DOI: 10.1002/cmdc.200700257

In vitro Effects of *Plasmodium falciparum* Dihydrofolate Reductase Inhibitors on Normal and Cancer Cell Proliferation

Tiziana Rossi,[b] Andrea Coppi,[b] Elisa Bruni,[b] Miriam Sgobba,[a] Gianluca Degliesposti,[a] and Giulio Rastelli*[a]

Two of the most important problems that have seriously compromised the utility of commonly used antimalarials are drug toxicity and drug resistance of malarial parasites, in particular for *Plasmodium falciparum*.[1–5] Malaria treatment requires a long-term therapy which, besides inducing resistant plasmodium strains, is characterised by nonselective toxicity towards human cells.[6–8] Drugs such as quinoline derivatives are characterised by a mechanism of action than can result both in DNA damage and in oxidative stress for human cells.[9–12] All these effects are considered very important steps of carcinogenesis. In addition, recent studies showed that malaria and cancer may be correlated,[13,14] and that antimalarial drugs pyrimethamine and chloroquine can act as promoting agents on the growth of MCF-7 cancer cells.[15] In particular, previous in vitro and in vivo studies performed in our laboratory confirmed that whereas certain antimalarials are able to induce a significant slowing of tumour progression, others act as tumour promoters.[15–18] These observations raise the possibility that antimalarial therapy may induce tumour progression, and further highlight that effective and safer compounds are surely needed. For these reasons, the selectivity of action and a possible interference with tumour cell proliferation are two important aspects that need to be evaluated.

In a previous work, a molecular docking strategy followed by structural refinement of the protein–ligand complexes led to the identification of new inhibitors of the *P. falciparum* bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) enzyme,[19] a well-characterised target of antimalarials. Further studies highlighted quantitative structure–activity relationships by pharmacophore analyses of classical and nonclassical Pf DHFR inhibitors.[20] These novel inhibitors, belonging to the urea, thiourea, dihydrazine, and N-hydroxyamidine classes of compounds, have structures completely unrelated to classical antifolates and importantly, they inhibit both Pf DHFR and its highly resistant mutants with micromolar and submicromolar affinities.[19] Therefore, they constitute interesting candidates for further evaluation and optimisation. Based on these premises, we have undertaken validation studies of these novel inhibitors by evaluation of their cytotoxicity and their potential effects on in vitro cancer cell growth.

In this study, we test and validate two new inhibitors chosen on the basis of their activity profile and solubility, one belonging to the *N*-hydroxyamidine and the other to the thiourea classes (molecules **1b**[19] and **6g**[19] in Figure 1) and, for comparison, pyrimethamine, chloroquine, mepacrine, and primaquine. Firstly, we evaluated their cytotoxicity on Vero cells and evaluated the apoptotic and/or proliferative markers p21 and p53, and A, B1, D1, and D2 cyclines. Secondly, we investigated a possible interaction of the compounds with the proliferation of tumour cell lines.

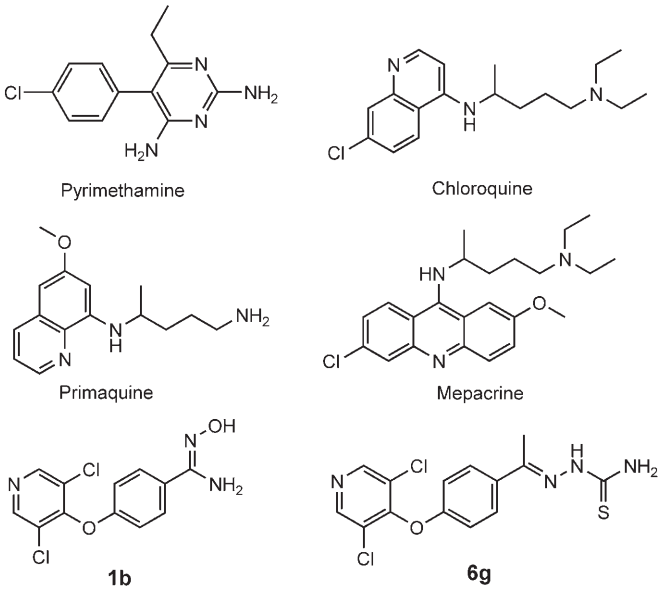

Figure 1. Antimalarial drugs and the two new Pf DHFR inhibitors **1b** and **6g**.

