ELSEVIER

European Journal of Pharmaceutics and Biopharmaceutics 47 (1999) 179–184

European Journal of Pharmaceutics and Biopharmaceutics

Note

Preliminary study on in vitro activity and cytotoxicity on cell cultures of a new polyene antifungal molecule (SPA-S-843)

Augusto Pessina*, Lorena Rossoni, Michela Piccirillo, Giuliana Sala, Maria Grazia Neri

Laboratory of Cell Culture, Institute of Microbiology, University of Milan, Milan, Italy

Received 15 December 1997; accepted 11 September 1998

Keywords: SPA-S-843; Polyenes; Amphotericin B; Cell cytotoxicity

1. Introduction

Polyenes are antibiotics with fungistatic and fungicidal activity against both fungi and yeast [1]. Their activity, related to their affinity for sterols of fungal cell membranes, is due to the alteration of cell permeability and leakage of potassium and other essential metabolites [2,3]. Since the mammalian cell membranes contain sterols, they may be a polyene target with different cytotoxic consequences.

This study considered a new polyene, (SPA-S-843) derived from mepartricine, the active structure of which is the macrolide ring with its rigid lipophilic and flexible hydrophilic parts.

In a first screening, we compared the fungicidal activity against *Candida albicans* of SPA-S-843 in comparison with Amphotericin-B by using both a standardized reference method (SRM) and a MTT microtiter test. Further, the polyene's toxicity was evaluated on different mammalian cell lines and on myeloid committed progenitors in order to calculate an in vitro therapeutic index for SPA-S-483 as a fungicidal drug in cell culture and to assess the capacity of SPA-S-483 to prevent cell culture contamination by *C. albicans*.

2. Materials and methods

The antifungal activity of SPA-S-843 (*N*-dimethylaminoacetil-partricin A 2-dimethylamide diascorbate) (Biospa, Milan, Italy) and Amphotericin B (Bristol-Myers-Squibb, USA) was tested on clinical isolate of *C. albicans* (type 1, Serotype 2576174). The antifungal test was performed in liquid suspension and followed by plating in Sabouraud dextrose agar (SDA) according to the SRM suggested by Galgiani et al. [6]. Antifungal activity was also tested by a 24 h MTT assay [7,8].

The drug's toxic effect on cell growth was studied on three murine established cell lines: WEHI-3B (D +) myelomonocytic leukaemia [3], L1210 (B leukaemia) (ATCC, CCL 219), SR-4987 (stromal cells) [4] and on myeloid precursors in fresh isolated murine bone marrow cells (mu-BMC) obtained from BDF-1 female mouse [5].

The cytotoxic effect was determined both in a cell proliferation MTT microtiter [7,8] assay and by an agar clonogenic test [9].

The effect on murine granulocyte-macrophage progenitors (GM-CFU) was assayed in a soft agar culture system [5].

Experimental cell cultures contamination with 10^2 CFU of *C. albicans* was done in multiwell plates after 24 h of drug pre-treatment and then the culture was checked for the presence of yeast until 11 days (two subcultures).

The IC_{10}, IC_{50} and IC_{90} values were determined by the Reed and Muench formula [10]. Data elaboration was done for determining the following arbitrary in vitro ratios: safety ratio = IC_{90} cells/IC_{10} cells; activity ratio = IC_{50} cells/IC_{50} yeast; therapeutic ratio = IC_{10} cells/IC_{90} yeast.

The dose response relationship was studied by linear regression and the calculation of correlation coefficient (r). Statistical analysis was performed using the Student's t-test; differences were considered significant at values of $P < 0.05$ in a bi-directional t-test.

* Corresponding author. Laboratory of Cell Culture, Institute of Microbiology, University of Milan, Via Pascal 36, 20133 Milan, Italy. Tel.: +39-2-2660-1222; Fax: +39-2-2660-1218.

