Novel non-substrate modulators of the transmembrane efflux pump P-glycoprotein (ABCB1)

Electronic supplementary information

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Experimental protocols

1. Chemistry

Commercial reagents were used without further purification. The ^{1}H-NMR spectra (400 MHz) were measured using tetramethylsilane as internal standard. TLC was performed on E. Merck 5554 silica gel plates. The EI mass spectra were measured with an AMD 402 mass spectrometer, the ESI spectra were recorded on a Finnigan LCQ Classic mass spectrometer. IR spectra were recorded on a FT-IR spectrometer. Elemental analysis indicated by the symbols of the elements was within ± 0.4% of the theoretical values and was performed using a Leco CHNS-932 apparatus.

1.1. General procedure for the formation of the N-substituted 4-aryl 1,4-dihydropyridines ***4a-r***

One equivalent of the respective aromatic aldehyde **1**, two equivalents of methyl propiolate **2** and one equivalent of the respective aliphatic amine **3** were dissolved in freshly distilled acetic acid. The solution was heated at 100 °C for 1 to 2 hours and then left standing until room temperature was reached. After the addition of water an extraction with several portions of chloroform followed. The volume of the unified organic layer was lowered under reduced pressure and the remaining browny and thicky product was treated with methanol under cooling. The separating product **4** was filtered off and dried.

1.1.1. Dimethyl 1-Benzyl-4-(3-chlorophenyl)-1,4-dihydropyridine-3,5-dicarboxylate ***4a***

Yield 36%; yellow solid; mp 151-152 °C; ^{1}H NMR (acetone-d_6) δ 7.47 (s, 2H, 2-, 6-H), 7.43-7.33 (m, 5H, benzylic H), 7.22-7.11 (m, 4H, phenylic H), 4.86 (s, 2H, *N*CH_2), 4.84 (s, 1H, 4-H), 3.55 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 397 [M^+]; IR (KBr): 2950, 1702, 1470 cm^{-1}. Anal. ($C_{22}H_{20}ClNO_4$) Calc. C 66.42, H 5.07, Cl 8.91, N 3.52; Found C 66.29, H 5.01, Cl 8.95, N 3.53.

1.1.2. Dimethyl 1-Benzyl-4-(3-bromophenyl)-1,4-dihydropyridine-3,5-dicarboxylate ***4b***

Yield 36%; yellow solid; mp 145-148 °C; ^{1}H NMR (acetone-d_6) δ 7.46 (s, 2H, 2-, 6-H), 7.40-7.43 (m, 5H, benzylic H), 7.38 (s, 1H, 2´-H), 7.27 (d, *J* = 7.8 Hz, 1H, 4´-H), 7.21 (d, *J* = 7.8 Hz, 1H, 6´-H), 7.13 ("t", *J* = 7.8 Hz, 1H, 5´-H), 4.86 (s, 2H, *N*CH_2), 4.83 (s, 1H, 4-H), 3.55 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 441 [M^+]; IR (KBr): 2951, 1697, 1582, 1471, 1281 cm^{-1}. Anal. ($C_{22}H_{20}BrNO_4$) Calc. C 59.74, H 4.56, Br 18.07, N 3.17; Found C 59.91, H 4.44, Br 18.10, N 3.17.

1.1.2. Dimethyl 1-Benzyl-1,4-dihydro-4-(3-trifluorophenyl)pyridine-3,5-dicarboxylate ***4c***

Yield 37%; yellow solid; mp 135-137 °C; ^{1}H NMR (acetone-d_6) δ 7.54 (s, 1H, 2´-H), 7.50 (d, *J* = 7.4 Hz, 1H, 4´-H), 7.49 (s, 2H, 2-, 6-H), 7.44 (d, *J* = 7.8 Hz, 1H, 6´-H), 7.40-7.42 (m, 5H, benzylic H), 7.38-7.36 (m, 1H, 5´-H), 4.94 (s, 1H, 4-H), 4.87 (s, 2H, *N*CH_2), 3.54 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 431 [M^+]; IR (KBr): 2952, 1698, 1583, 1496, 1283 cm^{-1}. Anal. ($C_{23}H_{20}F_3NO_4$) Calc. C 64.03, H 4.67, N 3.25; Found C 64.01, H 4.72, N 3.27.

