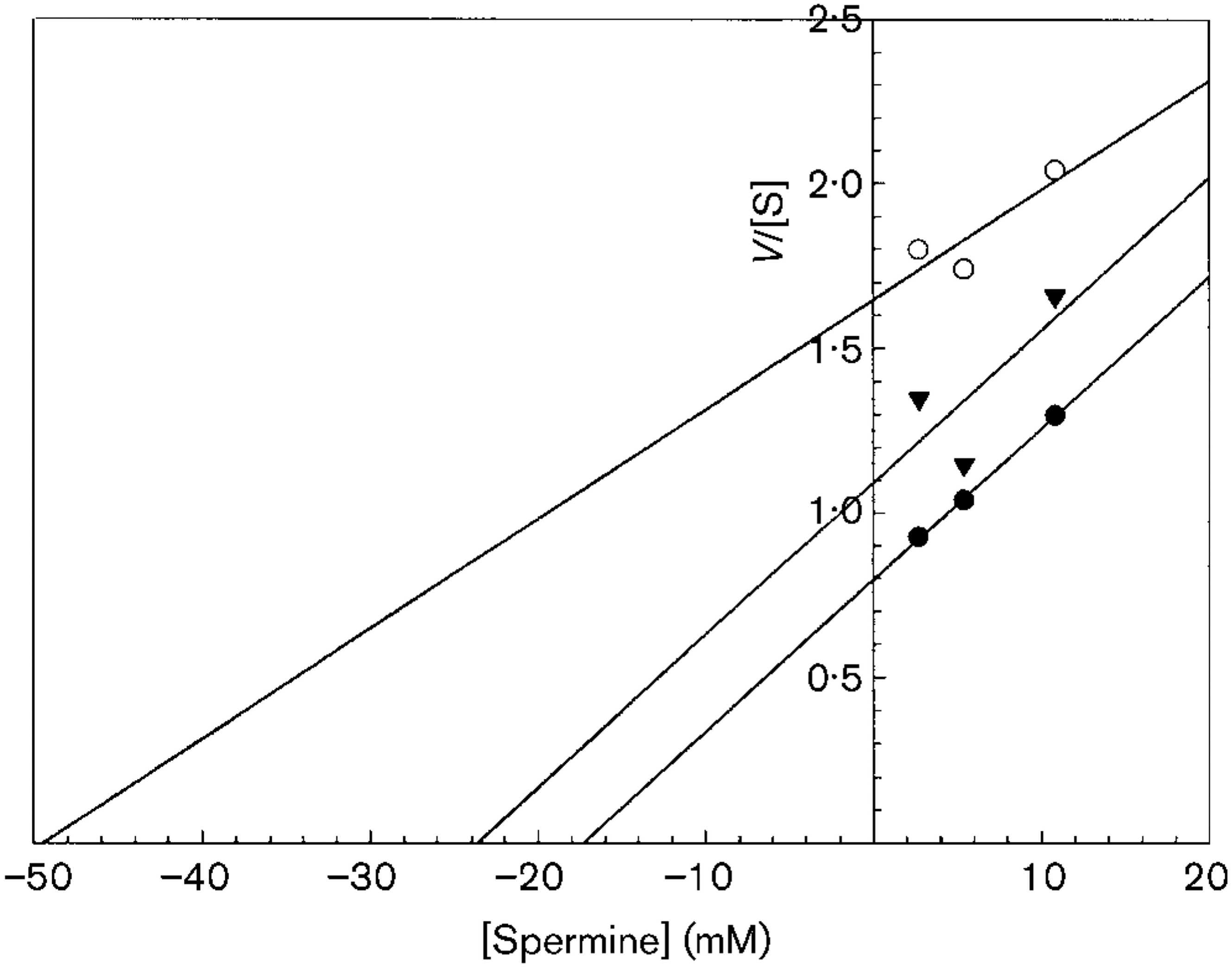

Fig. 2.

