

Effects of Anti-malarial Drugs on MCF-7 and Vero Cell Replication

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Abstract. *Background:* Previous *in vivo* studies performed in our laboratories demonstrated that anti-malarial drugs may or enhance or slow down Ehrlich's ascites tumour progression in infected mice. In the light of these observations, an *in vitro* study was undertaken to assess the response of human tumour cells to various anti-malarial drugs and consequently the safety of the anti-malarial therapy. *Materials and Methods:* MCF-7 cells and Vero cells (control line) were cultured in Eagle's minimum Essential medium (EMEM) and then subjected to graded concentrations of different anti-malarial drugs. Trypan-blue exclusion, MTT and Western blotting tests were performed. *Results:* The findings showed that pyrimethamine (12.5 mg/L), chloroquine (12.5 mg/L) and primaquine (1.56 mg/L) stimulated MCF-7 cell growth. The proliferative effect was inhibited by doxorubicin only in cultures treated with chloroquine and primaquine. These results might indicate that some anti-malarial drugs have a worrying tumour-promoting effect which should not be underestimated when undertaking anti-malarial prophylactic measures.

Malaria has been recognized as an important parasitic disease of humans for centuries, but despite the introduction of control programs, malaria is gradually returning to the world at large: every year about 300 million case of malaria and more than two million deaths are registered all over the world. The disease is transmitted to humans by the bite of an infected female *Anopheles* mosquito. Of the approximately 400 species of *Anopheles*, about two dozen serve as vectors: they infect humans and cause the infection in the human blood. A problem strictly related to malaria is a state of immunosuppression which

makes the patient more prone to bacterial infections; moreover, the treatment of the disease requires a long-term therapy which may induce a toxic response by healthy cells. In 1996, we started on studying the effects of some anti-malarial drugs on Ehrlich ascites tumour-infected mice (1). The results showed that mepacrine and primaquine significantly affected the survival pattern of infected mice, which died early with respect to controls. In an "*in vitro*" study, we observed that the oxygen consumption of tumoral cells cultured in the presence of the same anti-malarials was significantly higher if compared to that observed in control plates, or in cultures exposed to other anti-malarial drugs. We therefore proposed that the higher oxygen consumption was the consequence of an enhanced cellular metabolism and that some anti-malarials could promote tumoral progression in mice. In the light of these conflicting observations, an *in vitro* study was undertaken to assess the response of cultures of human tumour cells to various anti-malarial drugs alone and in combination with an antitumoral drug.

Materials and Methods

Compounds. Mepacrine, pyrimethamine, chloroquine (all from Sigma-Aldrich Milan, Italy), primaquine and doxorubicin (both from Pharmacia, Milan, Italy) were used as reference drugs.

Cells. MCF-7 (human breast adenocarcinoma) and Vero cells (kidney epithelial cells from African green monkey) were used. Vero cells were chosen as a control cell line.

Cell culture. All the cells were cultured in a thermostatically-controlled environment (37°C and 5% CO₂) in EMEM (Cambrex Bio Science, Milan, Italy) enriched with 5% foetal bovine serum (FBS, Cambrex Bio Science) and 1% antibiotic solution (penicillin 50 U/mL and streptomycin 0.5 mg/mL) and 1% L-glutamine. Once cells were grown to confluence (around 70%), they were transferred under sterile conditions, together with the culture medium, into disposable sterile dishes with 24 wells (initial inoculum 35x10⁴ cells). The plates were then incubated for 24 h at 37°C under the conditions described above. Subsequently, the programmed tests were performed.

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Assay for viable cell number. Cells were counted and tested for viability by the Trypan blue exclusion test.

MTT test. The test was performed to assess cell viability after treatment with increasing concentrations of the anti-malarial drugs. The MTT test is based on the reduction of the tetrazolium salt by mitochondrial enzymes, which is possible only in live cells therefore, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) was dissolved in sterile phosphate-buffered saline (PBS solution 5 mg/mL) and added to the cell cultures according to the method described by Mosmann (2). After incubation (3 h at 37°C), 1 N hydrogen chloride-isopropanol was added to each well and stirred to dissolve the dark-blue formazan crystals formed. Finally, the absorbance was measured with a spectrophotometer at $\lambda=570$, 630 and 690 nm. All tests were carried out in triplicate and compared with control wells in which no drug was added.