1.1.4. Dimethyl 1-Benzyl-1,4-dihydro-4-(4-methoxyphenyl)pyridine-3,5-dicarboxylate ***4d***

Yield 65%; light beige solid; mp 120-122 °C; ^{1}H NMR ($CDCl_3$) δ 7.43-7.23 (m, 7H, benzylic H, 2-, 6-H), 7.17 (m, 2H, 2´-, 6´-H), 6.74 (m, 2H, 3´-, 5´-H), 4.83 (s, 1H, 4-H), 4.55 (s, 2H, *N*CH_2), 3.73 (s, 3H, C4´-OCH_3), 3.59 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 393 [M^+]; IR (KBr): 2845, 1703, 1604 cm^{-1}. Anal. ($C_{23}H_{23}NO_5$) Calc. C 70.23, H 5.85, N 3.56; Found C 69.93, H 5.88, N 3.50.

1.1.5. Dimethyl 1,4-Dihydro-1-(4-methoxybenzyl)pyridine-4-phenyl-3,5-dicarboxylate ***4e***

Yield 49%; yellow solid; mp 118-120 °C; ^{1}H NMR (acetone-d_6) δ 7.41 (s, 2H, 2-, 6-H), 7.34 (m, 2H, 2-, 6-H of benzyl), 7.20-7.05 (m, 5H, phenylic H), 6.68 (m, 2H, 3-, 5-H of benzyl), 4.83 (s, 1H, 4-H), 4.75 (s, 2H, *N*CH_2), 3.80 (s, 3H, benzylic OCH_3), 3.53 (s, 6H, $COOCH_3$); MS (ESI), *m/z* = 416 [$M+Na^+$]; IR (KBr): 2949, 1686, 1660, 1570, 1514 cm^{-1}. Anal. ($C_{23}H_{23}NO_5$) Calc. C 70.23, H 5.85, N 3.56; Found C 69.95, H 5.88, N 3.21.

1.1.6. Dimethyl 1,4-Dihydro-1-(4-methoxybenzyl)-4-(4-methoxyphenyl)pyridine-3,5-dicarboxylate ***4f***

Yield 40%; yellow solid; mp 129-130 °C; ^{1}H NMR (acetone-D_6) δ 7.38 (s, 2H, 2-, 6-H), 7.33 (m, 2H, benzylic 2-, 6-H), 7.08 (m, 2H, 2´-, 6´-H), 6.98 (m, 2H, benzylic 3-, 5-H), 6.71 (m, 2H, 3´-, 5´-H), 4.76 (s, 1H, 4-H), 4.74 (s, 2H, *N*CH_2), 3.80, 3.73 (2 x s, 6H, C4´-, C4 of benzyl-OCH_3), 3.53 (s, 6H, $COOCH_3$); MS (ESI), *m/z* = 422 [$M-H^+$]; IR (KBr): 2951, 2838, 1690, 1574, 1440, 1284 cm^{-1}. Anal. ($C_{24}H_{25}NO_6$) Calc. C 68.07, H 5.95, N 3.31; Found C 68.15, H 5.88, N 3.21.

1.1.7. Dimethyl 1,4-Dihydro-1-(3-methoxybenzyl)-4-(4-methoxyphenyl)pyridine-3,5-dicarboxylate ***4g***

Yield 54%; yellow solid; mp 144-146 °C; ^{1}H NMR ($CDCl_3$) δ 7.30 (“t”, *J* = 7.9 Hz, 1H, benzylic 5-H), 7.24 (s, 2H, 2-, 6-H), 7.10 (m, 2H, 2´-, 6´-H), 6.87 (dd, *J* = 7.9, 2.4 Hz, 1H, benzylic 4-H), 6.82 (d, *J* = 7.9 Hz, 1H, benzylic 6-H), 6.76 (d, *J* = 2.4 Hz, 1H, benzylic 2-H), 6.74 (m, 2H, 3´-, 5´-H), 4.83 (s, 1H, 4-H), 4.52 (s, 2H, *N*CH_2), 3.80, 3.73 (2 x s, 6H, C4´-, C3 of benzyl-OCH_3), 3.59 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 423 [M^+]; IR (KBr): 2955, 2833, 1698, 1578, 1442, 1283 cm^{-1}. Anal. ($C_{24}H_{25}NO_6$) Calc. C 68.07, H 5.95, N 3.31; Found C 67.86, H 5.91, N 3.23.

