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Modulation of the multidrug resistance (MDR) phenotype in CEM MDR cells simultaneously exposed to anti HIV-1 protease inhibitors (PI's) and cytotoxic drugs

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Summary. - Vinblastine, vincristine and doxorubicyn are currently used in chemotherapeutic treatments of several malignancies including HIV-1 associated tumours Kaposi's sarcoma (KS) and non-Hodgkin lymphoma (NHL). Hence, AIDS patients also affected by KS and NHL may be simultaneously subjected to highly active antiretroviral therapy (HAART) and cytotoxic drugs to combat HIV-1 infection and cancer aggressiveness. In order to assess if the combination of these therapies may affect cell growth and survival of P-glycoprotein expressing MDR variants of the human $CD4^+$ T-lymphoblastoid CEM cell line, the protease inhibitors (PI's) ritonavir, saquinavir and indinavir were tested in an *in vitro* assay for their ability to potentiate the vinblastine, vincristine and doxorubicyn cytotoxicity. The results we obtained demonstrated that at the concentration of 10 μg/ml, ritonavir and in a lesser extent saquinavir act as MDR reversing agents. By contrast, the PI indinavir at least in the CEM cell system, does not affect the patterns of drug resistance. The level of chemosensitization exerted by ritonavir and saquinavir suggests that these PI's may render P-glycoprotein expressing MDR cells *de novo* susceptible to the antineoplastic drugs vinblastine, vincristine and doxorubicyn.

Key words: protease inhibitors, AIDS, tumours, P-glycoprotein, reversing agents.

Riassunto *(Modulazione della farmaco-resistenza multipla (MDR) in cellule CEM esprimenti fenotipo MDR trattate con composti citotossici ed inibitori delle proteasi (PI's)).* - Composti quali la vinblastina, vincristina e doxorubicina vengono correntemente utilizzati per il trattamento farmacologico di diversi tipi di tumori inclusi quelli che possono insorgere in associazione con l'infezione da HIV-1, come il Kaposi's sarcoma (KS) e non-Hodgkin lymphoma (NHL). Pertanto, i pazienti congiuntamente affetti da AIDS e KS o NHL possono essere sottoposti contemporaneamente a terapie ad alta attività antiretrovirale (HAART) ed agenti citotossici per combattere l'infezione da HIV-1 e l'aggressività delle cellule neoplastiche. Per verificare se la combinazione di questi farmaci possa interferire sulla crescita di varianti MDR di una linea $CD4^+$ di tipo linfoblastoide (CEM) esprimenti la P-glicoproteina, gli PI's ritonavir, saquinavir ed indinavir sono stati testati *in vitro* per la loro capacità di potenziare la citotossicità della vinblastina, vincristina e doxorubicina. I risultati ottenuti dimostrano che alla concentrazione di 10 μg/ml il ritonavir ed in misura ridotta il saquinavir, agiscono come agenti revertanti il fenotipo MDR. In contrasto, l'indinavir almeno nel sistema cellulare CEM da noi utilizzato sembra non interferire sulla farmaco resistenza. Il livello di chemosensibilizzazione indotto dal ritonavir e saquinavir, suggerisce che questi PI's possano rendere cellule MDR nuovamente suscettibili alla azione citotossica di composti antineoplastici quali vinblastina, vincristina ed doxorubicina.

Parole chiave: inibitori delle proteasi, AIDS, tumori, P-glycoproteina, agenti revertanti.

Introduction

Simultaneous resistance of tumour cells to multiple cytotoxic drugs (multidrug resistance, MDR) is a major limitation to the successful chemotherapeutic treatment of cancer. Subpopulations of MDR cells originating by an induction/selection mechanism exerted by anthracyclines, vinca alkaloids, epipodophyllotoxins and taxanes are usually cross-resistant to a large spectrum of cytotoxic compounds [1]. Studies on the molecular basis of MDR has revealed that genetic, biochemical and functional alterations associated with the MDR phenotype might be caused by an amplified gene designated MDR1 in human which encodes a 4.5 kb mRNA [2]. P-glycoprotein the product of this gene, is a 170 kDa transmembrane protein capable of pumping a wide range of relatively hydrophobic, amphipathic drugs out of cells. P-glycoprotein belongs to the ATP-binding cassette family of proteins, and ATP hydrolysis provide the energy for active drug extrusion, which can occur against steep concentration gradients [3].