0939-6411/99/$ - see front matter
PII: S0939-6411(98)00065-4

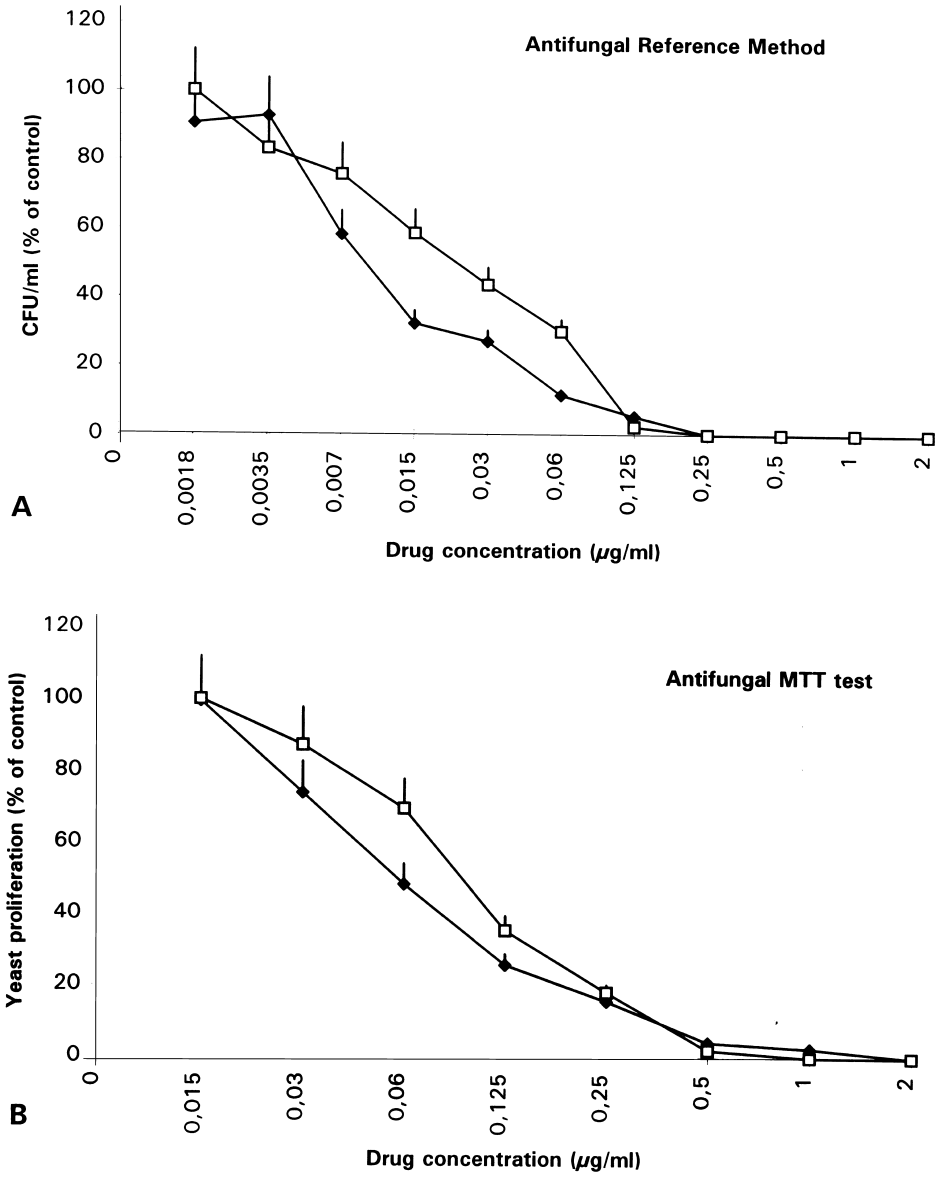

Fig. 1. Antifungal activity of Amphotericin B and SPA-S-843 on *Candida albicans*. A = reference method: growth is expressed as a percentage of the number of colonies (CFU) counted in untreated yeast cultures. B = MTT test: growth is expressed as a percentage of controls (untreated yeast). Each point represents the mean ± SEM of three experiments performed in duplicate. –♦–♦– (SPA-S-843) –□–□– (Amphotericin B).

