Mepacrine Antagonises Tumour Cell Growth Induced by Natural Polyamines

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Abstract. *Background: Mepacrine is an antiproliferative agent, characterised by an aliphatic chain similar to that of natural polyamines whose activation is closely associated with cell proliferation and may lead to malignant transformation and neurodegenerative diseases. This study aims to investigate a possible antagonism between mepacrine and polyamines in tumour proliferation. Materials and Methods: MCF-7 and Vero cells were cultured in Eagle's minimum essential medium and then subjected to graded concentrations of putrescine, spermine and spermidine alone and in combination with mepacrine. Methyl thiazole tetrazolium test and Western-blotting were performed. Results: Putrescine and spermidine at 0.5 mg/l significantly stimulated cell growth, whereas mepacrine treatment confirmed the enhanced p21 expression previously reported by a recent study and growth inhibition. When used in combination, mepacrine antagonized MCF-7 growth induced by polyamines. Conclusion: Our results suggest that mepacrine may represent a choice in the treatment of tumours induced by the modified concentration of polyamines.*

The natural polyamines putrescine, spermidine and spermine are small aliphatic amines whose concentration in biological tissues is crucial for cell growth since they interfere with nucleic acids activating both RNA*t* and RNA*r* (1). Considerable evidence has shown that polyamines play a critical role in breast cancer cell proliferation. In animal models and in *in vitro* studies, the depletion of polyamines resulted in a decrease of neoplastic cell proliferation (2-4). Mepacrine is an alkylating agent characterised by the presence of an aliphatic chain very similar to that found in natural polyamines (Figure 1). It is active against both resting and rapidly dividing cells, moreover it has been recognised as an inhibitor of ornithine decarboxylase (ODC) a crucial enzyme in the synthesis of natural polyamines (5, 6). On the basis of this knowledge and of previous results from our preliminary studies (7, 8), we investigated the activity of mepacrine on the replication of tumour cells induced by polyamines.

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Materials and Methods

Compounds. Putrescine, spermine, spermidine and mepacrine were obtained from Sigma-Aldrich Milan, Italy.

Cells and cell cultures. MCF-7 cells from human breast carcinoma and Vero cells were used. Vero cells were chosen as a control line. Both the cell lines were cultured in sterile flasks containing Eagle's minimum essential medium (EMEM) (Lonza, Milan, Italy) enriched with 10% fetal bovine serum (FBS; Lonza) and 1% antibiotic solution (penicillin 50 U/ml and streptomycin 0.5 mg/ml) and 1% L-glutamine and maintained at 37˚C in a fully humidified atmosphere of 5% CO_2 in air. Once cells were grown to confluence (around 70%), they were transferred under sterile conditions, together with the culture medium, into disposable sterile plates with 24 wells; living cells were counted by the Trypan blue exclusion test to assure an initial inoculum of 35×10^4 cells. The plates were then incubated for 24 hours under the same conditions and subsequently the programmed tests were performed.

Cytotoxicity test. After the above incubation time, mepacrine and polyamines were dissolved in sterile dimethylsulphoxide (final concentration 0.01-0.03%), sterilised by filtration and added to the wells at the following concentrations: polyamines from 0.5 to 100 mg/l; mepacrine from 4.0 to 136 mg/l.

After a further incubation period (24 h), the methyl thiazole tetrazolium test (MTT) was performed according to the method described by Mosmann (9) to assess cell viability. The MTT test was performed in triplicate on 24- and 48-hour cell cultures treated with the drugs alone and with their combination using mepacrine at the determined LD_{50} of each cell line. The results were compared with control wells in which the cells were cultured without drugs.

