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Apoptotic response and the mechanism of resistance to camptothecin: a study of gene expression and functional effects

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RC0.1, a camptothecin-resistant derivative of the DU145 prostate cancer cell line, known to contain an R364H point mutation in topoisomerase I (top1). To assess gene expression changes that might also be involved in the acquisition of RC0.1,s drug resistance, we used "Oncochip" cDNA microarrays to compare the two cell lines with respect to expression of 1,650 cancer-related genes. Differences in transcript expression level > 1.5-fold were found for 181 genes. This level of difference was demonstrated to be statistically reliable using the stratum-adjusted Kruskal-Wallis test, after taking into account a dye-dependent variable. These differences in expression were concentrated in a statistically significant way ($p<0.05$) in 5 out of 182 functional categories. The largest of these categories was that of "apoptosis" genes. To assess whether or not RC0.1 had developed generalized resistance to apoptosis, we assessed the cells' responses to multiple, disparate apoptotic triggers, including camptothecin, staurosporine, serum starvation, cisplatin, UV irradiation, and gamma irradiation. Flow cytometric Annexin-V and TUNEL assays provided indices of early and late stages of apoptosis, respectively. For all stimuli, reduced levels of both phosphatidylserine translocation (by Annexin-V) and DNA strand breakage (by TUNEL) were observed for RC0.1, providing functional evidence for generalized resistance to apoptosis. When the genes with expression levels altered in RC0.1 were located on maps of molecular interactions in the cell, it became evident that there were two coherent blocks of genes concentrated in the core apoptotic and Akt-related subsystems. The survival molecules PI3K, Akt and Bcl-2 were upregulated in RC0.1, and the of the apoptotic BAD and caspase 6 molecules down-regulated. As a first test of the functional significance of these observations, we exposed the cells to wortmannin, a natural product that blocks the activity of PI3K. Consistent with the proposed involvement of this portion of the pathway in resistance to the apoptotic triggers employed, wortmannin treatment increased the apoptotic response of DU145, but not RC0.1, following treatment with camptothecin. These findings demonstrate major differences between DU145 and RC0.1 in apoptotic machinery, independent of the mutation in topoisomerase 1.

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Role of MAPKs in heat-induced apoptosis

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External insults have been demonstrated to activate a cytoplasmic signaling system mitogen-activated protein kinase (MAPK) pathway, i.e. ERK1/2, JNK and p38 kinase. Although heat-shock has been demonstrated to stimulate MAPKs, the exact role of MAPKs in heat-induced cell death is unknown. In the present study, we have investigated possible relationship between the activation of MAPKs and apoptosis caused by heat-shock in HL60 human promyelocytic leukemia cells. The activation of MAPKs was examined with immunocomplex kinase assay and western blot analysis and the apoptosis was estimated from the DNA fragmentation using agarose gel electrophoresis and also from the cellular DNA content using flow cytometry. Heating HL-60 cells at 43°C for 1 h caused significant apoptosis and varying degrees of activations of the MAPKs. The most pronounced activation occurred in JNK, which was activated as much as 15 times at 1 h after heating. The heat-induced JNK activation could be suppressed by pre-treating the cells with JNK inhibitor II. Importantly, the JNK inhibitor also significantly suppressed the heat-induced apoptosis. The heat-induced activation of p38 was far less than that of JNK, and pre-treating the cells with p38 inhibitor SB203580 did not significantly suppress the heat-induced apoptosis. ERK1/2 were slightly activated by heat-shock and PD 98059, an inhibitor of ERK1/2, had little effect on the heat-induced apoptosis. It has been known that an acidic environment can induce apoptosis by itself and enhances heat-induced apoptosis. In the present study, the acidic stress alone did not significantly activate MAPKs, but it increased the heat-induced activation of MAPKs. It is concluded that among the MAPKs, JNKplays the major role in heat-induced apoptosis, and than an acidic environment increases the heat-induced apoptosis probably by increasing the activation of JNK.

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Aplidin induces the mitochondrial apoptotic pathway via oxidative stress-mediated JNK and p38 activation and protein kinase C delta

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Aplidin™, a new antitumoral drug presently in phase II clinical trials, has shown both *in vitro* and *in vivo* activity against human cancer cells. Aplidin™ effectively inhibits cell viability by triggering a canonical apoptotic program resulting in alterations in cell morphology, caspase cascade activation, and chromatin fragmentation. Pro-apoptotic concentrations of Aplidin™ induce early oxidative stress, which results in a rapid and persistent activation of both JNK and p38 MAPK and a biphasic activation of ERK. Inhibition of JNK and p38 blocks the apoptotic program induced by Aplidin™, demonstrating its central role in the integration of the cellular stress induced by the drug. JNK and p38 MAPK activation results in downstream cytochrome-c release and activation of caspases -9 and -3 and PARP cleavage, demonstrating the mediation of the mitochondrial apoptotic pathway in this process. We also demonstrate that protein kinase C delta (PKC-d) mediates the cytotoxic effect of Aplidin™ and that PKC-d is concomitantly processed and activated late in the apoptotic process by a caspase-3 mediated mechanism. PKC-d appears as a key component necessary for full caspase cascade activation and execution of apoptosis, which most probably initiates a positive feedback loop further amplifying the apoptotic process.

