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Synthesis and cytotoxicity of novel pyrido[1,2-*e*]purines on multidrug resistant human MCF7 cells

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The anticancer activity of 4-methylaminopyridol[1,2-*e*]purine **6a**, 4-(piperidin-1-yl)pyrido[1,2-*e*]purine **7a** and their 7-methyl derivatives **6b**, **7b** was investigated against the human MCF7 cancer cell line *in vitro*. The sensitive cell line showed a range of sensitivities to **6a**, **6b**, **7a**, **7b** (IC_{50}: 1.6 to 7.2×10^{-4} M) and sensitivity to doxorubicin (IC_{50}: 7.5×10^{-7} M). A resistant cell line with the multidrug resistant phenotype was sensitive to these derivatives (IC_{50}: 1.8 to 6.7×10^{-4} M), doxorubicin (IC_{50}: 5×10^{-5} M) and drug activity seems to be not affected by MDR resistance. Our data show that **6a**, **6b**, **7a** and **7b** appear to exert a low cytotoxicity on sensitive and MDR resistant MCF7 human cancer cell lines.

1. Introduction

Cancer cells can develop mechanisms of resistance allowing them to evade chemotherapy. The mechanisms of drug resistance are multifactorial: multidrug resistance (MDR) is the best known of all, since its molecular and genetic support has been identified [1]. MDR is associated with the expression of a membrane P-glycoprotein (PGP) of 170 kD [2]. PGP is an energy dependent efflux pump responsible for reducing intracellular drug concentration in resistant cells [3]. In addition, increased levels of PGP have been detected in several types of refractory tumors samples from untreated or relapsed patients, indicating a possible role of this transport protein in clinical drug resistance [4]. The development of acquired resistance and the severity of toxicity associated to the useful antineoplasic agents encourages the development of new drugs.

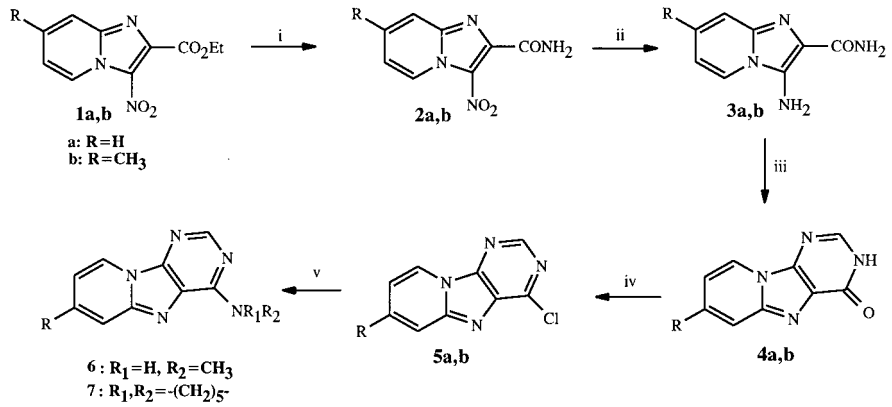
Recently a number of tricyclic nitrogen heterocycles were reported for their antineoplastic activities [5–7]. The same time, we have reported the synthesis of various derivatives in the pyrido[1,2-*e*]purine series [8], and the ability of two compounds of this ring system to intercale with synthetic oligonucleotides [9]. Additionally it was well established that a methyl substituent of the pyridine moiety of the imidazo[1,2-*a*]pyridine enhanced various pharmacological activities of the compounds, e.g. as antiviral agents [10]. From these results, we are now interested in quantification of the antineoplastic activity of these compounds on a breast tumour cancer cell line in culture in comparison to doxorubicin. In this work, we report the synthesis of new compounds in this series and their cytotoxicity on MCF7. We also investigated the ability of MDR to recognise and pump new compounds out of cells on a MDR human cell line: MCF7R.

