Results: The aberrant nuclear BCL10 expression was detected in six (86%) of 7 H pylori-independent high-grade gastric MALT lymphomas but none in 13 H pylori-dependent cases (P<0.001). All six patients with nuclear BCL10 expression had co-expression of nuclear NF-kB, while only two of 13 patients without nuclear BCL10 expression did so (P=0.005). Interestingly, the latter two patients were found to have tumor invasion of the gastric muscularis propria. There was a significant correlation between nuclear expression of BCL10 and NF-kB activation (P<0.001). Furthermore, the nuclear co-localization of RelA and BCL10 was confirmed by confocal immunofluorescence microscopy. The frequency of nuclear translocation of RelA was also significantly higher in H pylori-independent tumors than those H pylori-dependent tumors (6 of 7=86% versus 2 of 13=15%, P=0.004).
Conclusion: The results of this study suggest that nuclear BCL10 expression is closely associated with the nuclear NF-kB expression and support the hypothesis that nuclear BCL10 may activate NF-kB. Detection of the nuclear expression of either BCL10 or NF-kB is highly useful in the prediction of H pylori-dependent state of early-stage high-grade gastric MALT lymphoma. (This work was supported by grants from NSC91-3112-B-002-009, NHRI-91A1-CANT-1 and NTUH 91-N007)

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Identification of survivin as a transcriptional target of the Wnt pathway

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Survivin, a member of the inhibitor of apoptosis (IAP) family, is found in most cancers while undetectable in normal terminally-differentiated cells. Expressed in mitosis, survivin has been shown to inhibit apoptosis in cancer cells. Its expression is predictive of poor outcome in colorectal cancer, the second largest cause of cancer deaths in the United States. Most colorectal cancers have mutations in the Wnt pathway, where inactivation of the gatekeeper adenomatous polyposis coli (APC) inhibits its ability to bind and regulate beta-catenin. This allows abnormally high levels of beta-catenin to complex with T-cell factor (TCF) and transcriptionally activate target genes of the Wnt pathway. The objective of this study is to test a potential association between survivin and Wnt-dependent oncogenesis. Analysis of the survivin promoter yielded three potential TCF binding elements (TBE). Plasmids were constructed with increasing lengths of survivin promoter inserted upstream of a luciferase reporter gene. Promoter analysis resulted in 8 to 12-fold increased transcriptional activity in colorectal cancer cell line HCT-116, which has active Wnt signaling, as compared to HeLa cells, which have an inactive Wnt pathway. Co-transfecting plasmids containing TCF sites with beta-catenin cDNA in HCT-116 cells resulted in 2.5 to 4-fold increased transcriptional activity. Mutagenesis of each of the two most proximal TBE sites showed at least a 75% reduction in transcriptional activity compared to the wild-type construct. Transfection with a dominant-negative TCF-4 plasmid resulted in a 50% decrease in transcriptional activity as compared to control plasmid. Radioactive probes corresponding to each of the three TBE sites were shifted by nuclear HCT-116 extracts and were fully competed by molar excess of unlabeled probes in electrophoretic mobility shift assays. Survivin staining by immunohistochemistry is nearly absent in TCF-4 knockout embryonic mouse intestine. These data identify survivin as a direct transcriptional target of the Wnt/beta-catenin pathway. The overexpression of survivin in colorectal cancer via Wnt activation may contribute to modulation of apoptosis during linear step-wise tumorigenesis.

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Involvement of proapoptotic molecules Bax and Bak in TRAIL-induced apoptosis