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P-glycoprotein-mediated MDR is only one of the cellular mechanisms by which tumour cells may evade cytotoxic effects of anticancer agents, but is one of the best understood and most intensively studied form of mammalian MDR [4]. Besides its occurrence in cancer cells, P-glycoprotein is also expressed in normal tissues in a pattern that strongly suggest that this protein is involved in the protection of the host against xenotoxins, either by accelerating their excretion or by preventing their uptake from the gastro-intestinal tract following oral ingestion [5]. P-glycoprotein expression has been demonstrated in many solid tumours and haematological malignancies, including Kaposi's sarcoma (KS) and non-Hodgkin lymphoma (NHL) [1, 6]. Several studies have reported a prognostic significance of MDR1 since lower probability of achieving a complete remission was observed in patients with P-glycoprotein expressing tumours [1, 6]. These investigations have indicated that subpopulations of KS and NHL express high levels of P-glycoprotein which may play a role in the failure of chemotherapy [6]. Several antitumor agents including *Vinca* alkaloids and anthracyclines have led to good responses of patients with widespread KS and NHL [7, 8]. However, HIV-related bone-marrow suppression, the presence of multiple chronic opportunistic infections and expression of MDR1-P-glycoprotein may act in concert to limit the efficacy of anticancer therapy [9].

Numerous compounds have been identified which block the activity of P-glycoprotein and reverse drug-resistance to cytotoxic agents *in vitro* experimental systems [10]. This finding has suggested that clinical drug resistance in human tumours which often overexpress P-glycoprotein may be potentially circumvented through concomitant administration to patients of P-glycoprotien inhibitor and chemotherapeutic drugs [11]. Included in the P-glycoprotein blockers there are compounds showing a great variability in chemical structure, mechanism of action and biological origin. For example, inhibitors of the pump-efflux function may competitively or non-competitively inhibit drug transport through P-glycoprotein. In the former case, P-glycoprotein inhibitors may block cytotoxic drug efflux by binding to similar drug substrate binding sites; in the latter by binding to sites which cause allosteric changes resulting in inhibition of cytotoxic drug binding or transport [10, 11]. Previously studies have indicated that PI's currently used in HAART may also act as P-glycoprotein blockers [12, 13]. In theory, the combined delivering of PI's with *Vinca* alkaloids and anthracyclines would benefit the pharmacotherapy of HIV-infected patients also affected by AIDS-associated tumours [9, 14]. Hence, could be of very great clinical value to determine the ability of the PI's in rendering *de novo* susceptible MDR cells to the cytotoxic activity of antineoplastic drugs.

Materials and methods

Cells and chemicals

CEM cells and its MDR variants CEM-VBL10 and CEM-VBL100 were grown using standard conditions for cells cultured in suspension. The basic medium (BM) consisted in RPMI-1640 supplemented with 10% foetal calf serum (FCS), L-glutamine (2 mM) and antibiotics. All these ingredients were purchased from Hyclone (Logan, Utah, USA). Verapamil (isoptin) was provided by BASF-Knoll (Milan, Italy), ritonavir, by Abbott Laboratories (IL, USA), saquinavir by Roche Laboratories (Welwyn Garden City, UK), indinavir by Merck Laboratories (Rahway, NJ, USA), doxorubicyn by Farmitalia (Nerviano, Milan, Italy), vinblastine (Velbe) and vincristine by Eli Lilly (Paris, France). Vinblastine-bodipy was purchased from Molecular Probes (Eugene, OH, USA).