2. Investigations, results and discussion

2.1. Chemistry

7-Methyl-3-nitroimidazo[1,2-*a*]pyridine-2-carboxylate (**1b**) [11] was treated with liquid ammonia in a parr apparatus at 100 °C to give the corresponding amides **2b** in 70% yield. Reduction of the nitroamide to the corresponding aminoamide **3b** was achieved with tin in hydrochloric acid. The ring closure of **3b** was made by refluxing triethyl ortoformate in acetic acid media as reported for **4a** [8] to give the pyridopurinone derivative **4b**. The corresponding halides **5a**, **b** were obtained in refluxing phosphoryl chloride. It was well established that the 6-chloro substituent of purine can easily be removed by various nucleophiles [12]. Thus **5a**, **b** were allowed to react with methylamine (40% in water) or with piperidine at room temperature for 8 h to give the amino derivatives **6a**, **b** or **7a**, **b** in 63, 59, 57 and 55% yield, respectively.

Scheme



i: NH_3, parr apparatus, 100 °C, 2 h; ii: Sn, HBr, 1 h at 0 °C then 2 h RT; iii: $HC(OEt)_3$, reflux 15 h; iv: $POCl_3$, reflux, 5 h; v: piperidine or methylamine RT, 8 h

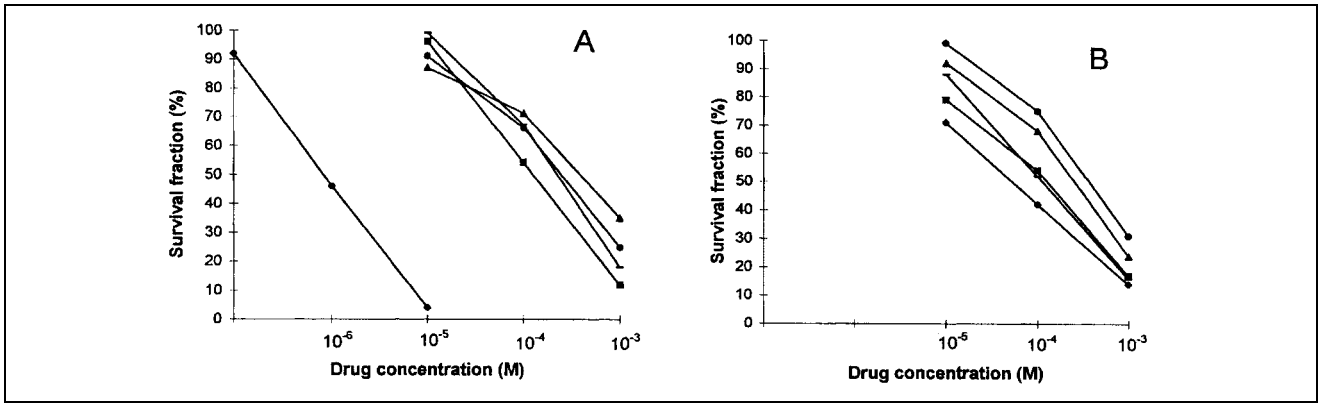


Fig.: Cytotoxicity of doxorubicin (◆), **6a** (●), **6b** (▲), **7a** (–) and **7b** (■) on exposure on MCF7 (A) and MCF7R (B) cell lines. Results were the mean of three values carried out in duplicate

The structural determination of all the new compounds was achieved by ^{1}H and ^{13}C NMR spectroscopy and if necessary by XHCOR and LRHETCOR.

2.2. Antineoplastic activities

2.2.1. Sensitivity of MCF7 and MCF7R to doxorubicin and analysis of P-glycoprotein

The concentration inducing 50% inhibition of cell proliferation for doxorubicin was 7.5×10^{-7} M for the parental cell line MCF7 and 5×10^{-5} M for the MCF7R cell. We showed that the MCF7R cell line were-66 fold more resistant to doxorubicin than parental MCF7 cells when the IC_{50} values of these tumor lines and the parent cells were compared.