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TRAIL/Apo-2L is a member of the tumor necrosis factor superfamily and has been demonstrated to induce apoptosis in a wide variety of cancer and transformed cells. Activation of BID, a 'BH3-domain-only' Bcl-2 family member, triggers the oligomerization of proapoptotic family members BAK or BAX, resulting in the release of cytochrome c from mitochondria to cytosol. In this study, we have shown the importance of BAX and BAK in TRAIL-induced apoptosis by studying in murine embryonic fibroblasts (MEFs) from Bax-/- Bak-/- animals. TRAIL induced cytochrome c release and apoptosis in wild type, Bid-/-, Bax-/-, or Bak-/- MEFs, but not in Bax-/- Bak-/- double knockout (DKO) MEFs. Bid, which functions upstream of cytochrome c release, was found to be cleaved in all the knock out cells except in Bid-/- MEFs. The release of cytochrome c was correlated with caspase-9 activity. TRAIL increased caspase-3 activity in all the cells except the Bax-/- Bak-/- DKO cells. TRAIL-induced mitochondrial membrane potential reduction was not observed in Bid-/-, Bax-/-, and DKO MEFs suggesting that preventing a mitochondrial membrane potential reduction alone was not sufficient to prevent TRAIL-induced apoptosis. However, microinjection of cytochrome c in DKO MEFs induced apoptosis indicating events downstream of cytochrome c are intact. Together the data suggest that activation of Bax or Bak appears to be an essential gateway to mitochondrial dysfunction required for apoptosis in response to TRAIL.

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Inhibition of PI3 kinase causes cell death through a PKB dependent mechanism and growth arrest through a PKB independent mechanism

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PTEN is one of the most commonly inactivated genes in human cancer. The mechanism by which PTEN exerts its tumor-suppressive effects has been elucidated. PTEN regulates the activity of the PI3K pathway. PI3K transduces cellular signals by phosphorylating the 3' position of phosphoinositides thus generating the second messengers PIP2 and PIP3. PTEN antagonizes this signal by dephosphorylating the 3' position of PIP3 and PIP2. PIP3 and PIP2 activate downstream effectors of PI3K. One effector of PI3K in this pathway is PKB. PKB mediates a discrete set of responses including cell survival,proliferation, migration and angiogenesis, implicating the PI3K/PKB pathway in cancer development. LNCaP is a human prostate carcinoma cell line isolated from lymph node metastasis. LNCaP is commonly used as a model for human prostate cancer because the cells are hormone dependent and express PSA. LNCaP cells have a mutation in the PTEN gene and subsequent activation of PKB. Introduction of wt PTEN into LNCaP cells, or treatment with the PI3K inhibitor LY294002, causes inactivation of PKB, growth arrest and apoptosis. While treatment with LY294002 causes inactivation of PKB and apoptosis the exact mechanism of apoptosis has not been elucidated. To specifically test the hypothesis that inhibition of PKB is responsible for the LY294002-induced apoptosis, LNCaP cells expressing a constitutively active form of PKB were generated. PKB is activated by phosphorylation on ser-473 and thr-308. Mutating these residues to aspartic acid (S473D/T308D) mimicsphosphorylation and results in activation of PKB. PKB activity is also regulated by the PH domain. Deletion of the PH domain (∗PH) leads to increased activity. Combining the S473D/T308D mutant with the ∗PH mutant generates a constitutively active PKB (∗PH-PKB-DD). The ∗PH-PKB-DD form of PKB cannot be inhibited by LY294002. LNCaP cells expressing the ∗PH-PKB-DD were generated. Activity of the ∗PH-PKB-DD protein and the inability to be inhibited by LY294002 will be confirmed by western blot analysis. Apoptosis and growth arrest were measured in the LNCaP ∗PH-PKB-DD cells and compared with control cells expressing vector alone. Both cell lines were treated with LY294002 and proliferation and cell death were measured. Expressing constitutively active PKB in LNCaP cells abrogated apoptosis induced by LY294002 but had no effect on the G1 arrest. These data identify PKB as a potential critical target for cancer therapy.

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Claudin-1 induced apoptosis in breast tumor spheroids

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Members of the claudin family together with occludin are the major constituents of the tight junction complex (TJ). The human homologue of the murine CLDN1, previously called SEMP1, was identified by molecular genetic analysis, and the CLDN1 mRNA was found to be downregulated or completely lost in human breast cancer cells *in vitro*. In this study we investigated the role of CLDN1 in CLDN1-transduced MDA-MB 361 breast tumor cells in adherent 2D and suspension 3D spheroid cell cultures. Retroviral-induced CLDN1 re-expression in breast cancer cells results in plasma membrane homing of the protein and reconstitution of paracellular flux inhibition, which is not dependent upon presence of occludin protein. There was