Characterization of the CEM cell systems

Before testing the MDR reversing ability of PI's, the human T-lymphoblastoid $CD4^+$ CEM cell line and its MDR variants CEM-VBL10 and CEM-VBL100 were characterized for P-glycoprotein expression and function. For P-glycoprotein expression the MAb MM4.17 to external P-glycoprotein domain was used. Living/intact CEM cells and its MDR variants in exponential phase of growth were collected, washed and incubated for 30' at 4 °C with 12.5 μg/ml of the MAb. At the end of incubation the cells were collected, washed again and incubated for an additional 30' with fluorescein (FITC) conjugated goat antimouse IgG (Cappel, West Chester, PA, USA). Specific binding of MAb to cells was revealed using standard methods for flow-cytometry analysis [15]. The function of P-glycoprotein was studied using dye-efflux assay performed as usual. Briefly, CEM, CEM-VBL10 and CEM-VBL100 cells ($1x10^6$) were loaded with doxorubicyn (10 μg/ml) in 1ml of BM for 1 hr at 37 °C. At the end of incubation, cells were washed in serum free medium (SFM) and resuspended in dye-free BM in the presence or in the absence 2.5 μg/ml of verapamil for a further 1 hr at 37 °C. Then cells were washed twice with ice-cold PBS/FACS, maintained in ice and analyzed in a flow-cytometer (FACscan, Becton-Dickinson).

Cytotoxic essay

CEM-VBL10 and CEM-VBL100 cells in exponential phase of growth were harvested and washed twice in SFM at 37 °C. Then, cells were adjusted in BM at the concentration of $5x10^4$/ml containing the PI to be tested or Verapamil to compare

with this potent P-glycoprotein blocker the level of the MDR modulation exerted by the PI's. Log-10^{-1} dilutions of vinblastine, doxorubicyn and vincristine (from 2 ng/ml to 20 μg/ml) were assessed in a volume of 100 μl in 96-wells Costar plates. Then 100 μl of BM containing cells and PI's (20 μg/ml) or verapamil (2.5 μg/ml as control) were added to wells. The cells were counted in a cell-counter after 72 hr of culturing. Each experiment was performed in triplicate and the cell growth was calculated using the expression (En - E_0) / (Cn - C_0) were E_0 and En were the initial and the final cell concentration in the drug-containing cultures and C_0 and Cn are the corresponding cell concentrations in untreated control cultures.

Results and discussion

Identification of the P-glycoprotein as one of the most important mechanisms for multidrug resistance provided a theoretical target to improve anticancer therapy. P-glycoprotein is also a factor that may limit the efficacy of antiviral therapy [9, 14]: in HIV-1-infected cells with a high P-glycoprotein content, both penetration and antiviral efficacy of indinavir, saquinavir, and ritonavir are diminished [12, 13]. P-glycoprotein is not only expressed in MDR tumours and lymphocytes, one of the main target of HIV [16, 17], but also in a range of pharmacological barriers that could give rise to potential tumours and HIV sanctuary sites in the body such as brain and testis [5, 18]. Hence, the simultaneous treatment of AIDS patients with PI's and anticancer drugs may affect biodisponibility and penetration of the compounds used for combatting HIV infection and tumour aggressiveness [9, 14]. Since the original observation that verapamil was capable of circumventing multidrug resistance [19], an increasing number of compounds capable of reversing multidrug resistance continues to be identified [10, 11]. P-glycoprotein blockers are as structurally diverse as the known P-glycoprotein substrates and many blockers are themselves transported to some extent by P-glycoprotein [1, 10, 11]. Co-administration of such blockers with conventional chemotherapy to cancer and/or AIDS patients might reverse the P-glycoprotein-mediated MDR of the tumour or HIV-target cells and thus enhance the response to anticancer and antiviral drugs.