The MCF7 and MCF7R cells were examined for the presence of P170, detected by immunochemical analysis using C219 and JSB1 monoclonal antibodies. As expected, only MCF7R cells showed a clear presence of P170, while the parental cell lines did not show any detectable red coloration. MCF7R was a resistant cell line expressing P-glycoprotein.

2.2.2. Sensitivity of MCF7, MCF7R to **6a**, **6b**, **7a**, **7b**, *and doxorubicin*

The effect of **6a**, **6b**, **7a** and **7b** on cell proliferation of sensitive and resistant cell lines after 96 h of exposure was assessed and compared with doxorubicin.

Cells (6000 per well) were incubated with various concentrations of drugs continuously during the incubation period. The IC_{50} data (M) from survival curves for the two cell lines treated with the derivatives are presented in the Table.

All breast cell lines showed low growth activity when treated under our conditions with **6a**, **6b**, **7a** and **7b**. Median concentrations needed to inhibit the growth of 50% of cells were 1.6 to 7.2×10^{-4} M for the evaluated compounds. The IC_{50} for doxorubicin were found in the range of 7.5×10^{-7} M for the sensitive cell line and 5×10^{-5} M for resistant cell lines. No significant difference in anticancer activity of **6a**, **6b**, **7a** and **7b**.

Table: IC_{50} values of the investigated compounds (MTT assay)

	Doxorubicin	**6a**	**6b**	**7a**	**7b**
MCF7	7.5×10^{-7}	4.6×10^{-4}	7.2×10^{-4}	3.6×10^{-4}	1.6×10^{-4}
MCF7R	5×10^{-5}	6.7×10^{-4}	4.9×10^{-4}	1.8×10^{-4}	2.3×10^{-4}

Standard deviation were within 15% of each values IC_{50} unducing 50% inhibition of cell proliferation. Results are the mean of three experiments

3. Discussion

The aim of this study was to investigate the potential anticancer activity of pyrido[1,2-*e*]purine analogs in MDR cell lines. Resistance to chemotherapy is an important therapeutic problem in medical oncology. The discovery of the P170 glycoprotein as a mediator of multidrug resistance (MDR) represents one of the most important research accomplishments in antineoplastic pharmacology during the last decade. Demonstration of P170 in epithelial tissues, untreated and chemotherapeutically pre-treated human malignancies and identification of various agents able to reversing resistance *in vitro* generated enthusiasm for clinical studies. We have synthesized various derivatives in the pyridol[1,2-*e*]purine series and have shown the ability of these compounds to intercale with synthetic oligonucleotides. These analogs were screened for activity against one cell lines, MCF7.

In this study, pyrido[1,2-*e*]purine was found to be active against on MDR cell lines, MCF7R, which were shown to have increased resistance to doxorubicin. Consequently, drug activity is not affected by MDR resistance. The activity of these new compounds to doxorubicin seems to be practically equivalent on MCF7R.

The pyrido[1,2-*e*]purines reported here showed low activity on MCF7 cell lines, and interesting activity on MCF7R. The drug activity is not affected by MDR Resistance. On the basis of these observations, we suggest that other derivatives must be synthesized and evaluated on diferent cell lines, particulary MDR cell lines to enhance activity. This pharmacomodulation may provide new leads against pleiotropic drug-resistant tumor cells.

4. Experimental

4.1. Chemistry

M.p.'s were recorded on a Kofler hotstage apparatus and are uncorrected. NMR spectra were recorded on a Bruker AC 100 or DPX 200. Chemical shifts are expressed in ppm from TMS as external reference in the ^{1}H NMR or the center resonance od $CDCl_3$ at 77.1 in ^{13}C NMR. The J values are expressed in Hz. MS were obtained on a LKB 2091 at 70 eV. All compounds gave satisfactory elemental analysis (±0.4% of the theoretical values). For compounds 6 and 7 attributions for G5a and G10a could be reversed.