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The impact of c-Src on drug sensitivity/resistance in human colon cancer cells

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c-Src expression level and/or activity is elevated early in the progression of colon cancer, a tumour that is commonly inherently resistant to chemotherapy. The participation of c-Src in signaling pathways may be important for the development of this malignancy, including those that promote proliferation, invasion and metastasis. v-Src both primes for and suppresses apoptosis in fibroblasts (Johnson et al 2000, Webb et al 2000). Here we have addressed the involvement of c-Src in determining drug sensitivity/resistance in the human colon carcinoma cell line KM12C transfected with constitutively activated c-Src (Y527F) to generate sublines 2C3 and 2C4 which had 4 and 10 fold higher levels of Src expression respectively than the empty vector control (2CV). 2C3 and 2C4 also expressed elevated levels of the anti-apoptotic Bcl-2 protein, though no other changes in the expression levels of Bcl-xL, Bax, Bak or Bad were observed. Elevated Bcl-2 expression suggested that 2C3 and 2C4 cells might display enhanced resistance to drug-induced apoptosis. However, increased sensitivity to a panel of anticancer drugs was observed as illustrated by the EC50 values below. The mode of oxaliplatin-induced KM12 cell death was confirmed as apoptosis based on changes in nuclear morphology. Sensitisation to oxaliplatin and cisplatin-induced apoptosis was also observed in KML4A cells (metastatic variant of KM12C) transfected with kinase-dead c-Src (MF-10) or the SH3-SH2 domains of c-Src (251-6, 251-13) as compared to the vector control (pBABE-1).

Src Construct	EC_{50} (μM)		
	Oxaliplatin	Cisplatin	Etoposide
Active – 2C3/2C4	65	75	4.8
Vector – 2CV	175	125	7.5
Kinase Dead – MF-10	75	70	–
SH3-SH2 – 251-6, 251-13	80	70	–
Vector – pBABE-1	175	95	–

When treated with a Src inhibitor (CGP77675, 1 uM) added 24h prior to and at 24h intervals during the course of oxaliplatin-exposure, oxaliplatin resistance was restored in all the src-transfected cell lines while the vector-only controls (2CV or pBABE-1) showed little or no change in oxaliplatin response. This suggests that c-Src is able to prime cells to oxaliplatin-induced apoptosis independently of its kinase domain. This priming for oxaliplatin induced apoptosis is inhibited by CGP77675 by an unclear mechanism that may involve a Src-like tyrosine kinase.

References

[1] Johnson et al (2000) Cell Death & Differentiation, 7: 685-696.
[2] Webb et al (2000) Molecular and Cellular Biology 20(24): 9271-80.

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Inhibition of MEK/ERK and PI3K/Akt pathways blocks apoptosis suppression signaling of FLT3/ITD

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Two kinds of FLT3 mutations have recently been detected in patients with acute myeloid leukemia (AML): (1) internal tandem duplication (inserted repeat spanning from as fewer than 7 to more than 30 amino acids) the jaxtamembrane domain in about 20% AML patients and (2) mutations in inactivation loop (generally D835Y) in about 7% of AML patients. Patients harboring FLT3/ITD mutations have relative poor prognosis, especially when the other allele is mutated and or lost. Both FLT3/ITD and FLT3/D835 mutations result in constitutive autophosphorylation of the receptor. Constitutive activation of FLT3 results in factor independent survival and growth in the transfected 32D and BA/F3 cell lines. Syngeneic mice injected of transfected cells develop myeloproliferative disease. Similarly, transplantation of FLT3/ITDs transfected bone marrow cells causes myeloproliferative disease in another mouse model. These data suggest that FLT3/ITDs inhibit apoptosis, promote proliferation and survival, and lead to leukemogenesis. Normal hematopoietic cells depend on the growth factor for survival and proliferation, whereas leukemic cell lines and primary leukemic cells often become partially or completely growth factor-independent. IL-3 dependent survival in BA/F3 cells is mediated by MEK/ERK pathway and protein kinase A (PKA) pathway. How BA/F3 cells transfected with FLT3/ITD or FLT3/D835 mutation become IL-3 independent is still largely unknown. To understand the mechanism of conversion of FLT3/ITD transfected BA/F3 cells towards IL-3-independence, we investigated (1) the activation of components in PI3K/Akt and MEK/ERK pathways by FLT3/ITD and (2) the effects of the inhibition of FLT3/ITD, PI3 Kinase or MEK on cell survival and proliferation. Our results: (1) FLT3/ITD constitutively activate PI3K/Akt and MEK/ERK pathways; (2) inhibition of FLT3/ITD autophosphorylation eliminated the activation of PI3K/Akt and MEK/ERK pathways and induced rapid apoptosis in most cells; (3) inhibition of PI3 Kinase block cell proliferation but only showed weak effect on apoptosis; (4) inhibition of MEK block proliferation and induced apoptosis; (5) combined inhibition of both PI3 Kanase and MEK had the same strong effects on apoptosis as inhibition of FLT3/ITD. Our results suggested that the mechanism of FLT3/ITD to suppress apoptosis and maintain survival involving both PI3-Kinase/ Akt and MEK/ERK pathways.