Western blotting test. Monolayer cultures in 60 mm dishes were washed with PBS and extracted by scraping with 100 μ l of extraction buffer consisting of 50 mM Tris-HCl (pH 8.5), 150 mM NaCl, 1% Na deoscolate, 1% Triton X-100, 0.1% SDS, 0.2% NaN₃ 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 Protein Assay Reagent (Protein Assay, Bio-Rad, CA, USA) in a total volume of 1 mL with bovine serum albumin as standard. Fifty μ g 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-p53 (1:400), anti-p21 (1:100), anti Cyclin-A (1:200), anti-Cyclin B1 (1:200), anti-Cyclin D1and D2 (1:200) (Bio Optica, Milan, Italy). Blots were washed three times in PBS/Tween and incubated with the secondary antibody: the 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. A statistical comparison of the data was carried out by the Bonferroni test. A *p*-value <0.05 was considered significant.

Results

MTT test results expressed as the percentage of the cell growth (mean values) are shown in Figures 1-4. Figure 1 and 2 show that chloroquine and pyrimethamine, blood schizonticidal agents effective against the erythrocytic stage of the plasmodia life cycle, have different effects on the two cultures. Both stimulated MCF-7 proliferation starting from a concentration of 6.25 mg/L; both were cytotoxic to Vero cells. In the plates treated with pyrimethamine, the Vero cell inhibition was dose-related. Mepacrine (Figure 3) significantly reduced the growth of both cells lines; the effect was dose-related, especially on Vero cells. An opposite effect was observed when primaquine was added to MCF-7 and Vero cultures: the stimulus of cell growth results as the main response (Figure 4).

International references demonstrate that a problem strictly related to malaria is a state of immunosuppression which makes the patient more prone both to bacterial infections and sometimes to tumours (3-5), consequently, there are times when anti-malarial and antitumor therapies could be administered simultaneously to the same patient. In order to investigate the effect of this combination, the antitumor drug doxorubicin was chosen as a reference drug, added to the cultures and investigated alone and in association with an anti-malarial drug.

The IC₅₀ values of the anti-malarial drugs and of doxorubicin on Vero cells were determined by the MTT test and the results are listed in Table I. Sub-inhibitory concentrations of the drugs were combined and added to the cultures which were then treated as previously described. The results are shown in Figures 5-8 and represent the percentage of MCF-7 cells cultured in the presence of concentrations of the anti-malarial (0.625, 1.25 or 2.50 mg/L) combined with two sub-inhibitory dosages of doxorubicin (0.156 and 0.625 mg/L). The outcome of the combination is that the proliferative effect of primaquine is completely inhibited by doxorubicin, without significant differences between the concentrations (Figure 5); while the combination chloroquine/doxorubicin results in a significant reduction (50%) of MCF-7 growth only on the addition of the highest doxorubicin concentration (Figure 6). The mepacrine/doxorubicin combination (Figure 7) is perhaps useful in avoiding the peak of MCF-7 growth (Figure 3) when the anti-malarial is added at a concentration of 3.125 mg/L. No anti-proliferative effect was exerted by doxorubicin on pyrimethamine-induced MCF-7 growth (Figures 2 and 8). Results from the Western blotting test (Figure 9) show that MCF-7 cell p21 expression was increased by mepacrine at 0.625 mg/L, as was D2 cyclin. Higher concentrations of mepacrine (1.25 and 2.5 mg/L) induced p53 expression, while expression of p21 and B1 and A cyclin were unchanged. No significant variations were observed after treatment with primaquine and chloroquine.

Discussion

In our study, we considered Vero cells as a control cell line (6, 7) and MCF-7 cells as tumour reference cells. The results from the MTT test show that the proliferative or anti-proliferative effects of the anti-malarial drugs on MCF-7 cells are dose-correlated. Drugs such as chloroquine and pyrimethamine have an anti-proliferative effect on Vero cells at the same concentrations at which they exert a stimulating effect on MCF-7 cells; this result must be considered a severe side-effect not to be underestimated when anti-malarial measures must be undertaken in an oncological patient, even if the results of our study demonstrate that combined therapy with doxorubicin at 0.625 mg/L may be effective in reducing the cellular