Figures 2 and 3 show the effects of mepacrine, chloroquine, primaquine, and pyrimethamine on Vero (control line) and MCF-7 (tumoural line) cells evaluated by the MTT test. The figures report the percentage of growth with respect to controls at different drug concentrations (mg L^{-1}). With the exception of primaquine, the other antimalarial drugs showed significant cytotoxicity on Vero cells (Figure 2). Moreover, chloroquine and pyrimethamine stimulated MCF-7 growth (Figure 3), an effect that in the case of pyrimethamine becomes evident even from the lower dosage (1.56 mg L^{-1}). Whereas mepacrine is a dose-dependent antiproliferative agent for both cell lines, primaquine exerts proliferative effects on both cell lines. Very interesting results came from the tests performed on compounds **1b** and **6g** (Figures 4 and 5). The addition of increasing concentrations of the *N*-hydroxyamidine derivative **1b** did not affect the growth or the number of treated cells, whereas the thiourea derivative **6g** behaved very similarly to chloroquine. Compound **6g** significantly stimulated Vero cell proliferation

[a] *Dr. M. Sgobba, Dr. G. Degliesposti, Prof. G. Rastelli*
Dipartimento di Scienze Farmaceutiche, Università di Modena e Reggio Emilia, via Campi 183, 41100 Modena (Italy)
Fax: (+39) 059-2055131
E-mail: giulio.rastelli@unimore.it

[b] *Prof. T. Rossi, Dr. A. Coppi, Dr. E. Bruni*
Dipartimento di Scienze Biomediche, Università di Modena e Reggio Emilia, via Campi 287, 41100 Modena (Italy)

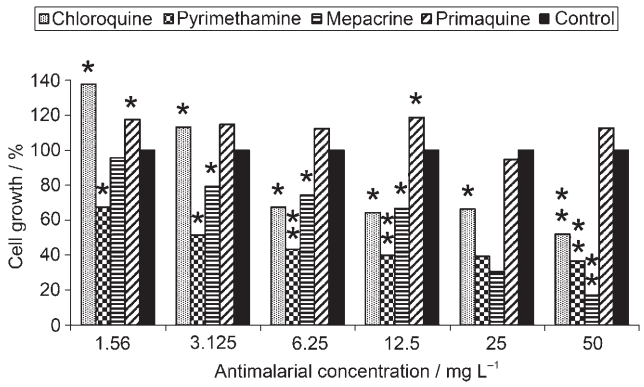

Figure 2. Antiproliferative activity of the antimalarials chloroquine, pyrimethamine, mepacrine, and primaquine on Vero cells evaluated by the MTT test. Data are expressed as percentage of growth inhibition with respect to control (0% inhibition). Significantly different *$P < 0.05$ and **$P < 0.01$ (Bonferroni Test).

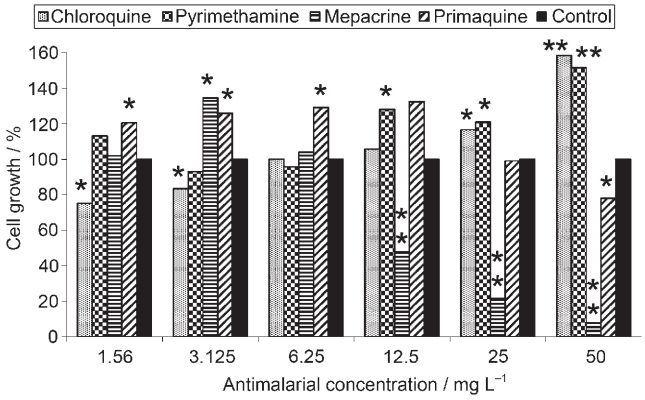

Figure 3. Antiproliferative activity of the antimalarials chloroquine, pyrimethamine, mepacrine, and primaquine on MCF-7 cells evaluated by the MTT test. Data are expressed as percentage of growth inhibition with respect to control (0% inhibition). Significantly different *$P < 0.05$ and **$P < 0.01$ (Bonferroni Test).

starting from the lowest dosage; the resulting effect is cell death when cultured in a small volume of the medium together with higher concentrations of the molecule (Figure 4). MCF-7 cultures are unaffected, and their growth parallels that of the control. Interestingly, the two new compounds **1b** and **6g** never inhibited the control line which was chosen as reference cells for healthy human cells, and did not stimulate the growth of tumour cells. Therefore, if compared to the classical antimalarials used in this study, **1b** demonstrated safer pharmacological behaviour. The effect of **6g** on Vero cell replication must be better investigated; if it will be demonstrated that its proliferative effect could represent a protection towards healthy cells of patients, however, we can conclude that the thiourea derivative displays an interesting pharmacological profile.