For the present study we have used a human T-lymphoblastoid cell system consisting of CEM cells and its MDR variants CEM-VBL10 and CEM-VBL100. These cells are characterized by a marked difference of the number of P-glycoprotein binding/sites per cell. While P-glycoprotein molecules are practically undetectable on the parental drug-sensitive CEM cell line, its number progressively increase in MDR variants: CEM-VBL10 and CEM-VBL100 express <1x10^4 and >1x10^6 P-glycoprotein binding/sites per cell, respectively [20]. This differences in P-glycoprotein content, parallel in these cells with binding of the P-glycoprotein specific MAb MM4.17 and efflux activity of the P-glycoprotein dye-substrate doxorubicyn (Fig. 1).

The ability of ritonavir, saquinavir, and indinavir to affect cell growth and survival in the MDR cells CEM-VBL10 and CEM-VBL100 was determined in the presence of Log-10^{-1} dilutions of vinblastine, vincristine and doxorubicyn. The concentration of PI's (10 μg/ml) and cytotoxic drugs used in this study are equivalent to that observed in the plasma of AIDS patients subjected to HAART and/or anticancer therapy [21]. The PI ritonavir, highly potentiates the cytotoxicity of both the *Vinca* alkaloid derivatives.

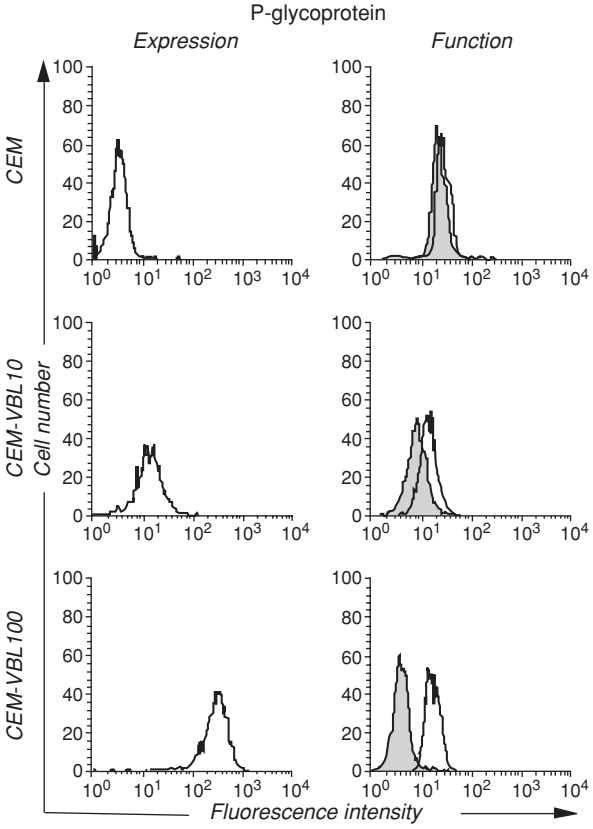

Fig. 1. - P-glycoprotein expression and function. MAb MM4.17 directed to an external domain of P-glycoprotein increases its specific binding to cells in parallel with the level to drug-resistance (CEM-VBL100 > CEM-VBL10 > CEM) (left part of the figure). The P-glycoprotein dye-substrate doxorubicyn (shaded profiles) is retained (CEM cells) or actively effluxed-out in P-glycoprotein-expressing MDR variants (CEM-VBL10 and CEM-VBL100 cells). The effect exerted by the potent P-glycoprotein blocker Verapamil on doxorubicyn efflux is also shown (bold profiles) (right part of the figure).

