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Antimalarial activity of methylpiperazinyl-substituted benzophenone-based farnesyltransferase inhibitors

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1. Introduction

Farnesyltransferase is a generally accepted novel target in the development of antimalarial agents (Medicines for Malaria Venture – annual report 2003). Farnesyltransferase catalyzes the post-translational modification of proteins characterized by a carboxy-terminal CAAX sequence (C, cysteine; A, amino acid with aliphatic side chain; X, serine or methionine) through the addition of a farnesyl residue to the thiol of the cysteine side chain (Fu and Casey 1999; Wittinghofer and Waldmann 2000). As a consequence of the increasing spread of *Plasmodium falciparum* (the causative parasite of malaria tropica) resistant to chloroquine and other commonly used antimalarials, malaria is one the world's most serious infectious diseases affecting approximately 40% of the world population (Sachs and Malaney 2002; Ridley 2002).

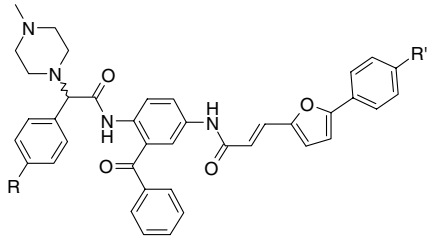
We have developed a novel type of benzophenone-based farnesyltransferase inhibitors with a methylpiperazinyl moiety as a distinctive structural feature. Inhibitors **3** and **4** (Table) represent the first farnesyltransferase inhibitors for which *in vivo* antimalarial activity has been described in the literature (Wiesner et al. 2004). In course of our studies towards the establishment of structure-activity relationships of this type of compounds we varied the para substituent of the phenylfurylacryloyl moiety, using substituents which led to high antimalarial activity in a structurally closely related type of farnesyltransferase inhibitors (Wiesner et al. 2002, 2003).

2. Investigations, results and discussion

The novel derivatives **5**–**8** were prepared from intermediates **1** and **2,** the synthesis of which is described elsewhere (Kettler et al. 2005), and appropriate 4-substituted phenylfurylacrylic acid chlorides, which were prepared according to Mitsch et al. (2004) (Scheme).

The farnesyltransferase inhibitory activity of the inhibitors was determined using the fluorescence enhancement assay as described by Pompliano et al. (1992). The assay employes yeast farnesyltransferase (FTase) fused to glutathione *S*-transferase at the N-terminus of the β-subunit (Del Villar et al. 1997). The heterologous expression of the farnesyltransferase genes from *P. falciparum* has not been

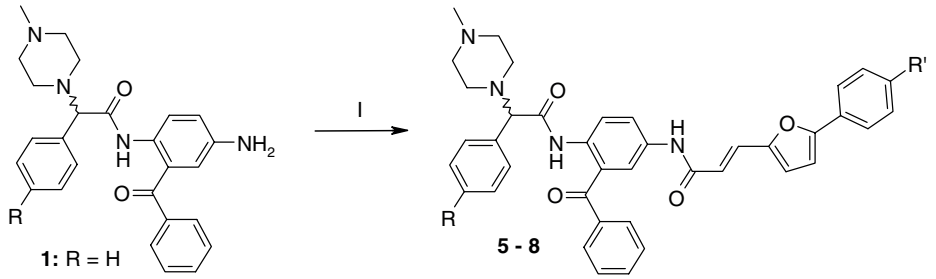
Table: Farnesyltransferase inhibition, antimalarial activity[a,b] and cytotoxicity[c] of compounds 3–8



	R	R′	IC_{50} (nM) FTase	IC_{50} (μM) Pfal	CC_{50} (μM) HeLa	CC_{50}/IC_{50}
3	H	NO_2	10 ± 3	0.27	37.0	137
4	Cl	NO_2	4 ± 2	0.21	38.6	184
5	H	−Cl	318 ± 40	0.46	6.8[d]	15
6	H	$-SO_2-CH_3$	594 ± 80	1.0	57.3	57
7	Cl	$-S-CH_3$	>10.000	1.8	39.0	21
8	Cl	$-SO_2-CH_3$	290 ± 36	2.6	22.0	8