Table 1

IC_{10}, IC_{50} and IC_{90} values of SPA-S-843 and Amphotericin B on cell proliferation and clonogenicity

	IC_{10} (μg/ml)		IC_{50} (μg/ml)		I C_{90} (μg/ml)	
	SPA-S-843	Amphotericin B	SPA-S-843	Amphotericin B	SPA-S-843	Amphotericin B
WEHI-3B (D+)						
Proliferation	3.2 ± 1.8	5.2 ± 2.6	5.04 ± 2.4	15.7 ± 6.2	8.03 ± 3.1	29.5 ± 4.5
Clonogenicity	0.4 ± 0.01	0.92 ± 0.1	0.78 ± 0.02	2.23 ± 0.005	1.13 ± 0.01	4.09 ± 0.64
L1210						
Proliferation	1.75 ± 0.5	0.81 ± 0.02	2.5 ± 0.7	3.91 ± 1.5	3.2 ± 0.6	12.43 ± 5.3
Clonogenicity	0.17 ± 0.02	0.28 ± 0.005	0.87 ± 0.01	1.37 ± 0.08	1.74 ± 0.02	8 ± 0.75
SR 4987						
Proliferation	1.24 ± 0.3	1.27 ± 0.2	3.83 ± 1.3	2.64 ± 0.4	7.65 ± 1.1	8.57 ± 2.8
BMC						
Clonogenicity	0.27 ± 0.09	0.22 ± 0.02	0.68 ± 0.01	1.3 ± 0.3	1.16 ± 0.01	3.2 ± 0.2

Each value represents the mean ± SEM of at least three experiments performed in duplicate.

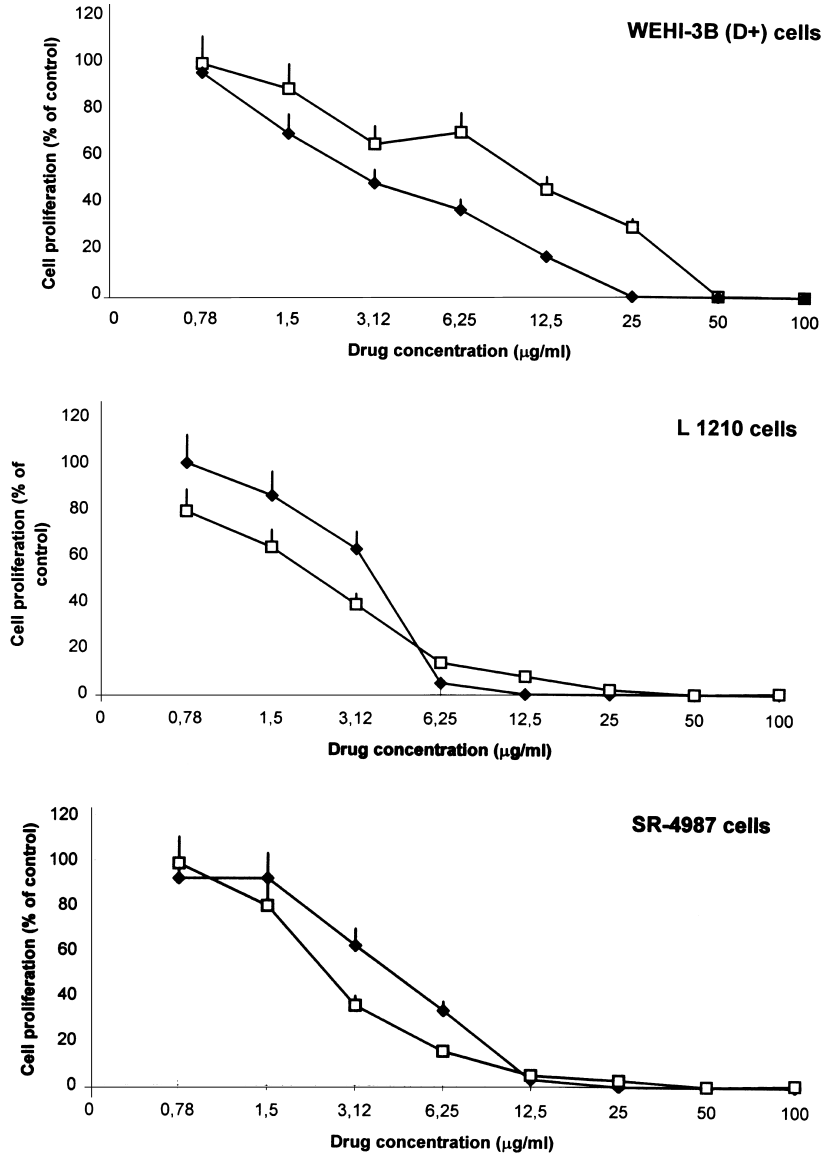

Fig. 2. Dose-response curve of cytotoxic effect of Amphotericin B and SPA-S-843 on in vitro cell proliferation (MTT test). Cell proliferation is expressed as a percentage of controls (untreated cells). The values of optical density in the controls were 1.33 ± 0.048 for WEHI-3B, 1.082 ± 0.106 for L1210 and 0.420 ± 0.065 for SR 4987. Each point represents the mean ± SEM of three experiments performed in triplicate. –♦–♦– (SPA-S-843) –□–□– (Amphotericin B).

