

Drug resistance and modifiers

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POSTER

Pharmacodynamic analysis of surrogate tissue responses to the demethylating agent 2'-deoxy-5-azacytidine (Decitabine)

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The DNA methyltransferase inhibitor 2'-deoxy-5-azacytidine (decitabine) can sensitise drug resistant tumour xenografts grown in nude mice to a range of cytotoxic chemotherapeutic drugs including carboplatin (Plumb et al, 2000, Cancer Res., 60, 6039). This treatment also induces reduced CpG-island methylation and increased expression of the *hMLH1* gene in drug resistant human tumour xenografts. These data have led to an ongoing Phase I clinical trial of decitabine and carboplatin in patients with advanced solid tumours. Decitabine was given IV over 6 hours on Day 1, Carboplatin at a fixed dose of AUC5 was given on Day 8 (IV over an hour) and treatment was repeated every 4 weeks. Patients have been treated at three dose levels of Decitabine: 45, 90 and 135mg/m². Maximum tolerated dose was identified as Decitabine 135mg/m² with Grade 4 febrile neutropenia (1 patient), and Grade 4 neutropenia necessitating delay of cycle 2 for >7 days (1 patient). Pharmacodynamic objectives of this trial were: 1) to study the time course and relationship between DNA methylation in surrogate tissues and the dose and pharmacokinetic behavior of decitabine. 2) to investigate changes in methylation of specific CpG-islands at gene promoters induced by decitabine in surrogate tissues. DNA extracted from peripheral blood mononuclear (PBM) cells showed a dose dependent decrease in levels of 5-methyl-2'-deoxycytidine up to day 10 which then reversed and had returned to near starting levels by day 22. The demethylation observed remains similar in subsequent cycles of decitabine treatment. The MAGE1A CpG-island is biallelically methylated in adult somatic normal tissues. Decitabine treatment induced dose dependent demethylation of the MAGE1A CpG-island in DNA isolated from PBM cells and buccal smears. The reduction in 5-methyl-2'-deoxycytidine levels in human PBM cells is equivalent or greater to that observed in murine PBM cells, at decitabine doses in mice when concomitant demethylation of the *hMLH1* gene and chemosensitisation occurs in drug resistant xenografts. Analysis of DNA methylation of tumours from patients before and after 90 mg/m² decitabine treatment is currently underway.

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Breast cancer resistance protein (BCRP) expression, function and promoter methylation in multiple myeloma: potential mechanism of drug resistance

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Background: The purpose of this study was to determine if BCRP is present and functional in human multiple myeloma (MM) cell lines, MM patient bone marrow aspirates, and whether BCRP expression is altered by microenvironment (cell density) or topotecan (TPT) exposure in MM. In addition, we investigated whether promoter methylation status controls BCRP expression.

Materials and methods: BCRP mRNA expression was assayed by quantitative PCR (QPCR), and protein expression by Western blot, flow cytometry and immunostaining. To determine if BCRP is functional in human plasma cells, TPT (a BCRP substrate) uptake, was examined by flow cytometry in the presence of a specific inhibitor of BCRP, tryprostatin A. Cells examined were 8226 and H929 MM cells, drug resistant 8226MR cells (positive control), and MM patient plasma cells isolated from bone marrow aspirates using CD138 magnetic beads. Bone marrow aspirates were collected pre- and during high-dose melphalan/TPT chemotherapy, and at relapse. Methylation of the BCRP promoter was determined using bisulfite DNA sequencing.