1.1.8. Dimethyl 1,4-Dihydro-1-(4-methoxybenzyl)-4-(3-methoxyphenyl)pyridine-3,5-dicarboxylate ***4h***

Yield 89%; yellow solid; mp 122-124 °C; ^{1}H NMR ($CDCl_3$) δ 7.24 (s, 2H, 2-, 6-H), 7.17 (m, 2H, benzylic 2-, 6-H), 7.11 (“t”, *J* = 7.9 Hz, 1H, 5´-H), 6.88 (m, 2H, benzylic 3-, 5-H), 6.84 (d, *J* = 7.9 Hz, 1H, 6´-H), 6.80 (d, *J* = 2.5 Hz, 1H, 2´-H), 6.66 (dd, *J* = 7.9, 2.5, 1H, 4´-H), 4.87 (s, 1H, 4-H), 4.48 (s, 2H, *N*CH_2), 3.80, 3.70 (2 x s, 6H, C3´-, C4 of benzyl-OCH_3), 3.60 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 423 [M^+]; IR (KBr): 2949, 2836, 1705, 1583, 1437, 1280 cm^{-1}. Anal. ($C_{24}H_{25}NO_6$) Calc. C 68.07, H 5.95, N 3.31; Found C 68.31, H 5.84, N 3.21.

1.1.9. Dimethyl 1,4-Dihydro-1-(3-methoxybenzyl)-4-(3-methoxyphenyl)pyridine-3,5-dicarboxylate ***4i***

Yield 34%; yellow solid; mp 138-140 °C; ^{1}H NMR ($CDCl_3$) δ 7.28 (“t”, *J* = 7.9 Hz, 1H, benzylic 5-H), 7.24 (s, 2H, 2-, 6-H), 7.11 (“t”, *J* = 7.9 Hz, 1H, 5´-H), 6.89-6.82 (m, 4H, 6´-H, benzylic 2-, 4-, 6-H), 6.76 (d, *J* = 2.6 Hz, 1H, 2´-H), 6.66 (dd, *J* = 7.9, 2.6 Hz, 1H, 4´-H), 4.88 (s, 1H, 4-H), 4.52 (s, 2H, *N*CH_2), 3.79, 3.71 (2 x s, 6H, C3´-, C3 of benzyl-OCH_3), 3.60

(s, 6H, $COOCH_3$); MS (EI), *m/z* = 423 [M^+]; IR (KBr): 2950, 2836, 1711, 1586, 1437, 1283 cm^{-1}. Anal. ($C_{24}H_{25}NO_6$) Calc. C 68.07, H 5.95, N 3.31; Found C 67.93, H 5.90, N 3.14.

1.1.10. Dimethyl 1,4-Dihydro-1-(3,4-dimethoxybenzyl)-4-(4-methoxyphenyl)pyridine-3,5-dicarboxylate ***4j***

Yield 93%; yellow solid; mp 161-164 °C; ^1H NMR ($CDCl_3$) δ 7.23 (s, 2H, 2-, 6-H), 7.16 (m, 2H, 2´-6´-H), 6.85 (d, *J* = 8.1 Hz, 1H, benzylic 5-H), 6.81 (d, *J* = 8.1 Hz, 1H, benzylic 6-H), 6.74-6.71 (m, 3H, 3´-, 5´-H, benzylic 2-H), 4.83 (s, 1H, 4-H), 4.48 (s, 2H, *N*CH_2), 3.88, 3.86, 3.73 (3 x s, 9H, C4´-, benzylic C3-, and C4-OCH_3), 3.59 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 453 [M^+]; IR (KBr): 2949, 2835, 1703, 1581, 1440, 1283 cm^{-1}. Anal. ($C_{25}H_{27}NO_7$) Calc. C 66.21, H 6.00, N 3.09; Found C 66.04, H 5.94, N 2.98.