[a] Activity was assayed by measuring radioactive hypoxanthine uptake by the multi-resistant *P. falciparum* (Pfal) strain Dd2. IC_{50} values (nM) for standard antimalarials were: chloroquine, 170; pyrimethamine, 2500; cycloguanile, 2200; quinine, 380; lumefantrine, 30; artemisinin, 18
[b] Antimalarial activity of compounds **3** and **4** has been published in (Wiesner et al. 2004)
[c] Assayed against HeLa cells
[d] GI_{50} against K-562 cells = 2.7 μM

Scheme



achieved so far and, therefore, no recombinant enzyme is available for routine screening (Chakrabarti et al. 2002).

Farnesylpyrophosphate and the dansylated pentapeptide Ds-GlyCysValLeuSer were used as substrates in the farnesyltransferase assay. Upon farnesylation of the cysteine thiol, the dansyl residue is placed into a lipophilic environment. The resulting enhancement of fluorescence at 505 nm is used to monitor the enzyme reaction.

Compounds were assayed for their inhibitory activity against intraerythrocytic forms of *P. falciparum* strain Dd2 using a semi-automated microdilution assay (Desjardins et al. 1979; Trager and Jensen 1976; Ancelin et al. 1998). The growth of the parasites was monitored through the incorporation of tritium labeled hypoxanthine. Comparability of different experiments was ensured by concurrent assay of standard compounds. The Dd2 strain used for the inhibition assays is resistant to several commonly used anti-malarial drugs (chloroquine, cycloguanile and pyrimethamine) (Table).

Cytotoxicity was evaluated against HeLa cells. Viability of the cells was determined after a 72 h incubation period using methylene blue staining and photometric evaluation.

Replacement of the para nitro group of the lead structures **3** and **4** by chlorine, methylthio or methylsulfonyl groups, respectively, resulted in any case in a marked decrease in farnesyltransferase inhibitory activity. Antimalarial activity was detrimentally influenced, too, although the reduction was less pronounced (Table). With exception of the chloro-compound **5**, cytotoxicity of the new derivatives was roughly in the same range as that of the lead compounds **3** and **4**. But through the decreased antimalarial activity also the cytotoxicity index (CC_{50}/IC_{50}) decreased markedly, indicating a loss of selectivity towards the parasites. The significantly higher cytotoxicity of inhibitor **5** is indicating a different activity profile of this compound, a result which is supported by the GI_{50}-value of 2.7 μM against the human leukaemia cell line K-562. The effect of the replacement of the nitro group of the lead structures **3** and **4** by the above mentioned substitutents is in marked contrast to the effects observed with the other benzophenone-based antimalarials which are basically differ from the ones described here by the lack of the methylpiperazinyl residue (Wiesner et al. 2002, 2003). On the first glance these results are somewhat unexpected since for the two types of inhibitors very similar binding modes were obtained by flexible docking especially in respect to the orientation of the para substituent of the terminal biaryl moiety (Mitsch et al. 2004; Wiesner et al. 2004). This shows that structure-activity relationships observed with one type of inhibitors are not readily applicable to another type even if the two classes of inhibitors are structurally very closely related and apparently possess the same binding mode.

However, these results also demonstrate that the activity profile of this type of inhibitor can be altered by comparatively small structural variations, thus it shall be possible to further enhance the selectivity towards malaria parasites.

3. Experimental

3.1. Preparation

^{1}H NMR spectra were recorded on a Jeol Lambda 500 delta, a Jeol JNM-GX-400, a Jeol Eclipse 500 and a Jeol Eclipse 400 spectrometer. Mass spectra were obtained with a Vacuum Generator VG 7070 H using a Vector 1 data acquisition system from Teknivent, an AutoSpec mass spectrometer from Micromass, an API 2000 LC/MS/MS – system of PE SCIEX using Analyst 1.2 of Applied Biosystems/MDS SCIEX and on a MStation JMS 700 of Jeol using Jeol Mass Data System MS – MP 9021D 2.30. IR spectra were recorded on a Nicolet 510P FT-IR-spectrometer and a Jasco FT/IR – 410 FT-IR-spectrometer.