In the second phase of this study, we analysed using Western blotting the cell apoptotic marker modulation and the proliferative marker modulation in MCF-7 cultures treated with the new compounds and with the antimalarial drugs which displayed the most interesting results in the MTT test. Results from the Western blotting test (Figure 6) show that MCF-7 cell

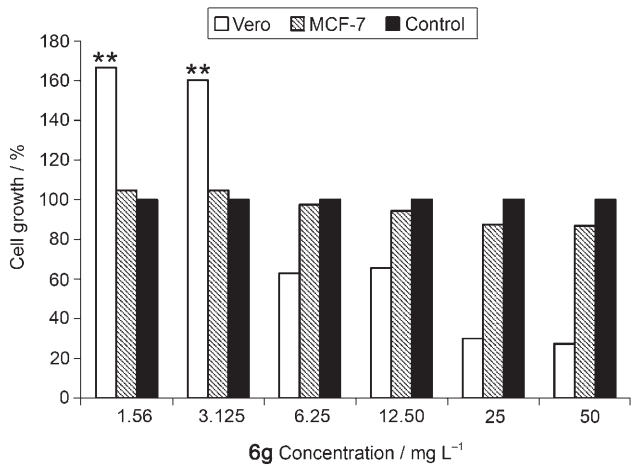

Figure 4. Antiproliferative activity of compound **6g** on Vero and MCF-7 cells evaluated by the MTT test. Data are expressed as percentage of growth inhibition with respect to control (0% inhibition). The significant increase in Vero cell replication at the lower dosages resulted in decreased survival of the cells in a monolayer culture (60 mm dishes) at the higher concentrations. Significantly different **$P < 0.01$ (Bonferroni Test).

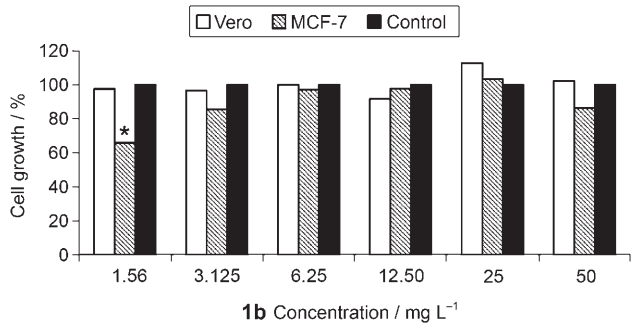

Figure 5. Antiproliferative activity of compound **1b** on Vero and MCF-7 cells evaluated by the MTT test. Data are expressed as percentage of growth inhibition with respect to control (0% inhibition). Significantly different *$P < 0.05$ (Bonferroni Test).

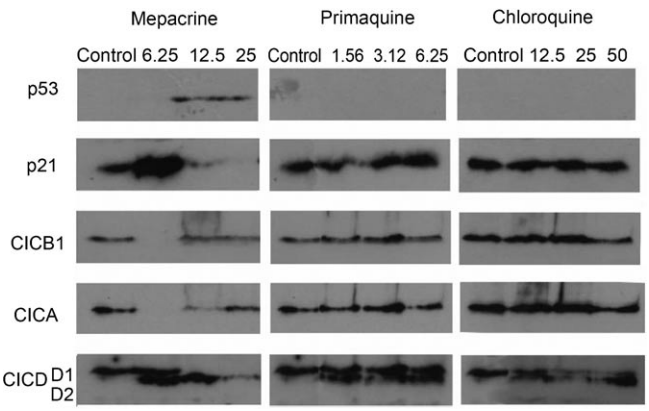

Figure 6. Apoptosis and cell proliferation marker modulation in MCF-7 cells treated with increased concentrations of mepacrine, primaquine, and chloroquine. The numbers on top of the lanes represent the concentration in mg L^{-1}.

p21 expression was increased by mepacrine at 6.25 mg L^{-1}, as was D2 cyclin. Higher concentrations of mepacrine (12.5 and 25 mg L^{-1}) induced p53 expression, while expression of p21, B1, and A cyclin were unchanged suggesting an arrest of the cell cycle in the G1 phase.