1.1.11. Dimethyl 1,4-Dihydro-1-(3,4-dimethoxybenzyl)-4-(3-methoxyphenyl)pyridine-3,5-dicarboxylate ***4k***

Yield 89%; yellow solid; mp 116-117 °C; ^1H NMR ($CDCl_3$) δ 7.25 (s, 2H, 2-, 6-H), 7.11 ("t", *J* = 7.9 Hz, 1H, 5´-H), 6.87-6.79 (m, 4H, 6´-H, benzylic 2-, 5-, 6-H), 6.73 (s, 1H, 2´-H), 6.66 (d, *J* = 7.9, 2.5 Hz, 1H, 4´-H), 4.88 (s, 1H, 4-H), 4.48 (s, 2H, *N*CH_2), 3.87, 3.86, 3.71 (3 x s, 9H, C3´-, benzylic C3-, and C4-OCH_3), 3.60 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 453 [M^+]; IR (KBr): 2949, 2836, 1709, 1582, 1437, 1280 cm^{-1}. Anal. ($C_{25}H_{27}NO_7$) Calc. C 66.21, H 6.00, N 3.09; Found C 66.35, H 6.03, N 3.13.

1.1.12. Dimethyl 1,4-Dihydro-1-(4-methoxybenzyl)-4-(3,4-dimethoxyphenyl)pyridine-3,5-dicarboxylate ***4l***

Yield 32%; yellow solid; mp 143-144 °C; ^{1}H NMR ($CDCl_3$) δ 7.24 (s, 2H, 2-, 6-H), 7.17 (m, 2H, benzylic 2-, 6-H), 6.90 (m, 2H, benzylic 3-, 5-H), 6.79 (d, *J* = 1.9 Hz, 1H, 2´-H), 6.75 (d, *J* = 8.2, 1.9 Hz, 1H, 6´-H), 6.70 (d, *J* = 8.2 Hz, 1H, 5´-H), 4.83 (s, 1H, 4-H), 4.48 (s, 2H, *N*CH_2), 3.80, 3.79, 3.73 (3 x s, 9H, C3´-, C4´-, benzylic C4-OCH_3), 3.60 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 453 [M^+]; IR (KBr): 2953, 2836, 1693, 1581, 1463, 1281 cm^{-1}. Anal. ($C_{25}H_{27}NO_7$) Calc. C 66.21, H 6.00, N 3.09; Found C 66.25, H 5.87, N 2.94.

1.1.13. Dimethyl 1,4-Dihydro-1-(3-methoxybenzyl)-4-(3,4-dimethoxyphenyl)pyridine-3,5-dicarboxylate ***4m***

Yield 44%; yellow solid; mp 159-160 °C; ^{1}H NMR ($CDCl_3$) δ 7.28 (“t”, *J* = 7.9 Hz, 1H, benzylic 5-H), 7.24 (s, 2H, 2-, 6-H), 6.87-6-77 (m, 5H, 2´-, 6´-, benzylic 2-, 4-, 6-H), 6.71 (d, *J* = 8.2 Hz, 1H, 5´-H), 4.84 (s, 1H, 4-H), 4.51 (s, 2H, *N*CH_2), 3.80, 3.79, 3.75 (3 x s, 9H, C3´-, C4´-, benzylic C3-OCH_3), 3.61 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 453 [M^+]; IR (KBr): 2950, 2837, 1690, 1579, 1444, 1280 cm^{-1}. Anal. ($C_{25}H_{27}NO_7$) Calc. C 66.21, H 6.00, N 3.09; Found C 66.42, H 6.00, N 2.99.