4.1.1. 7-Methyl-3-nitroimidazol[1,2-a]pyridine-2-carboxamide (**2b**)

A solution of nitroester **1b** (1.6 g, 6.4 mmol) in dry ethanol (40 ml) was introduced in a part. Liquid ammonia (18 ml) was added and the mixture was heated at 100 °C for 2 h. After cooling the suspension was filtered and the precipitate washed first with ethanol then with dichloromethane to give 1 g of **2b** (70%) as pale yellow plates; m.p. >260 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ : 2.52 (s, 3 H, CH_3), 7.40 (dd, 1 H, J = 7 Hz, J = 1.6 Hz, H-6), 7.80 (s, 1 H, H-8), 7.94–8.12 (2s, 2 H, NH_2), 9.22 (d, 1 H, J = 7 Hz, H-5).

4.1.2. 3-Amino-7-methylimidazo[1,2-a]pyridine-2-carboxamide (**3b**)

To hydrobromic acid (10 ml) cooled to −10 °C tin (1.3 g, 10.9 mmol) was added. The amide **2b** (1 g, 4.5 mmol) was added portionwise to avoid a temperature of more than 5 °C. The mixture was stirred at 0 °C for 1 h, allowed to stand at room temperature, then stirred for further 2 h. The suspension was made basic with sodium carbonate and was evaporated to dryness. The residue was chromatographed on neutral alumina eluted with dichloromethane/methanol (97/3 v/v) to give **3b** (0.8 g, 93%) as pale yellow plates; m.p.: >260 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ: 230 (s, 3 H, CH_3), 6.09 (s, 2 H, NH_2), 6.67 (d, 1 H, J = 7 Hz, H-6), 7.11 (s, 1 H, H-8), 7.32 (s, 2 H, NH_2), 8.04 (d, 1 H, J = 7 Hz, H-5).

4.1.3. 7-Methyl-4H-pyrido[1,2-e]purin-4-one (**4b**)

A suspension of aminoamide **3b** (0.7 g, 3.7 mmol) was refluxed in freshly distilled triethyl ortoformate for 15 h. After cooling the residue was evaporated to dryness. The residual solid was suspended in dichloromethane, filtered and washed to give **4b** (0.5 g, 71%) as pale yellow plates; m.p.: >260 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) δ: 2.44 (s, 3 H. CH_3), 6.98 (d, 1 H, J = 7 Hz, H-8), 7.49 (s, 1 H, H-6), 8.14 (s, 1 H, H-2), 8.57 (d, 1 H, J = 7 Hz, H-9), 12.57 (br.s., 1 H, NH); ^{13}C NMR (DMSO-d_6, 50 MHz) δ: 21.7 (CH_3), 116.3 (C-6*), 116.5 (C-8*), 124.0 (C-9), 127.2 (C-4a), 140.7 (C-7), 141.9 (C-10a), 143.9 (C-2), 145.3 (C-5a), 158.0 (C-4).

4.1.4. General procedure for chlorination

A solution of 4*H*-pyrido[1,2-*e*]purin-4-one derivative **4a** or **4b** (6 mmol) was refluxed in freshly distilled phosphoryl chloride (25 ml) for 5 h. After cooling, the solution was poured on ice and the resulting solution was made alkaline with sodium carbonate then extracted with dichloromethane. The organic layers were dried over calcium chloride, filtered and evaporated in vacuo to leave a residue which was chromatographed on neutral alumina eluted with dichloromethane.

4.1.4.1. 4-Chloropyrido[1,2-*e*]purine (**5a**)

Yield: 66%, m.p.: 250 °C, ^{1}H NMR ($CDCl_3$, 100 MHz) δ: 7.04 (m, 1 H, H-8), 7.71 (m, 2 H, H-6,7), 8.73 (d, 1 H, J = 7.2 Hz, H-9), 8.83 (s, 1 H, H-2); ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 112.8 (C-8), 119.0 (C-6), 125.2 (C-9), 132.8 (C-4a), 133.4 (C-7), 146.4 (C-10a), 149.1 (C-5a), 149.2 (C-2), 151.0 (C-4); MS (EI) m/z (%): 206 (M + 2, 20), 204 ($M^{+\cdot}$, 50), 158 (52), 78 (57).