Table 2

Comparison of in vitro therapeutic ratio, activity ratio and safety ratio of SPA-S-843 and Amphotericin B

	Safety index (IC_{90} cell/IC_{10} cell)		Activity index (IC_{50} cell/IC_{50} yeast)		Therapeutic index (IC_{10} cell/IC_{90} yeast)	
	SPA-S-843	Amphotericin B	SPA-S-843	Amphotericin B	SPA-S-843	Amphotericin B
WEHI-3B (D +)						
Proliferation	2.50	5.7	420	654	41	54.7
Clonogenicity	2.51	4.42	65	93	5.76	9.74
L1210						
Proliferation	1.82	15.52	207	162	22.43	8.42
Clonogenicity	9.94	28.2	73	57	2.24	3
SR 4987						
Proliferation	6.17	6.74	319	110	15.89	13.37
BMC						
Clonogenicity	4.25	14.82	56.6	54.2	3.5	2.28
Mean ± SEM	4.53 ± 1.26	12.55 ± 3.66	190.1 ± 62.4	188.4 ± 94	15.13 ± 6.1	15.25 ± 8.11

3. Results and discussion

3.1. In vitro antifungal activity

As shown in Fig. 1 the antifungal activity of the two drugs is dose-dependent and no significant differences are observed between the two different antifungal assays. The linearity test gave an identical coefficient of correlation ($r = -0.97$ for SPA-S-843; $r = -0.98$ for Amphotericin B) both in the SRM and in MTT microtiter test (although this last test is three times less sensitive). The IC_{50} values ± s.d. determined in SRM were: 0.012 ± 0.0034 μg/ml for SPA-S-843 and 0.024 ± 0.014 μg/ml for Amphotericin B. However, the higher potency of SPA-S-843 is not statistically significant ($P > 0.05$) if compared with Amphotericin B potency.