Western blotting. Monolayer cultures in 60 mm dishes were washed with phosphate-buffered saline (PBS) and extracted by scraping with 100 μl of extraction buffer consisting of 50 mM Tris-HCl (pH 8.5), 150 mM NaCl, 1% Na deossicolate, 1% Triton® X-100, 0.1% sodium dodecylsulfate (SDS), 0.2% NaN_3 with the protease inhibitors aprotinin (0.2 TIU/ml; Sigma), leupeptin (0.01 mg/ml; Sigma) and phenyl methyl sulphonyl fluoride (PMSF, 4 mM; Sigma). Protein concentration was measured by means of the

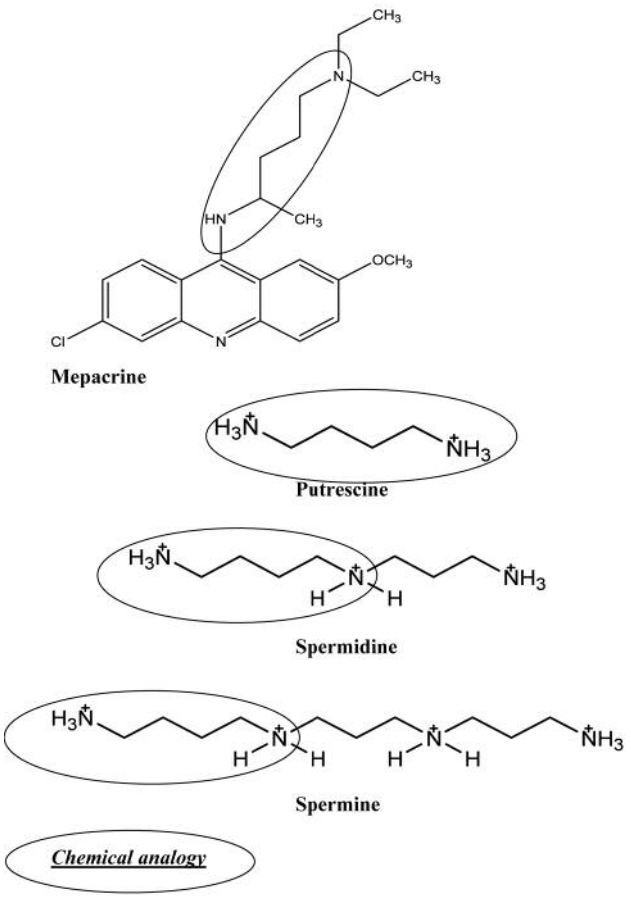

Figure 1. *Chemical structure of mepacrine and natural polyamines.*

Protein Assay Reagent (Protein Assay; Bio-Rad, CA, USA) in a total volume of 1 ml with bovine serum albumin as standard. Fifty microgrammes of protein per lane were loaded onto a 13% polyacrylamide gel and transferred to nitrocellulose. To verify equal loading of total protein in all lanes, the membrane was stained with red ponceau. The blot was incubated with anti-p21 (1:100), anti-cyclin-A (1:200), anti-cyclin B1 (1:200), anti-cyclin D1 and D2 (1:200) (Bio Optica, Milan, Italy). Blots were washed three times in PBS/Tween and incubated with the secondary antibody: purified goat anti-mouse IgG H+L horseradish peroxidase conjugate (Bio-Rad). Detection was performed using the ECL chemiluminescent system (Amersham, IL, USA) and autoradiographic film (Hyperfilm-ECL; Amersham).

Statistical analysis. The program SPSS for Windows, Release 10 was used for statistical analysis. The comparison of data was carried out using the Bonferroni test. The results were considered statistically significant when $p<0.05$.