1.1.14. Dimethyl 1,4-Dihydro-1-(3,4-dimethoxybenzyl)-4-(3,4-dimethoxyphenyl)pyridine-3,5-dicarboxylate ***4n***

Yield 38%; yellow solid; mp 170-172 °C; ^{1}H NMR ($CDCl_3$) δ 7.25 (s, 2H, 2-, 6-H), 6.86-6.80 (m, 3H, benzylic 2-, 5-, 6-H), 6.74 (dd, *J* = 8.2, 1.9 Hz, 1H, 6´-H), 6.72 (d, *J* = 1.9 Hz, 1H, 2´-H), 6.69 (d, *J* = 8.2 Hz, 1H, 5´-H), 4.84 (s, 1H, 4-H), 4.48 (s, 2H, *N*CH_2), 3.87, 3.85, 3.79, 3.75 (4 x s, 12H, C3´-, C4´-, benzylic C3-, C4-OCH_3), 3.61 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 483 [M^+]; IR (KBr): 2947, 2837, 1711, 1585, 1441, 1280 cm^{-1}. Anal. ($C_{26}H_{29}NO_8$) Calc. C 64.59, H 6.05, N 2.90; Found C 63.99, H 6.06, N 2.77.

1.1.15. Dimethyl 1-Benzyl-4-(3-benzyloxy-4-methoxyphenyl)-1,4-dihydropyridine-3,5-dicarboxylate ***4o***

Yield 24%; yellow solid; mp 87-88 °C; ^{1}H NMR ($CDCl_3$) δ 7.37-7.25 (m, 10H, benzylic H, benzyloxy H), 7.21 (s, 2H, 2-, 6-H), 6.83-6.80 (m, 2H, 2´-, 6´-H), 6.74 (d, *J* = 7.9 Hz, 1H, 5´-H), 5.00 (s, 2H, OCH_2), 4.79 (s, 1H, 4-H), 4.51 (s, 2H, CH_2), 3.80 (s, 3H, C4´-OCH_3), 3.56 (s, 6H, $COOCH_3$); MS (ESI), *m/z* = 498 [M-H$^+$]; IR (KBr): 2948, 2836, 1703, 1579, 1439, 1282 cm^{-1}. Anal. ($C_{30}H_{29}NO_6$) Calc. C 72.13, H 5.85, N 2.80; Found C 72.19, H 5.75, N 2.46.

1.1.16. Dimethyl 1-Benzyl-4-(4-benzyloxy-3-methoxyphenyl)-1,4-dihydropyridine-3,5-dicarboxylate ***4p***

Yield 36%; yellow solid; mp 121-124 °C; ^{1}H NMR ($CDCl_3$) δ 7.40-7.25 (m, 10H, benzylic H, benzyloxy H), 7.24 (s, 2H, 2-, 6-H), 6.83 (s, 1H, 2´-H), 6.71-6.70 (m, 2H, 5´-, 6´-H), 5.06 (s, 2H, OCH_2), 4.83 (s, 1H, 4-H), 4.54 (s, 2H, CH_2), 3.74 (s, 3H, C3´-OCH_3), 3.60 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 499 [M$^+$]; IR (KBr): 2948, 2838, 1693, 1574, 1439, 1271 cm^{-1}. Anal. ($C_{30}H_{29}NO_6$) Calc. C 72.13, H 5.85, N 2.80; Found C 72.31, H 5.79, N 2.61.

1.1.17. Dimethyl 1-Benzyl-1,4-dihydro-4-(1-naphtyl)pyridine-3,5-dicarboxylate ***4q***

Yield 34%; yellow solid; mp 185-189 °C; ^{1}H NMR (acetone-d_6) δ 8.62 (d, *J* = 8.6 Hz, 1H, naphtl 9-H), 7.76 (d, *J* = 7.8 Hz, 1H, naphtyl 4-H), 7.64 (d, *J* = 7.9 Hz, 1H, naphtly 6-H), 7.49-7.45 (m, 7H, 2-, 6-, benzylic H), 7.41 (m, 2H, naphtyl 7-, 8-H), 7.34 (d, *J* = 7.8 Hz, 1H, naphtyl 2-H), 7.28 (“t”, *J* = 7.8 Hz, 1H, naphtyl 3-H), 5.67 (s, 1H, 4-H), 4.90 (s, 2H, CH_2),

3.38 (s, 6H, $COOCH_3$); MS (EI), *m/z* = 413 [M^+]; IR (KBr): 2949, 1694, 1582, 1452, 1281 cm^{-1}. Anal. ($C_{26}H_{23}NO_4$) Calc. C 75.53, H 5.61, N 3.39; Found C 75.71, H 5.51, N 3.03.