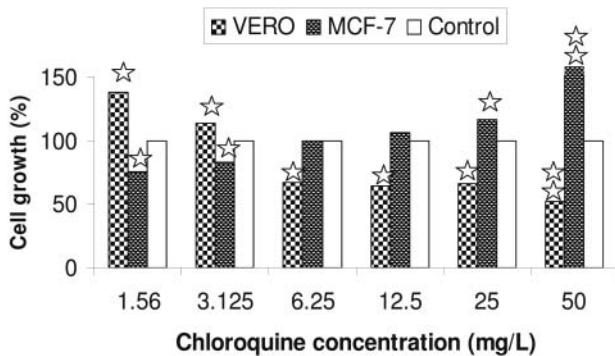


Figure 1. Antiproliferative activity of chloroquine evaluated by MTT test on Vero and MCF-7 cells. Data are expressed as percentage of growth inhibition with respect to control (0% inhibition). Significantly different $\star p < 0.05$ and $\star\star p < 0.01$ by Bonferroni test.

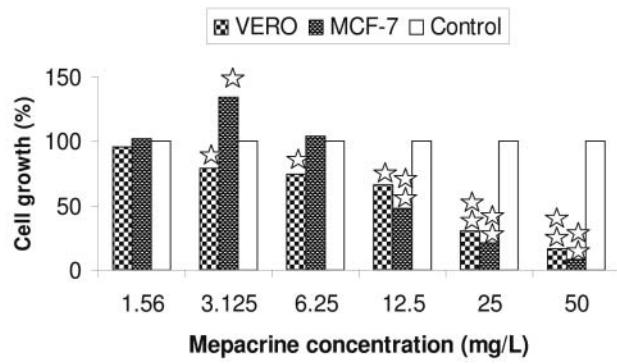


Figure 3. Antiproliferative activity of mepacrine evaluated by MTT test on Vero and MCF-7 cells. Data are expressed as percentage of growth inhibition with respect to control (0% inhibition). Significantly different $\star p < 0.05$ and $\star\star p < 0.01$ by Bonferroni test.

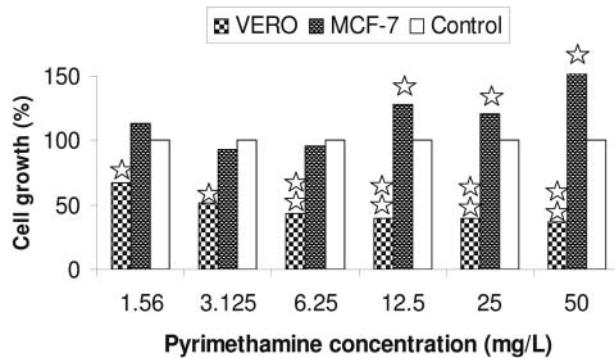


Figure 2. Antiproliferative activity of pyrimethamine evaluated by MTT test on Vero and MCF-7 cells. Data are expressed as percentage of growth inhibition with respect to control (0% inhibition). Significantly different $\star p < 0.05$ and $\star\star p < 0.01$ by Bonferroni test.

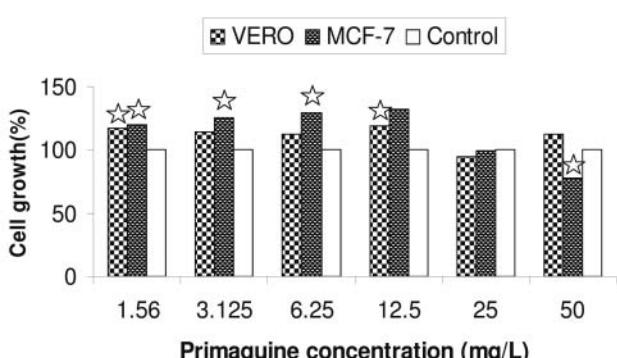


Figure 4. Antiproliferative activity of primaquine evaluated by MTT test on Vero and MCF-7 cells. Data are expressed as percentage of growth inhibition with respect to control (0% inhibition). Significantly different $\star p < 0.05$ and $\star\star p < 0.01$ by Bonferroni test.

proliferation induced by the anti-malarials under study (Figures 5-8). From the Western blotting analysis, we observed that the apoptotic markers are unchanged after the treatment with primaquine and chloroquine, whereas the lowest concentration of mepacrine strongly induced p21 expression in MCF-7 cells with a consequent reduction of cyclin B1 and A, which led us to suppose an arrest of the cell cycle in the G1 phase (Figure 9).