No significant variations were observed after the treatment with primaquine, chloroquine, **6g**, or **1b** (Figure 7), thus confirming the results obtained in the MTT test.

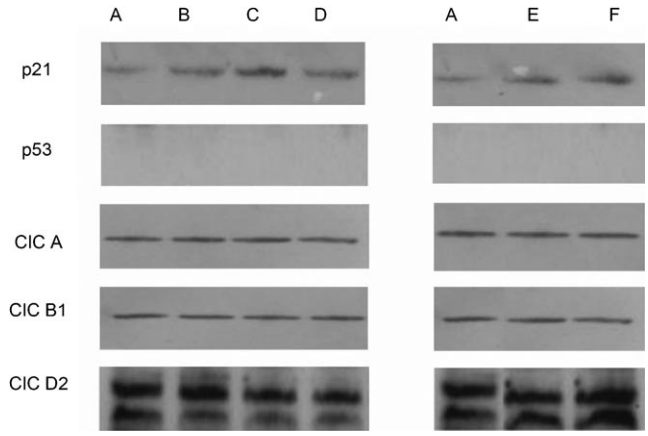

Figure 7. Apoptosis and cell proliferation marker modulation in MCF-7 cells treated with increased concentrations of compounds **1b** and **6g**. Lane A: MCF-7 control; lane B: MCF-7 + **1b** (1.56 mg L^{-1}); lane C: MCF-7 + **1b** (3.12 mg L^{-1}); lane D: MCF-7 + **1b** (25 mg L^{-1}); lane E: MCF-7 + **6g** (3.12 mg L^{-1}); lane F: MCF-7 + **1b** (25 mg L^{-1}).

In conclusion, we selected two of the most active compounds among the novel inhibitors of the *P. falciparum* DHFR-TS enzyme (**1b** and **6g**) and we evaluated their cytotoxicity on Vero cells and their apoptotic and/or proliferative markers p21, p53, and A, B1, D1, and D2 cyclines. Secondly, we investigated a possible interaction of the compounds with the proliferation of MCF-7 tumour cell lines. The results have been compared with mepacrine, chloroquine, primaquine, and pyrimethamine used as reference compounds. Toxicological evaluations resulted in interesting observations: the N-hydroxyamidine derivative **1b** did not affect Vero and MCF-7 cell growth whereas pyrimethamine (a known antimalarial drug) exerted a significant stimulating effect on MCF-7 replication. The thiourea derivative **6g** did not affect the growth of MCF-7 cells, but affected Vero cultures in a concentration-dependent way. In conclusion, the most interesting molecule seems to be the N-hydroxyamidine derivative **1b** in that it is active towards highly resistant *P falciparum* strains without affecting healthy cell survival or increasing tumoural cell replication at any investigated concentration. If these preliminary results are confirmed by further studies, we suggest that this molecule can be considered an interesting new candidate for further development.

Experimental Section

Compounds: **1b** (4-(3,5-dichloropyridin-4-yloxy)-*N*-hydroxybenzamidine) and **6g** (2-(1-{4-[(3,5-dichloro-4-pyridil)oxy]phenyl}ethylidene)hydrazine-1-carbothioamide) were purchased from Maybridge; chloroquine, mepacrine, and primaquine were from Sigma-Milan, Italy, and pyrimethamine was obtained from the Wellcome foundation Ltd London, UK.

Cell and cell cultures: MCF-7 (human breast adenocarcinoma) and Vero cells (kidney epithelial cells from African green monkey) were used. Vero cells were chosen as a control line. All the cells were cultured in a thermostatically-controlled environment in Eagle Minimum Essential Medium (EMEM) enriched with 5% foetal bovine serum (Lonza, Milan, Italy) and 1% antibiotic solution (penicillin 50 U mL^{-1} and streptomycin 0.5 mg mL^{-1}) 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; living cells were counted by the Trypan blue exclusion test to assure an initial inoculum of 35×10^4 cell/well. The plates were then incubated for 24 h at 37 °C at the conditions described above. Subsequently the programmed tests were performed.

Cytotoxicity test: After the incubation time, the molecules under investigation were dissolved in the culture medium and added to the wells at the following concentrations: 1.56; 3.125; 6.25; 12.5; 25, and 50 mg L^{-1}. After a further incubation period (24 h), the MTT test was performed according to the method described by Mosmann[21] to assess cell viability. All tests were carried out in triplicate and compared with four control wells in which the cells were cultured without drugs.