Microanalyses were obtained by a CH analyzer according to Dr. Salzer from Labormatic, from a Hewlett-Packard CHN-analyzer type 185 and from a Vario EL of Elementar and are within ±0.4% of the calculated values. Melting points were obtained with a Reichert Austria microscope and are uncorrected. Column chromatography was carried out using silica gel 60 (0.062–0.200 mm) from Macherey-Nagel and silica gel 60 (0.040–0.063) of Merck.

3.1.1. (E-R,S)-N-{3-Benzoyl-4-[2-(4-methyl-1-piperazinyl)-2-phenylacetylamino]phenyl}-3-[5-(4-chlorophenyl)-2-furyl]acrylic acid amide (**5**)

(*E*)-3-[5-(4-Chlorophenyl)-2-furyl]acrylic acid chloride (187 mg, 0.75 mmol) was dissolved in dioxane (30 mL) and added to a solution of (*R,S*)-*N*-(4-amino-2-benzoylphenyl)-2-(4-methyl-1-piperazinyl)-2-phenylacetamide (**1**) (321 mg, 0.75 mmol) in hot toluene (50 ml). The mixture was heated under reflux for 2 h. Then, the solvent was removed in vacuo and the crude product was purified by recrystallization from ether: *n*-hexane (1 : 1). Yellow solid: yield 145 mg (29%); m.p. 217 °C. IR (KBr): $\tilde{\nu}$ (cm^{-1}) = 3395, 2935, 1679, 1632, 1596, 1537, 1503, 1478, 1453, 1401, 1333, 1286, 1244, 1194, 1165, 1145, 1093, 1024, 1012, 830, 788, 704. ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 2.10 (s, 3 H), 2.29 (s, 8 H), 3.94 (s, 1 H), 6.66 (d, J = 16 Hz, 1 H), 6.92–7.30 (m, 7 H), 7.34 (d, J = 16 Hz, 1 H), 7.51–7.88 (m, 11 H), 8.04–8.06 (m, 1 H), 10.29 (s, 1 H), 10.88 (s, 1 H). MS (ESI): m/z (%) = 661 (48, $[M + H]^+$), 659 (100, $[M + H]^+$), 429 (2), 189 (4), 100 (3). $C_{39}H_{35}ClN_4O_4$ (659.2)

3.1.2. (E-R,S)-N-{3-Benzoyl-4-[2-(4-methyl-1-piperazinyl)-2-phenylacetylamino]phenyl}-3-[5-(4-methylsulfonylphenyl)-2-furyl]acrylic acid amide (**6**)

(*E*)-3-[5-(4-Methylsulfonylphenyl)-2-furyl]acrylic acid chloride (219 mg, 0.75 mmol) was dissolved in dioxane (30 mL) and added to a solution of (*R,S*)-*N*-(4-amino-2-benzoylphenyl)-2-(4-methyl-1-piperazinyl)-2-phenylacetamide (**1**) (322 mg, 0.75 mmol) in hot toluene (50 ml). The mixture was heated under reflux for 2 h. The product crystallized from the mixture. Yellow solid: yield 385 mg (63%); m.p. 163 °C. IR (KBr): $\tilde{\nu}$ (cm^{-1}) = 3420, 1696, 1596, 1496, 1449, 1409, 1296, 1181, 1148, 1117, 1014, 954. ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 2.48 (s, 3 H), 2.71 (s, 8 H), 3.11 (s, 3 H), 4.51 (s, 1 H), 7.13–7.38 (m, 9 H and d, J = 16 Hz, 1 H), 7.40–7.55 (m, 3 H and d, J = 16 Hz, 1 H), 7.63–7.66 (m, 2 H), 7.72–7.76 (m, 4 H), 7.92–7.95 (m, 1 H), 10.98 (s, 1 H), 11.12 (s, 1 H). MS (ESI): m/z (%) = 703 (26, $[M + H]^+$), 529 (100), 484 (13), 429 (96), 242 (11). $C_{40}H_{38}N_4O_6S$ (702.8)

3.1.3. (E-R, S)-N-{3-Benzoyl-4-[2-(4-chlorophenyl)-(4-methyl-1-piperazinyl) acetylamino]phenyl}-3-[5-(4-methylsulfanylphenyl)-2-furyl]acrylic acid amide (**7**)