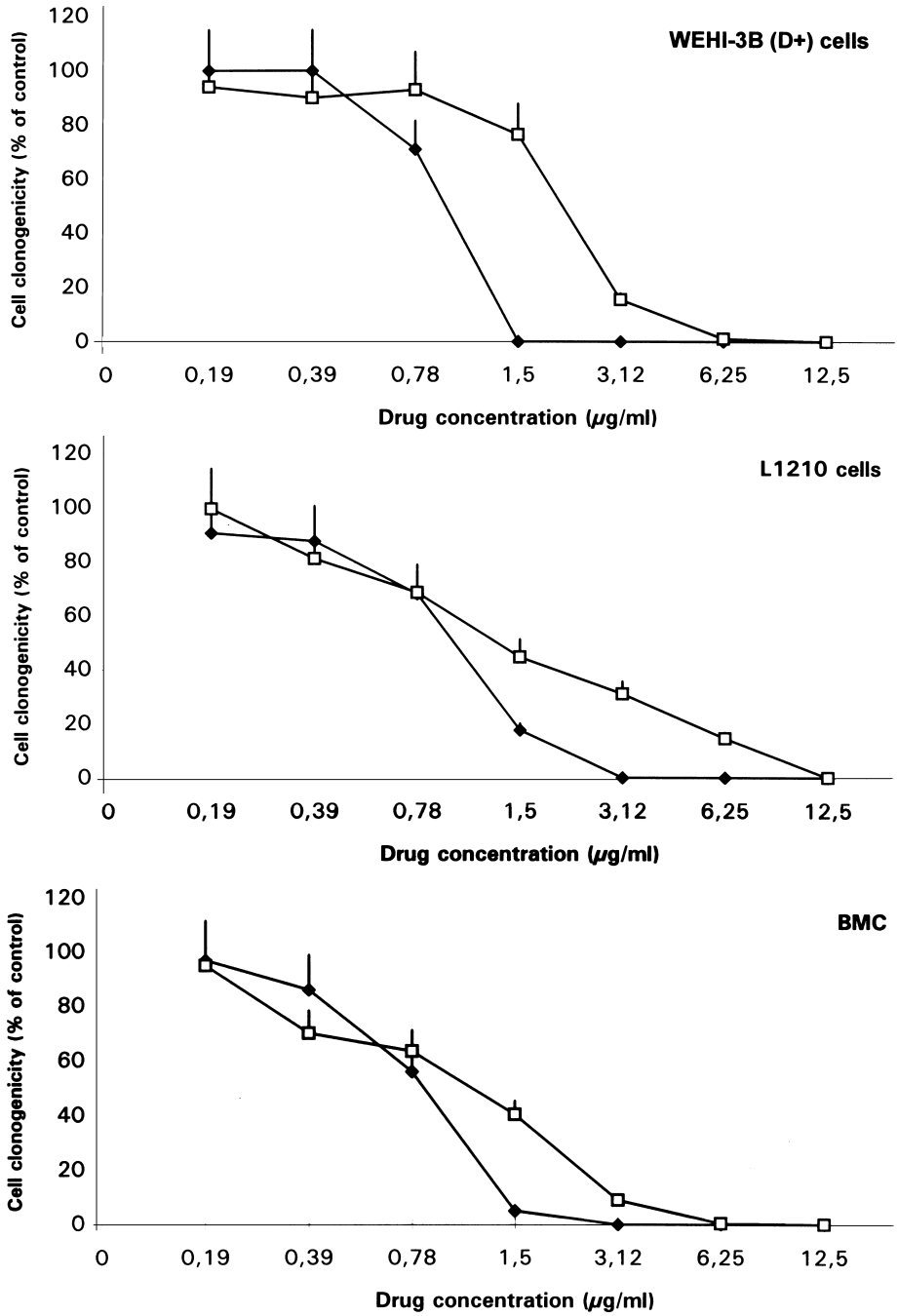

Fig. 3. Dose-response curve of cytotoxic effect of Amphotericin B and SPA-S-843 on cell clonogenicity (Agar test). Clonogenic capacity is expressed as a percentage of controls (untreated cells). The number of colonies in the controls were 212 ± 62 for WEHI-3B, 153.3 ± 31.0 for L1210 and 129 ± 10.5 for granulocyte macrophage progenitors (GM-CFU). Each point represents the mean ± SEM of two experiments performed in duplicate. –♦–♦– (SPA-S-843) –□–□– (Amphotericin B).

3.2. In vitro toxicity on cell proliferation and clonogenicity

The dose-response curves of drug toxicity on cell proliferation are reported in Fig. 2. As confirmed by linear regression analysis, the toxic effect of Amphotericin B and SPA-S-843 is dose-dependent and the activity of the two drugs appears substantially comparable. The IC_{10}, IC_{50} and IC_{90} values of the drugs toward the cell lines (Table 1) do not differ significantly. Only the IC_{90} values on WEHI-3B and L1210 indicate a significant ($P < 0.05$) greater activity of SPA-S-843.

The dose-response curves of the two drugs tested on cell clonogenicity are reported in Fig. 3. The kinetics for L1210 cells and GM-CFU appear to be similar whereas the two drugs exert a significantly different kinetic pattern of toxicity on WEHI-3B.

The IC_{10}, IC_{50} and IC_{90} values of the drugs to each cell line (Table 2) indicate that in WEHI-3B cells the IC_{50} value for SPA-S-843 is lower than Amphotericin B ($P \geq 0.05$) and the comparison of IC_{90} values confirm the data of cell proliferation experiments in which SPA-S-843 proved more active ($P < 0.05$) than Amphotericin B in all the cell models.

3.3. Comparison of toxicity parameters

In general the cytotoxicity is better expressed by the clonogenic assay than in the proliferation, one both for SPA-S-843 and Amphotericin B in all the cell lines tested. Further SPA-S-843 seems to exert a cytotoxic effect stronger than that exerted by Amphotericin B and this agrees with the expression of a higher antifungal activity. In fact, as reported in Table 2, the therapeutic and activity indices of SPA-S-843 and Amphotericin B are almost identical whereas the difference observed between the safety ratios are not statistically significant ($P > 0.05$).