Results

Mepacrine (Figure 2a) was confirmed to be cytotoxic against both cell lines (LD_{50}=50 mg/l and 34 mg/l on Vero and MCF-7 cells, respectively). Results from the MTT test confirmed that variation in polyamine concentrations can stimulate tumour cell growth. In our experiments, putrescine and spermine at 0.5 mg/l significantly stimulated MCF-7 cells (putrescine +57.5% and spermine +34% with respect to control) while, with the exception of putrescine at 0.25 mg/l, Vero cell replication was reduced in a dose-dependent manner (Figures 2b-d) thus showing a cytotoxic effect of spermine and spermidine against the control line. The aim of the present study was to investigate the effect of the combination between mepacrine and natural polyamines on tumour cell proliferation, therefore, we combined the same concentrations of putrescine, spermine and spermidine with mepacrine at the LD_{50}. It is evident from the histograms of Figure 2b-d that the combination resulted in an antiproliferative effect which can be considered useful only to inhibit MCF-7 cells, but was toxic to Vero cultures whose replication appeared further reduced with respect both to the control and to polyamine alone. However, in the case of putrescine at 0.5 mg/l combined with mepacrine at the LD_{50}, the proliferative activity of putrescine was inhibited by mepacrine without exerting selective toxicity on Vero cultures (42% growth inhibition on both cell lines). Contrasting results came from Vero cultures treated with spermidine: the combined treatment with mepacrine significantly improved cell replication both with respect to control and to the treatment alone (Figure 2c). This could be ascribed to chemical interactions between the molecules, but a more detailed study must be performed to explain these results.

Western blot analysis of the MCF-7 cells treated with mepacrine showed the up-regulation of p21 production. Briefly, MCF-7 cell p21 expression was increased by mepacrine at 6.25 μg/ml, as was D2 cyclin. In a recent study (10), we observed that higher concentrations of mepacrine (12.5 and 25 μg/ml) induced p53 expression, while p21 and B1 and A cyclin were unchanged. These results suggested that the antiproliferative activity of mepacrine may be due to the arrest of the cell cycle in the G_2-M phase.

Discussion

Polyamines are essential for the maintenance of cell growth, but among their negative effects are stimulation and proliferation of cancer cells, therefore, variation in their concentration in case of neurodegenerative illness is a real risk for survival of patients (11-13). Regulation of polyamine biosynthesis is complex and the key biosynthetic enzyme, ODC, is one of the most highly regulated enzymes since it allows the conversion of ornithine into putrescine (14-16). Our results demonstrated that putrescine stimulates both Vero and MCF-7 cell proliferation. Mepacrine has been recognised as a potent and specific inhibitor of phospholipase A2 (17-20) and consequently of ODC activity. Moreover, data from literature confirm that polyamine analogues reduce ODC