Western blotting test: Monolayer culture 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% SDS, 0.2% NaN_3 with the proteases inhibitors aprotinin (0.2 TIU mL^{-1}, Sigma), leupeptin (0.01 mg mL^{-1}, Sigma) and PMSF (4 mM, Sigma). Protein concentration was measured by means of the protein assay reagent (Protein Assay, Bio-Rad) in a total volume of 1 mL with bovine serum albumin as standard. 50 μg 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 (Bio-Optica, Milano), anti Cyclin-A 1:200, anti Cyclin B1 1:200, anti-Cyclin D1,and D2 1:200 (BioOptica, Milano). Blots were washed three times in PBS/Tween and incubated with the secondary antibody. Detection was performed using the ECL chemiluminescent system (Amersham, IL) and autoradiographic film (Hyperfilm-ECL, Amersham).

Keywords: cancer · cytotoxicity · DHFR inhibitors · malaria

[1] World Health Organization. The world health report 2002: reducing risks, promoting healthy life. Geneve, World Health Organization, **2002**.
[2] J. G. Breman, M. S. Alilio, A. Mills, *Am. J. Trop. Med. Hyg.* **2004**, *71*, 1–15.
[3] R. W. Snow, J. F. Trape, K. Marsh, *Trends Parasitol.* **2001**, *17*, 593–597.
[4] J. May, C. G. Meyer, *Trends Parasitol.* **2003**, *19*, 432–435.
[5] N. White, *Br. Med. Bull.* **1998**, *54*, 703–715.
[6] K. V. Gurova, J. E. Hill, C. Guo, A. Prokvolit, L. G. Burdelya, E. Samoylova, A. V. Khodyakova, R. Ganapathi, M. Ganapathi, N. D. Tararova, D. Bosykh, D. Lvovskiy, T.R. Webb, G. Stark, A. V. Gudkov, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 17448–17453.
[7] W. Wang, W. C. Ho, D. T. Dicker, C. MacKinnon, J. D. Winkler, R. Marmorstein, W. S. El Deiry, *Cancer Biol. Ther.* **2005**, *8*, 893–898.
[8] J. Sotelo, P. Guevara, B. Pineda, C. Diaz, *Surgery* **2004**, *136*, 700–707.
[9] D. J. Back, H. S. Purba, B. K. Park, S. A. Ward, M. L. Orme, *Br. J. Clin. Pharmacol.* **1983**, *16*, 497–502.
[10] D. J. Back, H. S. Purba, C. Staiger, M. L. Orme, A. M. Breckenridge, *Biochem. Pharmacol.* **1983**, *32*, 257–263.
[11] M. Murray, *Biochem. Pharmacol.* **1984**, *33*, 3277–3281.

[12] S. M. Toler, D. Noe, A. Sharma, *Neurosurg. Focus* **2006**, *15*, 1734–1743.
[13] M. Odida, R. Schmauz, S. K. Lwanga, *Int. J. Cancer* **2002**, *99*, 737–741.
[14] A. I. Ojesina, E. E. Akang, K. O. Ojemakinde, *Ann. Trop. Paediatr.* **2002**, *22*, 159–163.
[15] T. Rossi, A. Coppi, E. Bruni, A. Ruberto, S. Santachiara, G. Baggio, *Anticancer Res.* **2007**, *27*, 2555–2560.
[16] M. Castelli, G. Baggio, A. I. Ruberto, A. Tampieri, P. L. Tartoni, T. Rossi, M. R. Bossa, I. Galatulas, *Anticancer Res.* **1996**, *16*, 2673–2676.
[17] T. Rossi, A. Coppi, A. I. Ruberto, G. G. Baggio, *Boll. Soc. Med. Chir. Modena.* **2004**, *1–6*, 1–4.
[18] T. Rossi, A. Coppi, G. Zandomeneghi, S. Lodi, A. Ruberto, G. Baggio, *Anticancer Res.* **2004**, *24* (5D), 3614.
[19] G. Rastelli, S. Pacchioni, W. Sirawaraporn, R. Sirawaraporn, M. D. Parenti, A. M. Ferrari, *J. Med. Chem.* **2003**, *46*, 2834–2845.
[20] M. D. Parenti, S. Pacchioni, A. M. Ferrari, G. Rastelli, *J. Med. Chem.* **2004**, *47*, 4258–4267.
[21] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55–63.

Received: September 14, 2007
Revised: October 1, 2007
Published online on October 31, 2007