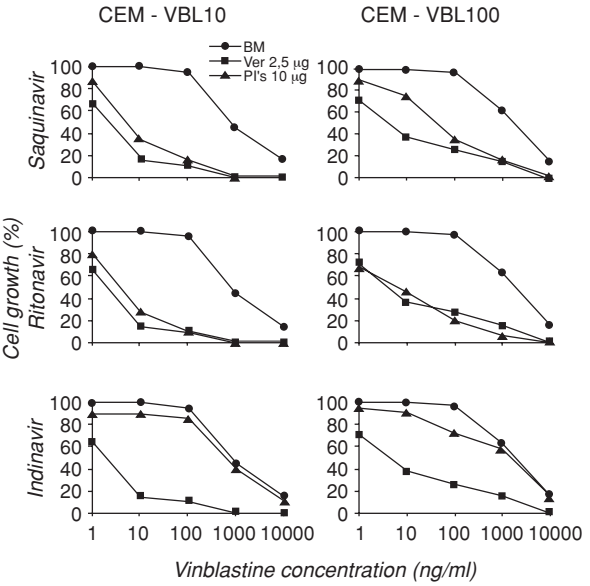

Fig. 2. - Effect of PI's on the potentiation of vinblastine and cytotoxicity on CEM-VBL10 and CEM-VBL100 cells. The final concentration of PI's ritonavir, saquinavir and indinavir was 10 μg/ml. Verapamil was used as MDR modifier agent at the final concentration of 2.5 μg/ml. The cells were counted after 72 hr of cell growth and expressed as % relative of control cells grown in BM + Log-10^{-1} drug dilution. The mean of triplicate measurements is shown; standard deviation was <15% of each single value.

However, the MDR reversing ability of ritonavir appears to be more pronounced in combination with vinblastine than vincristine as it is evidentiated by comparing cell growth curves of CEM-VBL10 and CEM-VBL100 (Figs 2 and 3). To note that the reduction of the levels of drug resistance exerted by 10 μg/ml of ritonavir (in vinblastine containing medium) was in the order of that observed with 2.5 μg/ml of the potent P-glycoprotein inhibitor verapamil. Saquinavir (although less than ritonavir) also acts as an MDR reversing agent; the combination of this PI with vinblastine and vincristine affects cell growth and survival of CEM-VBL10 and CEM-VBL100 cells. In agreement with the hypothesis that the reversion of the MDR phenotype exerted by ritonavir and saquinavir could be the result of competition between PI's and cytotoxic compounds to gain P-glycoprotein binding sites, ritonavir and saquinavir have a more pronounced effect on CEM-VBL10 than CEM-VBL100 (Fig. 2). The PI Indinavir although currently included in forming part of the large array of P-glycoprotein substrates [12, 13] does not significantly potentiates vinblastine and vincristine cytotoxicity neither at very high concentrations nor in CEM-VBL10 cells (Figs 2-4). This may suggest the existence of different P-glycoprotein binding sites for *Vinca* alkaloids and indinavir.

The MDR reversing ability of ritonavir, saquinavir and indinavir was also tested in combination with doxorubicyn. This anthracyclin derivative appears to be less cytotoxic than vinblastine and vincristine if we compare their respective IC-50 values [22]. However, doxorubicyn cytotoxicity increases if combined with ritonavir or saquinavir. By contrast, the PI Indinavir does not affect cell growth and survival if combined with doxorubicyn. The similar level of chemosensitization to doxorubicyn exerted by ritonavir and saquinavir in CEM-VBL10 and CEM-VBL100 cells suggests that the amounts of this anticancer compound even at the highest concentrations does not saturate all P-glycoprotein molecules acting as drug transporter (this may be also caused by the low affinity to P-glycoprotein drug binding sites). Several lines of evidence suggest that the P-glycoprotein inhibition may be the result of different mechanism of actions including direct competition of P-glycoprotein blockers and anticancer compounds to same P-glycoprotein drug binding sites or by binding to sites which cause allosteric changes resulting in inhibition of cytotoxic drug binding or transport [10, 11]. It has been also reported that a variety of P-glycoprotein point mutations may affect drug resistance. A common observation found with point mutations is a differential