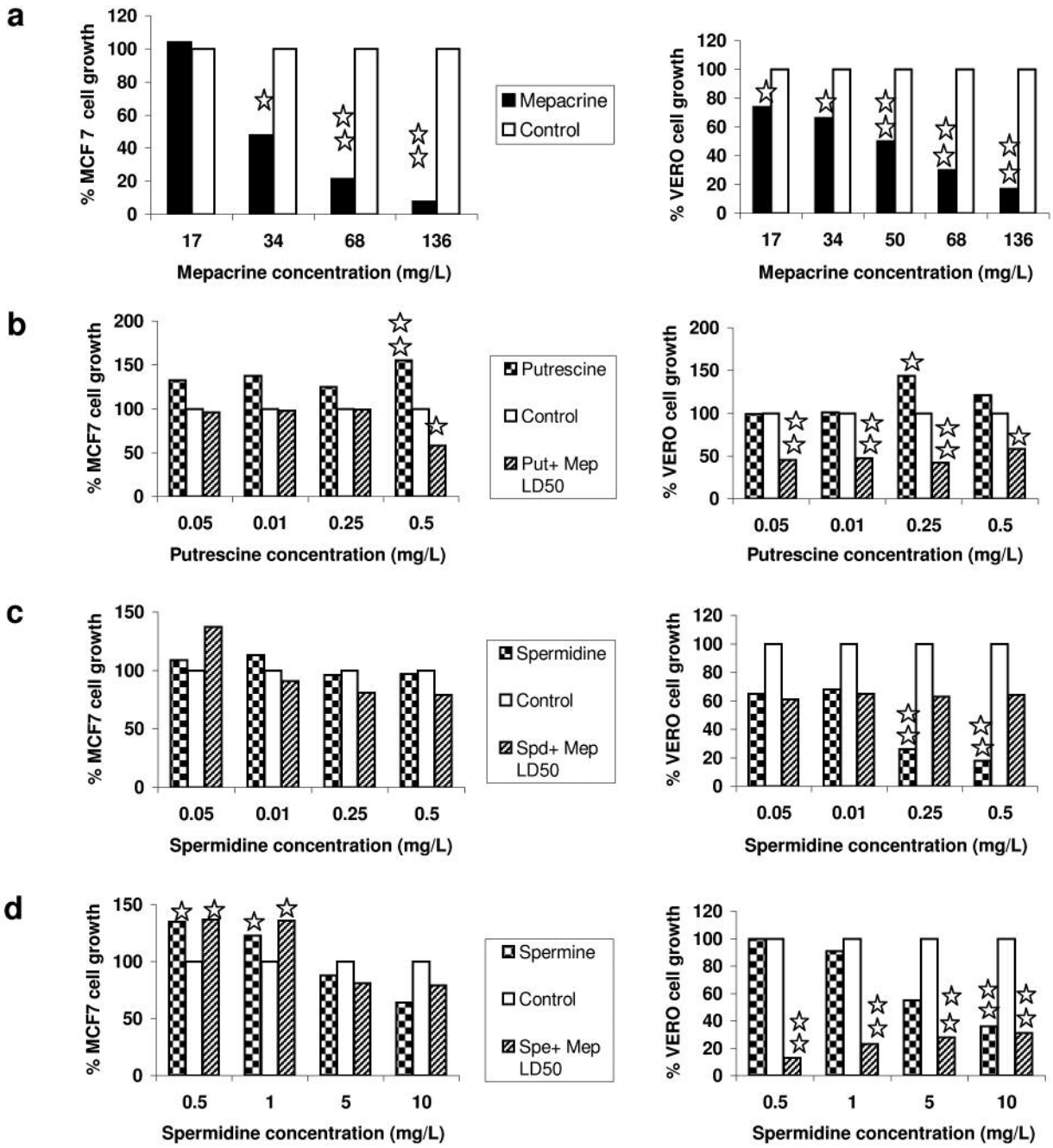

Figure 2. *Antiproliferative activity of a) mepacrine and b) putrescine, c) spermidine and d) spermine evaluated by MTT test on MCF-7 and Vero cells. Data are expressed as % cell growth with respect to control (100% growth). In the third column are represented the effects of the combinations: putrescine +mepacrine LD_{50} (Put+ Mep LD_{50}), spermidine +mepacrine LD_{50} (Spd+ Mep LD_{50}) and spermine+mepacrine LD_{50} (Spe+ Mep LD_{50}). Significantly different with respect to control (100% growth): ☆$p<0.05$ and ☆☆$p<0.01$ by Bonferroni test*

activity and deplete cellular polyamines (21-24). In the present study, we assumed that the chemical analogy between the aliphatic chain of mepacrine and that of polyamines could represent a hindrance in the pathway for the biosynthesis of polyamines, therefore, we treated breast human cancer cells (MCF-7) with increasing concentrations of putrescine, spermine and spermidine. Vero cells were chosen as control line. Mepacrine LD_{50} was determined on both cell lines, but mepacrine cytotoxicity was higher in Vero cells (Figure 2a). The results showed that putrescine and spermine stimulated MCF-7 cell growth in a dose-dependent manner. Vero cultures were stimulated by putrescine and inhibited by

spermine and spermidine thus showing their selective cytotoxicity against the control line. The Western blotting suggested that low concentrations of mepacrine (6.25 μg/ml) may cause the arrest of the cell cycle by up-regulating p21 expression. Finally, the effects we observed in the cultures treated with putrescine combined with mepacrine confirmed that a polyamine analogue, such as mepacrine, may interfere with putrescine activity in cell proliferation.

Conclusion

In the light of these preliminary results and in accordance with recent literature data (25), we conclude that mepacrine could represent a new opportunity to treat tumour proliferation induced by modifying the concentration of natural polyamines. Unfortunately, polyamines showed a selective cytotoxicity to Vero cells: only putrescine concentration may be modified by mepacrine without severe and selective cytotoxic effects against healthy cells.

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