Hanes–Woolf plot of *Enc. cuniculi* SSAT activity with spermine as substrate versus SL-11158 as inhibitor. Inhibitor concentrations: ●, none; ▼, 0·54 mM; ○, 1·08 mM. Spermine concentrations were 2·1, 5·4 and 10·8 mM. The non-converging lines show a mixed type of inhibition. The K_m value for spermine was 17·3 mM and the K_i value of 0·24 mM was obtained for SL-11158. Reactions were in triplicate.

Table 1

Activities of enzymes of polyamine metabolism in freshly isolated mature spores of *Enc. cuniculi*

Spores were obtained aseptically from supernatants of infected HEK or RK-13 cells. Enzyme preparations were made using glass beads as described in Methods. Data are the mean of (*n*) 4–13 preparations, assayed in duplicate. Ranges are in parentheses. Data for ODC, arginine decarboxylase (ADC) and AdoMetdc were reported previously (Bacchi *et al.*, 2001) and are included for comparative purposes.

Enzyme	**Substrate**	***n***	**Activity [nmol (mg protein)$^{-1}$ (2 h)$^{-1}$]**
ODC	1-[^{14}C]Ornithine	4	25·35 (18·6–32·1)
ADC	[U-^{14}C]Arginine	4	<0·0036
AdoMetdc	*S*[carboxyl-^{14}C]AdoMet	4	123·6 (28·6–242·3)
SSAT	[1-^{14}C]Acetyl-CoA	13	24·12 (5·6–52·8)
PAO	*N*^{1}-Acetylspermine	5	438·15 (81·02–818·3)

Table 2
Polyamine and amino acid uptake by *Enc. cuniculi* pre-emergent pores

Organisms were harvested from HEK cells as described. The spore suspension (240 μg protein) was incubated in W-T medium for 15 min with 25–500 μM of [1-^{14}C]spermine, [terminal methylenes-^{3}H(N)]spermidine, [1,4-^{14}C]putrescine or L-[2,3-^{3}H]ornithine. Cells were centrifuged through 200 μl mineral oil; the supernatant medium was aspirated. Tips of tubes were cut off and counted in Beckman Ready Protein. Michaelis–Menten kinetics were used to analyse data.

Substrate	K_m **(nmol)**	V_{max} **[nmol (15 min)$^{-1}$]**	V_{max}/K_m
[1-^{14}C]Spermine	12·5	10 000	800
[terminal methylenes-^{3}H(N)]Spermidine	90	660	7·3
[1,4-^{14}C]Putrescine	285	2 220	7·7
L-[2,3-^{3}H]Ornithine	250	1 810	7·2

Table 3

Effects of polyamine analogues on uptake and interconversion of [1-^{14}C]spermine by *Enc. cuniculi* pre-emergent spores

Preparations were incubated for 1 h in W-T medium with [1-^{14}C]spermine. At 1 h, incubation mixtures were microfuged (12 000 *g*, 1 min) and the supernatant premium was aspirated and frozen. Pellets were extracted with 10% TCA overnight and frozen. Pellet extracts and supernatant medium were assayed by HPLC as described in Methods. Results are from duplicate incubations. Retention time values: unknown (possibly acetamidopropionaldehyde), 3–5 min; spermidine, 26 min; spermine, 30 min. All analogues were added at 10 μM.