4.1.4.2. 4-Chloro-7-methylpyrido[1,2*e*]purine (**5b**)

Yield: 70%, m.p.: 236–238 °C; ^{1}H NMR ($CDCl_3$, 100 MHz) δ: 2.49 (s, 3 H, CH_3), 6.84 (d, 1 H, J = 7 Hz, H-8), 7.45 (s, 1 H, H-6), 8.55 (d, 1 H, J = 7 Hz, H-9), 8.75 (s, 1 H, H-2); ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 22.4 (CH_3), 115.8 (C-8), 116.7 (C-6), 124.0 (C-9), 132.9 (C-4a), 145.4 (C-7), 146.4 (C-10a), 148.6 (C-2), 149.5 (C-5a), 150.1 (C-4).

4.1.5. General procedure for nucleophile substitution

A mixture of the chloro derivative **5a** or **5b** (0.55 mmol) and a suitable amine (5 Eq.) was stirred at room temperature for 8 h. The solution was extracted with dichloromethane, the organic layers were dried over calcium chloride. Usual work up gave a residue which was chromatographed on neutral alumina eluted with dichloromethane.

4.1.5.1. 4-Methylaminopyrido[1,2-*e*]purine (**6a**)

Yield: 63%, m.p.: 205–207 °C, ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 3.28 (d, 3 H, J = 4.8 Hz, VH_3), 6.49 (br. s., 1 H, NH), 6.95 (dt, 1 H, J = 6.7 Hz, J = 1.0 Hz, H-8), 7.47 (ddd, 1 H, J = 9.3 Hz, J = 6.7 Hz, J = 1.3 Hz, H-7), 7.65 (dt, 1 H, J = 9.3 Hz, J = 1.0 Hz, H-6), 8.56 (s, 1 H, H-2), 8.64 (dt, 1 H, J = 6.8 Hz, J = 1.2 Hz, H-9); ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 27.8 (CH_3), 111.7 (C-8), 118.2 (C-6), 122.0 (C-4a), 124.0 (C-9), 129.8 (C-7), 142.8 (C-10a), 145.5 (C-5a), 151.3 (C-2), 156.0 (C-4).

4.1.5.2. 4-Methylamino-7-methylpyrido[1,2-*e*]purine (**6b**)

Yield: 59%, m.p.: 202–204 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 2.51 (s, 3 H, CH_3), 3.29 (d, 3 H, J = 5 Hz, CH_3), 6.25 (br.s., 1 H, NH), 6.81 (dd, 1 H, J = 7.0 Hz, J = 1.4 Hz, H-8), 7.40 (d, 1 H, J = 1.3 Hz, H-6), 8.52 (d, 1 H, J = 7.0 Hz, H-9), 8.54 (s, 1 H, H-2); ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 22.0 (CH_3), 27.7 ($NHCH_3$), 114.5 (C-8), 116.1 (C-6), 122.0 (C-4a), 123.5 (C-9), 141.1 (C-7), 142.9 (C-10a); 146.0 (C-5a), 150.9 (C-2), 155.8 (C-4).

4.1.5.3. 4-(Piperidin-1-yl)pyrido[1,2-*e*]purine (**7a**)

Yield: 57%, m.p.: 124–126 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.80 (s, 6 H, piperidine), 4.43 (s, 4 H, piperidine), 6.93 (m, 1 H, H-8), 7.43 (m, 1 H, H-7), 7.67 (t, 1 H, J = 9.6 Hz, H-6), 8.46 (s, 1 H, H-2), 8.61 (m, 1 H, H-9); ^{13}C NMR ($CDCl_3$, 25 MHz) δ: 24.9 (C-4′), 26.4 (C-3′,5′), 46.7 (C-2′,6′), 111.4 (C-8), 118.4 (C-6), 122.2 (C-4a), 124.2 (C-9), 129.2 (C-7), 143.9 (C-5a), 144.9 (C-10a), 150.7 (C-2), 154.5 (C-4).