Conclusion

The present *in vitro* study confirms that some anti-malaria drugs can promote the growth of tumoral cells such as MCF-7. On the other hand it could be expected since malaria requires a long term therapy with drugs which are characterised by no selective toxicity towards human cells (8-

10) and by a mechanism of action than can result both in a DNA damage and in an oxidative stress for human cells (11): all these effects are considered very important steps of the carcinogenesis. Therefore the risk for humans affected by malaria is just in the treatment with quinoline derivatives, which must be prolonged for years thus exposing human cells to a continuous damage which could result in a progressive mutation of cells towards carcinogenesis. The effect could be enhanced on initiated cells just as we observed in Ehrlich tumour infected mice (1). On the contrary, the antiproliferative effect of mepacrine could be advantageous in reducing tumoral cell replication and for this reason the molecule is actually under study as a potential antitumor drug (12-14), however, we must state that at the dosages we used in the present study, mepacrine shows no selective toxicity towards control cells. On the basis of our results the drugs

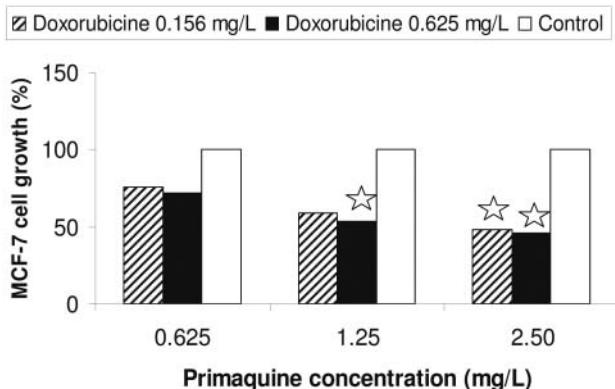


Figure 5. Effect of sub-inhibitory concentrations of primaquine and doxorubicine added in combination to MCF-7 cell cultures. Significantly different $\star p < 0.05$ and $\star\star p < 0.01$ by Bonferroni test.

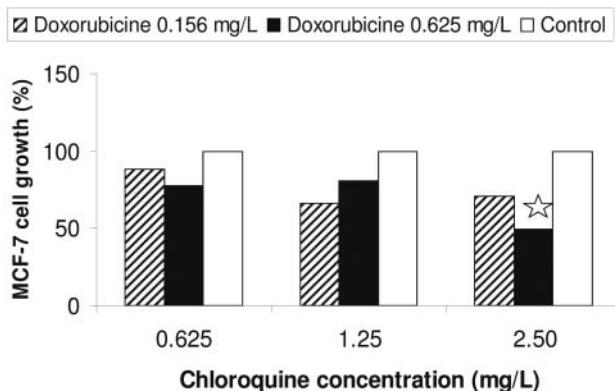


Figure 6. Effect of sub-inhibitory concentrations of chloroquine and doxorubicine added in combination to MCF-7 cell cultures. Significantly different $\star p < 0.05$ and $\star\star p < 0.01$ by Bonferroni test.

under study show a worrying tumour-promoting effect which should be taken into account when undertaking anti-malarial prophylactic measures. Further investigations on other tumoral cell lines and with other drugs should be performed to validate these preliminary results.

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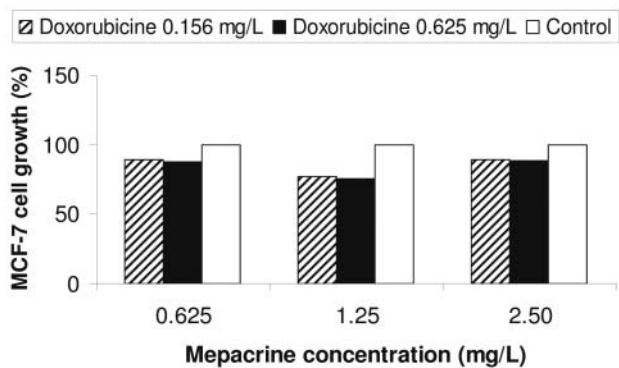


Figure 7. Effect of sub-inhibitory concentrations of mepacrine and doxorubicine added in combination to MCF-7 cell cultures.