Preparation	**Activity [nmol (mg protein)$^{-1}$ (2 h)$^{-1}$]**			
	Unknown	**Spermidine**	**Spermine**	**Total**
Control pellet	8·16	1·45	14·6	24·25
+SL-11061	1·93 (−76·3%)	0·25 (−82·6%)	5·25 (−64 %)	7·44 (−69·3%)
+SL-11158	0·58 (−92·8%)	0·23 (−83·5%)	3·71 (−74·5%)	4·54 (−81·3%)
+BW-1	0·77 (−90·5%)	0·06 (−95·6%)	5·59 (−61·7%)	6·43 (−73·4)
Control supernatant	3·81	–	5·86	10·35
+SL-11061	4·29 (+12·5%)	0·37	5·03 (−14·1%)	8·25
+SL-11158	3·04 (−20·2%)	–	3·96 (−32 %)	5·33
+BW-1	2·78 (−27 %)	–	6·24 (+6·4%)	4·11

Table 4

Characteristics of mammalian and *Enc. cuniculi* PAO activity

Data for mammalian enzyme compiled from Seiler (1987, 1995) and Bitonti *et al.* (1990). *Enc. cuniculi* PAO prepared from freshly harvested mature spores broken using the mini beadbeater technique (Weiss *et al.*, 1994b); assays as described in legend to Table 5. +, Activity; −, no activity; ND, not determined.

Substrates	**Mammalian PAO**	***Enc. cuniculi* PAO**
N^1-Acetylspermine	+	+
Spermine	+	−
Spermidine	+	−
N^1,N^{12}-Diacetylspermine	+	ND
N^1-Acetylspermidine	+	ND
N^8-Acetylspermidine	−	+
bis(Benzyl)putrescine	+	−
MDL 27695	+	+(Inhibits at 100–500 μM)
Benzylamine	+	+(16% at 100 μM)
Aminoguanidine	−	+ (1–100 μM)
Cofactor		
FAD	+	−
pH optimum	10·0	7·0–8·0
Inhibitors		
MDL 72521	K_i 0·3 μM	Stimulates activity at 10–100 μM
MDL 72527	K_i 0·1 μM	Stimulates activity at 10–100 μM
Pargyline	−	80% at 50 μM
Aminoguanidine	−	30–90% (at 100–500 μM)

Table 5

Polyamine analogues as substrates or inhibitors of *Enc. cuniculi* PAO

Enzyme reactions contained 0·1 M glycine buffer pH 7·0, 10 units horseradish peroxidase (Sigma), 5 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 20–200 μg *Enc. cuniculi* mature spore cell-free homogenate, in a final volume of 1 ml. Assays of analogues as substrates had no N^1-acetyl-spermine and analogues were added to start the reaction; assays of analogues as inhibitors had 1 mM N^1-acetylspermine added to start the reaction. Assays were linked to reduction of ABTS by peroxidase and measurement of the oxidized product at 420 nm (Childs & Bardsley, 1975). All activities were determined in duplicate and corrected for blanks run with extract, but without N^1-acetylspermine. Data are compiled from eight separate experiments in which the mean specific activity of full activity controls was 21·1 nmol (mg protein)$^{-1}$ min^{-1} [range: 5·0–75 nmol (mg protein)$^{-1}$ min^{-1}]. +, Stimulation of activity; ND, not determined.

Compound	Concentration	Percentage of control activity (substrate)	Percentage inhibition of activity
N^1-Acetylspermine (control)	1 mM	100	0
bis(Benzyl)putrescine	1 mM	0	ND
SL-11061	100 μM	0	+6
SL-11093	100 μM	0	13
SL-11144	100 μM	0	75
SL-11158	100 μM	0	57
MDL 27695	1 mM	33·9	ND
Aminoguanidine	1 μM	56·1	15
Aminoguanidine	10 μm	38·8	22
Aminoguanidine	100 μm	33·7	33
Aminoguanidine	500 μm	0	88
Benzylamine	100 μM	16·4	ND
MDL 72527	10 μM	ND	+7·5
MDL 72527	50 μM	ND	+29
MDL 72527	100 μM	91·3	+30
MDL 72521	100 μM	101·3	+55
MDL 72521	10 μM	103	+49
MGBG[*]	100 μM	ND	+360
BW-1	1 mM	54·9	+260
Pargyline	50 μM	ND	80

[*] MGBG, Methylglyoxal-bis(guanylhydrazone).