Results: QPCR data established that BCRP mRNA is expressed in MM patient samples and MM cell lines. BCRP mRNA expression data demonstrate a strong correlation with Western blot and immunofluorescence (see table). TPT export correlated with mRNA and protein data; high expressors of BCRP exported more TPT than low expressors (see table). However, even low level expression of BCRP was able to confer resistance to TPT uptake in wild-type 8226 cells. To further confirm that BCRP was responsible for export we used the specific BCRP inhibitor, tryprostatin A, which was able to reverse TPT efflux. BCRP expression was affected by microenvironment and methylation, high density cells in log phase growth had greater BCRP expression, and expression was

strongly affected by promoter methylation. Human MM patient cells (CD138 selected) expressed BCRP protein and mRNA. As was seen in MM cell lines, BCRP efflux of TPT correlated well with protein and mRNA expression in patient bone marrow aspirates. BCRP protein increased in response to chemotherapy with melphalan and TPT, and BCRP protein and mRNA were increased at relapse.

Conclusions: Based on data from Q-PCR, Western blot, flow cytometry, and immunofluorescence staining, we found that BCRP is expressed and is functional in MM cells, both *in vivo* and *in vitro*, and therefore may contribute to drug resistance.

Cell/tissue type	RNA (QPCR) (copies/GAPDH)	BCRP protein (FACscan)	BCRP activity (TPT uptake)
Multiple myeloma (n=7)	263.01±239.78	No data	29.0 to 210.3 (range)
Normal peripheral blood lymphocytes	1.10±0.99	No data	No data
8226MR myeloma	4402.7±195.5	238.0	0.5
8226 myeloma	827.4±30.6	92.0	10.4
H929 myeloma	56.1±3.1	17.0	58.6

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POSTER

Analogies in imatinib-resistant threonine-to-isoleucine mutation in BCR-ABL, KIT and PDGFRA: a combined experimental/computational approach

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Background: Currently, there is an increasing interest in therapies targeting critical molecular pathways for tumors carrying pivotal molecular alterations. The paradigm of this new treatment trend is represented by BCR-ABL in positive chronic myelogenous leukemia (CML) where the therapy with Imatinib, an ATP-competitor, induces dramatic and often durable clinical responses in most patients. An analogous response has been observed in c-KIT mutated gastrointestinal stromal tumors (GIST), and in FIP1L1-PDGFRα positive idiopathic hypereosinophilic syndrome (HES).

In all these tumors, Imatinib is able to switch off the pathologically activated tyrosine kinases, ABL, KIT and PDGFRA, respectively, and to block the activation of a cascade of intracellular proteins that promote tumor cell survival and proliferation.

However, in CML, GIST and HES, development of drug-resistant phenotypes have been observed, mainly sustained by point mutations. These mutations involving the ATP-binding pocket of the kinases are claimed to induce a structural modification of ATP pocket conformation, and to dramatically lower affinity of the receptor for the drug. While several aminoacidic changes have been detected in resistant BCR-ABL clones in CML, a single mutation has been reported to be responsible for acquired resistance in GIST and HES, i.e. T670I in KIT and T674I in PDGFRA. Surprisingly, the alignment of KIT and PDGFRA sequences indicate that these mutations are homologous to the T315I of BCR-ABL.

Materials and Methods: Computational free energy perturbation techniques were applied both to calculate the stability of wild-type and T-to-I mutants of BCR-ABL, KIT and PDGFRA, and to predict the relative binding affinities between the mutant forms and Imatinib.

Results: We were able to qualify and quantify the crucial molecular parameters for protein stability, and the differential contacts between wild-type and the mutated kinases and Imatinib. For instance, mutating T to I at position 315 in the BCR-ABL receptor resulted in a calculated ΔΔG of binding of 1.74 kcal/mol with respect to the corresponding wild-type structure, in very good agreement with the reported experimental finding of 1.07–2.0 kcal/mol. Further, a plethora of van-der-Waals and hydrophobic interactions are drastically, unfavorably changed in the mutant trajectory. As an example for all, the role played by the conformation of F382, thought crucial for the proper binding of Imatinib, is no longer maintained, resulting in the net loss of a favorable stabilizing interaction.

Conclusions: Our results show that three apparent different mutations in three different histotypes, affecting three different kinases but all responsible for Imatinib-acquired resistance in addition to involve the same residue of the corresponding ATP pockets share similar protein stabilities and mechanisms of decreasing drug binding affinity.