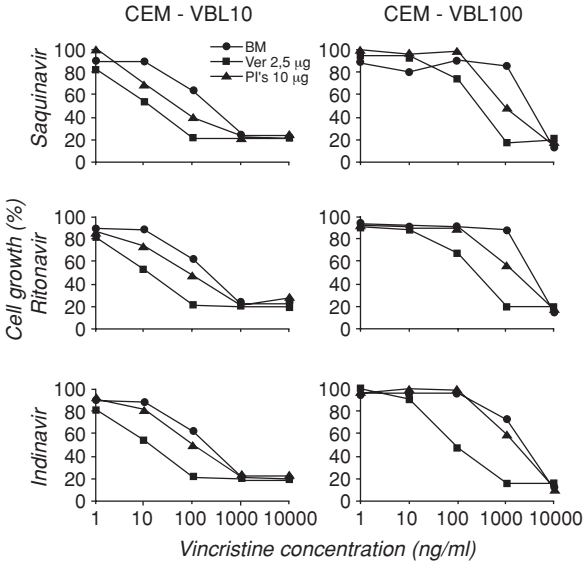

Fig. 3. - Effect of PI's on the potentiation of vincristine cytotoxicity on CEM-VBL10 and CEM-VBL100 cells. The final concentration of PI's ritonavir, saquinavir and indinavir was 10 μg/ml. Verapamil was used as MDR modifier at the final concentration of 2.5 μg/ml. The cells were counted after 72 hr of cell growth. The mean of triplicate measurements is shown; standard deviation was <15% of each single value.

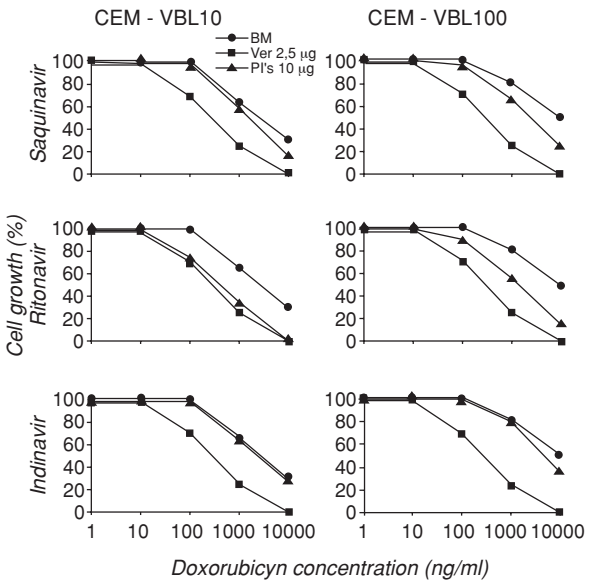

Fig. 4. - Effect of PI's on the potentiation of doxorubicyn cytotoxicity on CEM-VBL10 and CEM-VBL100 cells. The final concentration of PI' s ritonavir, saquinavir and indinavir was 10 μg/ml. Verapamil was used as MDR modifier at the final concentration of 2.5 μg/ml. The cells were counted after 72 hr of cell growth. The mean of triplicate measurements is shown; standard deviation was <15% of each single value.

effect on drug resistance with cells expressing the mutant P-glycoprotein inducted by specific cytotoxic compounds [23, 24]. To this regard, the use of vinblastine as selective agent to isolate CEM MDR variants and the high susceptibility of CEM cells to *Vinca* alkaloid derivatives may be strictly correlated. The results herein described demonstrate that *in vitro* assay the PI's ritonavir and in a lesser extent saquinavir may act as MDR reversing agents by potentiating vinblastine, vincristine and doxorubicyn cytotoxicity. However, by considering the functional etherogeneity of P-glycoprotein [25, 26], it cannot in principle be excluded that the PI indinavir also may act as P-glycoprotein blocker if tested in a different MDR cell system or in combination with other anti-cancer compounds. In conclusion, we have demonstrated that in *in vitro* model PI's may act as efficient MDR reversing agents. *In vivo*, the potential benefits of co-administration of PI's and anticancer compounds may be several. For example, blocking of P-glycoprotein in pharmacological barriers and T-lymphocytes would increase PI penetration and retention in the putative pharmacological sanctuaries, MDR tumours and HIV-1 target cells. However the safety of the combination of PI's and anticancer compounds should be carefully investigated in preclinical studies before its clinical use.

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