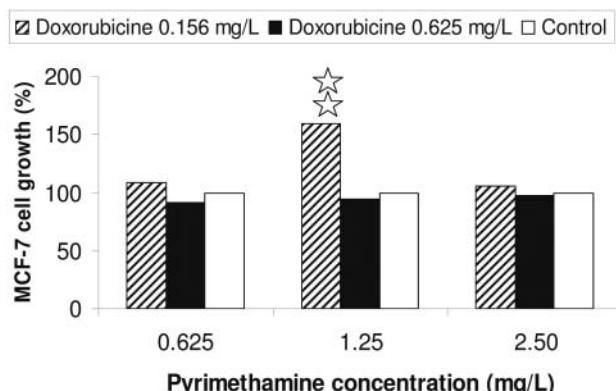


Figure 8. Effect of sub-inhibitory concentrations of pyrimethamine and doxorubicine added in combination to MCF-7 cell cultures. Significantly different $\star p < 0.05$ and $\star\star p < 0.01$ by Bonferroni test.

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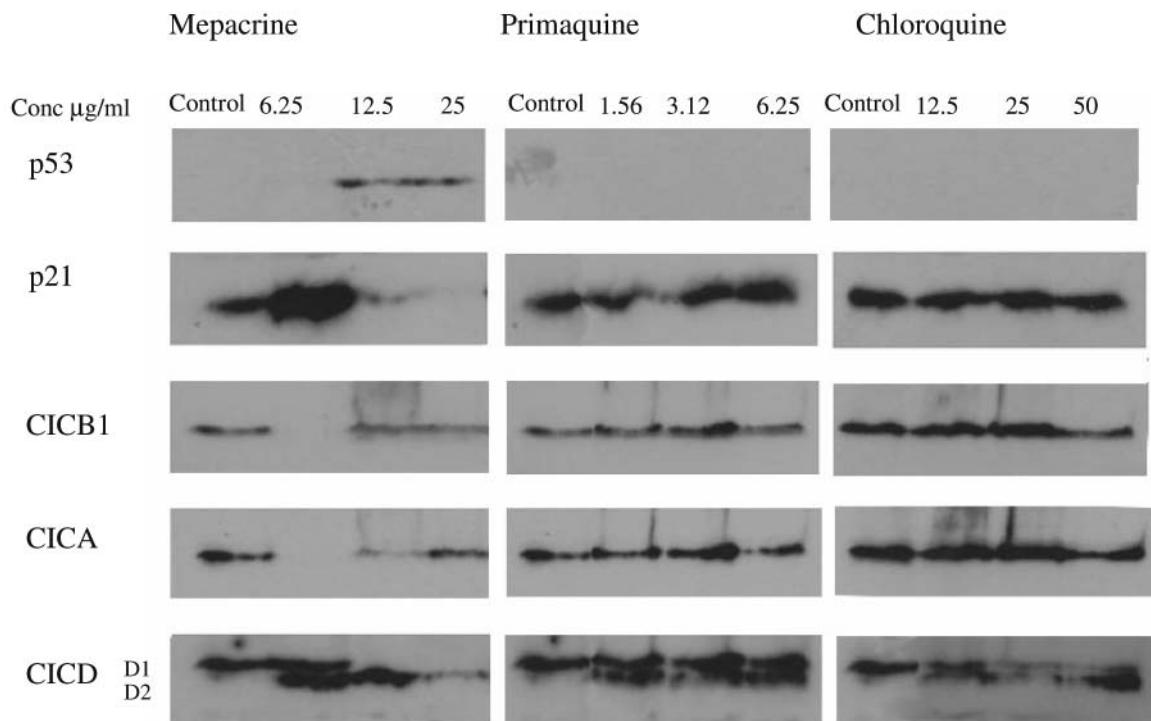


Figure 9. Apoptosis and cell proliferation marker modulation in MCF-7 cells treated with increasing concentrations of mepacrine, primaquine and chloroquine.

Table I. IC_{50} values of the anti-malaria drugs and doxorubicine on Vero cells.

Drugs	IC_{50}
Mepacrine	12.26 mg/L
Pyrimethamine	03.50 mg/L
Primaquine	>50 mg/L
Chloroquine	>50 mg/L
Doxorubicine	>2.5 mg/L

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