(*E*)-3-[5-(4-Methylsulfanylphenyl)-2-furyl]acrylic acid chloride (195 mg, 0.75 mmol) was dissolved in dioxane (30 mL) and added to a solution of

(*R,S*)-*N*-(4-amino-2-benzoylphenyl)-2-(4-chlorophenyl)-2-(4-methyl-1-piperazinyl)acetamide (**2**) (348 mg, 0.75 mmol) in hot toluene (50 ml). The mixture was heated under reflux for 2 h. The product crystallized from the mixture. Yellow solid: yield 324 mg (61%); m.p. 151 °C. IR (KBr): $\tilde{\nu}$ (cm^{-1}) = 3401, 1695, 1663, 1595, 1577, 1493, 1449, 1411, 1370, 1318, 1294, 1181, 1092, 1015. ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 2.48 (s, 3 H), 2.50 (s, 3 H), 2.69 (m, 4 H), 3.07 (m, 4 H), 4.41 (s, 1 H), 7.31–7.41 (m, 6 H and d, J = 16 Hz, 1 H), 7.47–7.57 (m, 4 H and d, J = 16 Hz, 1 H), 7.62–7.65 (m, 7 H) , 7.88–7.91 (m, 1 H), 10.89 (s, 1 H), 11.01 (s, 1 H). MS (ESI): m/z (%) = 707 (3, $[M + H]^+$), 705 (9, $[M + H]^+$), 565 (29), 563 (63), 463 (100).
$C_{40}H_{37}ClN_4O_4S$ (705.3)

3.1.4 (E-R,S)-N-{3-Benzoyl-4-[2-(4-chlorophenyl)-(4-methyl-1-piperazinyl) acetylamino]phenyl}-3-[5-(4-methylsulfonylphenyl)-2-furyl]acrylic acid amide (**8**)

(*E*)-3-[5-(4-Methylsulfonylphenyl)-2-furyl]acrylic acid chloride (219 mg, 0.75 mmol) was dissolved in dioxane (30 mL) and added to a solution of (*R,S*)-*N*-(4-amino-2-benzoylphenyl)-2-(4-chlorophenyl)-2-(4-methyl-1-piperazinyl)acetamide (**2**) (348 mg, 0.75 mmol) in hot toluene (50 ml). The mixture was heated under reflux for 2 h. The product crystallized from the mixture. Yellow solid: yield 322 mg (58%); m.p. 170 °C. IR (KBr): $\tilde{\nu}$ (cm^{-1}) = 3381, 2979, 1752, 1702, 1661, 1622, 1596, 1577, 1528, 1493, 1449, 1410, 1369, 1317, 1294, 1224, 1183, 1149, 1092, 1016, 960, 835. ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 2.42 (s, 3 H), 2.71 (s, 8 H), 3.24 (s, 3 H), 4.45 (s, 1 H), 7.33–7.42 (m, 8 H and d, J = 16 Hz, 1 H), 7.48–7.54 (m, 3 H and d, J = 16 Hz, 1 H), 7.62–7.73 (m, 5 H), 7.88–7.89 (m, 1 H), 7.96–7.97 (m, 1 H), 10.99 (s, 1 H), 11.10 (s, 1 H). MS (FAB): m/z (%) = 739 (4, $[M + H]^+$), 737 (10, $[M + H]^+$), 663 (3), 563 (100), 354 (2), 223 (18).
$C_{40}H_{37}ClN_4O_6S$ (737.3)

3.2. Enzyme preparation

Yeast farnesyltransferase was used as a fusionprotein to glutathione S-transferase at the N-terminus of the β-subunit. The recombinant farnesyltransferase was produced in *Escherichia coli* DH5α grown in LB media containing ampicillin and chloramphenicol for co-expression of pGEX-DPR1 and pBC-RAM2 (Del Villar 1997). The enzyme was purified by standard procedures with glutathione-agarose beads for selective binding of the target protein.