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Hypoxia increases potency of the proteasome inhibitor VELCADETM (bortezomib) for injection: potential for a hypoxic cell cytotoxin in solid tumors

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The 26S proteasome is a ubiquitous enzyme that plays an essential role in the regulation of cellular protein degradation, gene expression and cell cycle transition. In cancer cells these events can be deregulated and it has been hypothesized that antagonism of proteasome function might provide therapeutic benefit. VELCADE, (formerly know as PS-341), a potent and selective inhibitor of the proteasome, induced apoptosis in a variety of human tumor cells and in pre-clinical cancer animal models. In Phase I clinical trials, VELCADE demonstrated promising anti-tumor activity in multiple cancer types and is now in Phase II and III trials. To understand the potential contribution of proteasome function to the observed biological responses to VELCADE treatment, we adapted multiple myeloma cells to proliferate in high concentrations of the drug and generated stable cellular clones with greater than 500-fold resistance. The parental and VELCADE-adapted myeloma cell lines were subsequently grown as xenografts in mice to evaluate whether the *in vitro* adaptation translates into *in vivo* resistance. Surprisingly, VELCADE showed similar efficacy against tumors generated from both cell lines, suggesting that the tumor microenvironment might influence the efficacy of VELCADE. One parameter evaluated was the potential contribution of tumor hypoxia, a feature of rapidly growing solid tumors which is thought to contribute to the clinical failure of many chemotherapeutics. We found the adapted cells to no longer be resistant to VELCADE-induced apoptosis in hypoxia. Importantly, the parental myeloma cells as well as human lung and colon tumor cells were more sensitive to VELCADE-induced apoptosis under similar hypoxic conditions. These effects were specific for VELCADE, as other proteasome inhibitors and several standard chemotherapeutic agents did not demonstrate increased potency under hypoxic conditions. Since tumor cells often undergo cell cycle arrest under hypoxia, we examined whether VELCADE could induce apoptosis in non-cycling cells. Unlike other chemotherapeutics, VELCADE induced apoptosis equally well in G1-arrested and cycling colon tumor cells. This raises the possibility that VELCADE might work well in combination with hypoxia-inducing anti-angiogenic agents in solid tumors. These findings suggest that VELCADE represents a novel class of cancer therapeutics that offers significant advantages over other therapeutic modalities for the treatment of solid tumors.

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Determinants of AKT-dependent resistance to postmitochondrial apoptosis induction

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In the present study, we examined the molecular mechanisms underlying the chemoresistance of human cancer cells against the topoisomerase II inhibitor etoposide. Using pharmacological inhibitors and after adenoviral gene transfer of AKT signaling mutants, we found that inhibition of AKT activity rapidly and effectively sensitized cancer cells to etoposide-induced apoptosis. To identify the molecular targets of AKT-dependent chemoresistance, we first examined the release of mitochondrial apoptotic complementation factors after etoposide treatment or following AKT inhibition. By using cell fractionation techniques and immunofluorescence analysis, we measured the release of mitochondrial cytochrome c in cancer cell lines including LNCaP prostate carcinoma cells and observed that etoposide treatment alone caused mitochondrial damage independently of AKT inhibition. The subsequent activation of caspase-3/7 and apoptosis, however, was not induced efficiently unless AKT activity was also suppressed. Thus, we concluded that at least in some cancer cell lines, AKT inhibition was required to facilitate caspase activation by apoptogenic mitochondrial factors. To determine the mechanism underlying the AKT-dependent postmitochondrial resistance, we then examined whether downstream caspases were inhibited by direct AKT-dependent phosphorylation. By using transient transfection assays and *in vitro* reconstitution experiments using AKT-specific phosphorylation site mutants, we determined that the direct inhibition of caspases by AKT did not play a significant role in determining the AKT-dependent outcome after etoposide treatment. We also found that the AKT-dependent sensitization to apoptosis induction, even though it required ongoing macromolecular synthesis, did not depend on p53-dependent transcriptional activity. Supported in part by DAMD17-99-1-9153 and DAMD17-00-1-0214, and by the Speaker's Fund for Biomedical Research.

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Synergistic therapy of head and neck cancer with EGFR blockade and paclitaxel is mediated through abrogation of PI3kinase/AKT signaling

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Purpose: Survival for patients with oral cancer has not improved over the past 25 years and new approaches for treatment are needed. Targeted molecular therapy against epidermal growth factor receptor (EGFR) has shown promise as an adjuvant therapy in preliminary studies in several solid tumors, including head and neck cancer. The objective of this study is to determine the efficacy of paclitaxel and PKI166, a novel inhibitor of EGFR, in oral cavity cancer.
Experimental Design and Results: Using propidium iodide assay, JMAR human oral cancer cells were evaluated for the induction of apoptosis when treated with paclitaxel [0.001 to 0.1 mM] in the absence or presence of