4.1.5.4. 7-Methyl-4-(piperidin-1-yl)pyrido[1,2-*e*]purine (**7b**)

Yield: 55%, m.p.: 164–166 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.79 (s, 6 H, piperidine), 2.48 (s, 3 H, CH_3), 4.40 (s, 4 H, piperidine), 6.75 (d, 1 H, J = 7 Hz, H-8), 7.40 (s, 1 H, H-6), 8.44 (s, 1 H, H-2), 8.47 (d, 1 H, J = 7 Hz, H-9); ^{13}C NMR ($CDCl_3$, 25 MHz) δ: 21.7 (CH_3), 24.6 (C-4′), 26.0 (C-2′,6′), 46.3 (C-3′,5′), 114.5 (C-8), 116.0 (C-6), 121.9 (C-4a), 123.0 (C-9), 140.2 (C-7), 144.1 (C-5a), 144.6 (C-10a), 150.0 (C-2), 154.5 (C-4).

4.2. Biological activity

4.2.1. Chemicals

Commercial doxorubicin (from Pharmacia, Saint Quentin en Yvelines, France, Lot n° 6001BA) was used. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St Louis, USA). All the other chemicals and solvents were purchased from commercial sources and of the highest available analytical grade.

4.2.2. Cells and culture

A solid tumor, MCF7 human breast cells was obtained from the A.T.C.C. (American Type Culture Collection, Rockville, Md, USA). The resistant subline was kindly provided by Dr. Pierre Canal (Toulouse, France). This subline have been established by continuous exposure of cells to gradually increasing concentrations of doxorubicin and were maintened in doxorubicin supplemented medium. Cultures were grown in RPMI 1640 (Gibco laboratories, France) supplemented with 10% (v/v) fetal bovine serum, 2 nM glutamine and penicillin/streptomycin. Cell lines were cultured at 37 °C under 95% relative humidity in an atmosphere containing 5% CO_2 in air.

4.2.3. Cytotoxicity assays

Cells at a density of 6×10^3 cells/well were seeded in 96 well microtiter plates in a final volume of 200 µl of complete medium. The cells were exposed to different concentrations of drugs (doxorubicin: 10^{-3} to 10^{-6} M for MCF7 and 10^{-3} to 10^{-5} M for MCF7R; evaluated compounds: 10^{-3} to 10^{-6} M for MCF7 and MCF7R) for 96 h at 37 °C. After 4 days the cells were washed and 50 µl of tetrazolium dye (MTT) solution at 1 mg/ml in RPMI/SVF (90/10) was added for each well. The plates were incubated for 4 h at 37 °C. At the end of the culture period, culture medium was carefully aspirated and 100 µl of isopropanol alcohol was added. Complete and homogeneous solubilization of formazan crystals was achieved after 10 min of incubation and vigourous shaking of well contents. The absorbance was measured on a microculture plate reader (Dynatech MR 5000, France) at 570 nm. The data were expressed in terms of IC_{50} which correspond to a 50% reduction in the cell number as compared with controls. The resistance factor (RF) was calculated from the ratio between the IC_{50}% growth-inhibitory concentration (IC_{50} values) recorded from MCF7R and MCF7 cells respectively.

4.2.4. Monoclonal antibodies

Purified mouse monoclonal C219, JSB1 antibody against an internal cell surface epitope of P-glycoprotein was purchased respectively from Centocor Inc. (Malvern, USA) and Tebu (Le Perray en Yvelines, France). The relative amount of P-glycoprotein (P170) in the resistant cell lines compared to the sensitive cell line was determined by immunochemical detection using a monoclonal antibody against the P-glycoprotein: C219, JSB1 [13].

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