3.3. Farnesyltransferase assay

The assay was conducted as described by Pompliano et al. (1992). Farnesylpyrophosphate (FPP) was obtained as a solution of the ammonium salt in methanol-10 mM aqueous NH_4Cl (7 : 3) from Sigma-Aldrich. Dansyl-GlyCysValLeuSer (Ds-GCVLS) was custom synthesized by ZMBH, Heidelberg, Germany. The assay mixture (100 µl volume) contained 50 mM Tris/HCl pH 7.4, 5 mM $MgCl_2$, 10 µM $ZnCl_2$, 5 mM dithiothreitol (DTT), 7 µM Ds-GCVLS, 20 µM FPP and 5 nmol (approx.) yeast GST-farnesyltransferase and 1% of various concentrations of the test compounds dissolved in dimethylsulfoxide (DMSO). The progress of the enzyme reaction was followed by monitoring the enhancement of the fluorescence emission at 505 nm (excitation 340 nm). The reaction was started by addition of FPP and run in a Quartz cuvette thermostated at 37 °C. Fluorescence emission was recorded with a Perkin Elmer LS50B spectrometer. IC_{50} values (concentrations resulting in 50% inhibition) were calculated from initial velocity of three independent measurements of four to five different concentrations of the respective inhibitor.

3.4. Cytotoxicity assay

HeLa (DSM ACC 57) cells were grown in RPMI 1640 culture medium (GIBCO BRL 21875–034) supplemented with 25 µg/ml gentamicin sulfate (BioWhittaker 17–528 Z), and 10% heat inactivated fetal bovine serum (GIBCO BRL 10500-064) at 37 °C in high density polyethylene flasks (NUNC 156340). The test substances were dissolved in DMSO (10 mg/ml) before being diluted in the cell culture medium (1 : 200). The adherent HeLa cells were harvested at the logarithmic growth phase after soft trypsinization, using 0.25% trypsin in PBS containing 0.02% EDTA (Biochrom KG L2163). For each experiment approximately 10,000 cells were seeded with 0.1 ml RPMI 1640 (GIBCO BRL 21875-034), containing 25 µg/ml gentamicin sulfate (BioWhittaker 17–528 Z), but without HEPES, per well of the 96-well microplates (NUNC 167008). For the cytotoxic assay HeLa cells were preincubated for 48 h without the test substances. The dilutions of the test substances were carried out carefully on the monolayers of HeLa cells after the preincubation time. The HeLa cells were further incubated for 72 h at 37 °C in a humidified atmosphere and 5% CO_2. The adherent HeLa cells were fixed with 25% glutaraldehyde and stained with a 0.05% solution of methylene blue for 15 min. After gently washing the stain was eluted with 0.2 ml of 0.33 N HCl per well. The optical densities were measured at 660 nm in SUNRISE microplate reader (TECAN). For data analysis the Magellan software (TECAN) was used.

3.5. In vitro measurement of P. falciparum parasite growth inhibition

Compounds were tested by a semiautomated microdilution assay against intraerythrocytic forms of *P. falciparum* (Desjardins et al. 1979). The *P. falciparum* strain Dd2 was cultivated by a modification of the method described by Trager and Jensen (1976). The culture medium consisted of RPMI 1640 supplemented with 10% human type 0^+ serum and 25 mM HEPES. Human type 0^+ erythrocytes served as host cells. The cultures were kept at 37 °C in an atmosphere of 5% O_2, 3% CO_2, and 92% N_2.
Drug testing was carried out in 96-well microtiter plates. The compounds were dissolved in DMSO (10 mM) and prediluted in complete culture medium (final DMSO concentrations ≤1%) (In order to avoid a loss of lipophilic test compounds by adsorbance to the plastic material used for the assay, complete culture medium containing erythrocytes was used to dilute the DMSO stock solutions). Infected erythrocytes (200 µl per well, with 2% hematocrit and 0.4% parasitemia, predominantly ring-stage parasites) were incubated in duplicate with a serial dilution of the drugs for 48 h (Ancelin 1998). After the addition of 0.8 µCi [^{3}H]-hypoxanthine in 50 µl medium per well, the plates were further incubated for 24 h. Cells were collected on glass fiber filters with a cell harvester (Micromate 196, Packard) and incorporated radioactivity measured using a β-counter (Matrix 9600, Packard).

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