1.1.18. Dimethyl 1,4-Dihydro-1-(-napht-1-ylmethyl)-4-(1-phenyl)pyridine-3,5-dicarboxylate ***4q***

Yield 51%; yellow solid; mp 130-133 °C; ^1H NMR (acetone-d_6) δ 8.12 (d, *J* = 8.2 Hz, 1H, naphtyl 9-H), 7.98 (d, *J* = 7.6 Hz, 1H, naphtyl 5-H), 7.93 (dd, *J* = 7.9, 1.3 Hz, 1H, naphtly 4-H), 7.65-7.52 (m, 4H, naphtyl 2-, 3-, 7-, 8-H), 7.50 (s, 2H, 2-, 6-H), 7.18-7.03 (m, 5H, benzylic H), 5.33 (s, 2H, CH_2), 4.86 (s, 1H, 4-H), 3.52 (s, 6H, $COOCH_3$); MS (ESI), *m/z* = 436 [M-Na^+]; IR (KBr): 2949, 1706, 1578, 1437, 1282 cm^{-1}. Anal. ($C_{26}H_{23}NO_4$) Calc. C 75.53, H 5.61, N 3.39; Found C 75.40, H 5.71, N 3.15.

2. Bioanalysis

2.1. Cell culture

Both cell line were cultured in McCoy´s 5A medium which was supplemented with 10% calf serum and L-glutamin (2 mM) at 37 °C under a carbon dioxide atmosphere (5%). The P-gp expressing subline was cultured under addition of colchicine in a concentration of 60 ng/ mL to ensure a survival of only such P-gp overexpressing cells. The cell suspensions were diluted three times a week with fresh medium in a relation of 1 to 20.

2.2. Evaluation of the P-gp modulating properties using flow cytometry

Both cell lines were adjusted to a number of one million cells per mL. After centrifugation at 2000 rpm the upper layer was removed and the remaining cells were resuspended in medium. Each 0.5 mL of the cell suspensions were placed in an eppendorf tube and supplemented with the modulator in the respective concentration from stock solutions. Incubation followed for 20 min. Then 0.5 µL of a rhodamine 123 stock solution (0.5 mM) was added and incubation was continued for additional 40 min. Then centrifugation followed again with 2000 rpm. The medium was removed and the cell suspension was washed with PBS buffer for two times at a pH of 7.4. Then the fluorescence was measured in both cell lines and the fluorescence values were each related to the fluorescence of the untreated control to give the final *FAR* value by relating both corrected fluorescence values of the P-gp overexpressing cell line and the parental cell line. The determination of the *FAR* value followed the equation:

$$FAR = \frac{\text{MDR treated / MDR untreated control}}{\text{Parental treated / Parental untreated control}}$$

2.3. Determination of cellular toxicity in the MTT assay for modulators and daunorubicin

100 µL of the cell suspension of each cell line with a number of one hundred thousand cells per mL were placed in wells of a 96-well plate. After the addition of the modulator or daunorubicin in the relevant increasing concentration the well plates were incubated for 24 hours at 37 °C in the 5% carbon dioxide atmosphere. Then 10 µL of a 5 mg/mL solution of MTT were added and incubation continued for 4 h. Cell lysis was then practiced by the use of 100 µL of a buffer containing 50 g of SDS, 374 mL of dmso and 125 mL of acetic acid. The formed formazan dye was measured at 570 nm and the resulting value was corrected by substraction of the modulator or daunorubicin untreated control. The percentual cell vitality was calculated in relation to the modulator or daunorubicin untreated control.

2.4. Determination of the modulator uptake in the relevant cell line using flow cytometry

Cultured cells were adjusted to a concentration of one million cells per mL. 0.5 ML of this cell suspension were placed in an eppendorf tube and 5 µL of the modulator in the relevant final concentration were added from a stock solution. Cells were cultured under the described conditions for 60 min. Then the suspension was centrifuged for 2 min and the upper medium was removed and replaced with PBS. An additional washing procedure followed and the samples were measured in 0.5 mL of PBS buffer. The fluorescence values were determined using flow cytometry and the fluorescence values of both cell lines were related to one another after each relation to the untreated control cell line giving the final *FAR* values.