The studies on the prevention of *C. albicans* contamination are reported in Table 3. In the cell cultures without drugs, a mean of 89.5 ± 5.9 yeast colonies/culture were counted after only 24 h from the experimental contamination whereas no yeast growth is observed either in SPA-S-843 or in Amphotericin B pre-treated cultures until 96 h of observation.

Table 3
Prevention of experimental contamination

Cell line	[a]Drug pretreatment 24h (1μg/ml)	Yeast growth (CFU/culture) 24 h
WEHI-3B (D+)	Untreated	94 ± 4
	SPA-S-843	0
	Amphotericin B	0
L1210	Untreated	90 ± 3
	SPA-S-843	0
	Amphotericin B	0
SR 4987	Untreated	85 ± 5
	SPA-S-843	0
	Amphotericin	0

0, no yeast colonies were detected by microscope.
[a]After 24 h of drug pretreatment the cell cultures were inoculated with 10^2 CFU of *C. albicans*, and then checked after further 24 h.

4. Conclusion

These observations clearly suggest that SPA-S-843 can be considered a very interesting molecule entitled to be developed as a prophylactic agent likely to be useful in preventing yeast contamination in cell cultures and perhaps also able to cure cell cultures infected by fungi. Due to its high water solubility and its chemical stability (in comparison with Amphotericin B) SPA-S-843 could represent an advantage making the substance easier to handle and easier in allowing for the preparation of more accurate dilutions in culture medium. These observations on cytotoxicity need to be confirmed on more cell lines and further studies are needed on drug stability in cell culture media (in relation to the pH change due to cell growth) and on protein binding capacity (which could modify the drug availability for the cells). It will be also important to consider problems related with yeast drug resistance if such a drug should be used routinely in cell cultures.

Acknowledgements

The authors thank Professor Martin Clynes for critically reading this manuscript.

References

[1] A.M. Sugar, The polyene macrolide antifungal drugs, in: P.K. Peterson, J. Verhoef (Eds.), Antimicrob. Agents Annual 2, Elsevier, Amsterdam, 1987, pp. 228–237.
[2] S.M. Hammond, Biological activity of polyene antibiotics, Prog. Med. Chem. 4 (1977) 105–177.
[3] L.N. Warner, M.A.S. Moore, D. Metcalf, A transplantable myelomonocytic leukemia in Balb/C mice: cytology, caryotype and neuraminidase content, J. Natl. Cancer Inst. 43 (1969) 963–982.
[4] A. Pessina, E. Mineo, M.G. Neri, L. Gribaldo, R. Colombi, P. Brambilla, G. Zaleskis, Establishment and characterisation of a new murine cell line (SR-4987) derived from marrow stromal cells, Cytotechnology 8 (1992) 93–102.
[5] A. Pessina, M.G. Neri, A. Muschiato, P. Brambilla, A. Marocchi, P. Mocarelli, Stimulating factor produced by murine adrenocortical tumour cells, J. Natl. Cancer Inst. 76 (1986) 1095–1099.
[6] J.N. Galgiani, M.S. Bartlett, A. Espinel-Ingroff, R.A. Fromtling, M.A. Pfaller, M.G. Rinaldi, Reference method for broth dilution antifungal susceptibility testing of yeasts; proposed standard, The National Committee for Clinical Laboratory Standards 12 (1995) 1–22.
[7] A. Pessina, L. Gribaldo, E. Mineo, M.G. Neri, In vitro short-term and long-term cytotoxicity of fluoroquinolones on murine cell lines, Ind. J. Exp. Biol. 32 (1994) 113–118.
[8] T. Mossman, Rapid colorimetric assay for cellular growth and sur-

vival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.

[9] A. Pessina, E. Mineo, M. Masserini, M.G. Neri, C.E. Cocuzza, Inhibition of murine leukemia (WEHI- 3B and L1210 proliferation by cholera toxin B subunity, Biochem. Biophys. Acta 1013 (1989) 206–211.

[10] L.J. Reed, H. Muench, A simple method of estimating fifty per cent endpoints, Am. J. Hyg. 27 (1938) 493.