no difference in proliferation and cell death characteristics in proliferating, adherent monolayer cell cultures of CLDN1-positive compared to control CLDN1-negative and mock-transduced cell cultures. MDA-MB 361 parental cells exhibited no changes in cell death induction either in 2D monolayer or in 3D spheroid cell cultures. In contrast, clonal CLDN1-transduced derivatives displayed a significant elevation of apoptosis which became evident as early as 2 days after 3D spheroid culture onset. The clonal MDA-MB 361 CLDN1-positive cultures which exhibited a more prominent cell membrane localization showed a pronounced increase of apoptosis in tumor spheroids up to 8-fold over the CLDN1-negative control. In parallel, inhibition of the paracellular flux rate was observed. These findings support the potential role of the TJ protein CLDN1 in breast cancer cells to restrict nutrient and growth factor supplies, and indicate that the loss of the cell membrane localization of the TJ protein CLDN1 in carcinomas is likely a crucial step during the process of tumorigenesis.

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DNA damage induces a novel p53-survivin signaling pathway regulating cell cycle and apoptosis in acute lymphoblastic leukemia cells

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Survivin is a novel member of the inhibitor of apoptosis protein (IAP) family. Here we report that the chemotherapeutic drug doxorubicin, a DNA-damaging agent, activates a p53-survivin signaling pathway inducing cell cycle arrest and apoptosis in childhood acute lymphoblastic leukemia (ALL). Treatment of wild-type (wt) p53 ALL cells (EU-3 cell line) with doxorubicin caused accumulation of p53, resulting in dramatic downregulation of survivin, depletion of cells in G2/M and apoptosis (increased sub-G1 compartment). In contrast, doxorubicin treatment of mutant (mut) p53 cells (EU-6/ALL line) upregulated survivin and induced G2/M arrest without inducing apoptosis. However, treating EU-6 with anti-survivin antisense re-sensitized these cells to doxorubicin, resulting in apoptosis. With a p53-null cell line (EU-4), although doxorubicin treatment arrested cells in G2/M, survivin expression was unchanged, and cells underwent only limited apoptosis. However, re-expression of wt-p53 in EU-4 cells could restore the doxorubicin-p53-survivin pathway, resulting in significantly decreased survivin expression and increased apoptosis in these cells after doxorubicin treatment. Following cotransfection of p53-null EU-4 cells with survivin promoter-luciferase constructs and either wt-p53 or different mut-p53 expression vectors, wt-p53 inhibited survivin promoter activity; p53-mediated inhibition could be abrogated by overexpression of MDM2 protein. Together, these studies define a novel p53-survivin signaling pathway activated by DNA damage that results in downregulation of survivin, cell-cycle arrest and apoptosis. Furthermore, our data indicate that loss of wt-p53 function in tumor cells may contribute to upregulation of survivin and resistance to DNA-damaging agents.

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Small molecule inhibitors of BCL-2 protein-protein interactions show anti-tumor activity in nude mice

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Oncogenes are potent activators of the apoptotic response and cells must abrogate the cell death response in order to survive and form tumors. Many cancers achieve this through upregulation of the BCL-2 family dominant suppressors of apoptosis. These proteins function through protein-protein interactions to ablate the oncogene-induced pro-apoptotic BAX/BAK mediated release of signals from mitochondria, resulting in activation of caspases. As previously shown, the prodigiosin GX01 compound series (Mr < 400) inhibit BCL-2/BAX interactions, and while these compounds readily enter both normal and cancer cells, the cancer cells show a significantly greater apoptotic response at low nM concentrations. Here we show that when Jurkat cells were treated with 100 nM GX015-000, loss of cell viability as measured by ViaLight ATP detection was seen over a period of 72 hours. Consistent with a BCL-2 inhibition, cell cycle analysis by flow cytometry did not reveal any major accumulation at a distinct cell cycle phase, suggesting that GX015 does not cause a cell cycle arrest. At the 72 hour point, there were a large number of apoptotic sub-diploid cells and degraded cell debris was apparent. To determine the anti-cancer activity of GX015 compounds, murine xenograft tumor models were used. Nude mice (15 mice per group) were injected subcutaneously with C33-A human cervical carcinoma cells or human prostatic PC3 carcinoma cells, both of which over-express BCL-2 family members. Once the tumors became palpable, an oil emulsion of GX015-000 was administered subcutaneously at a site distal from the tumor. The mice tolerated doses up to 10 mg/kg per day and doses equal to 3.4 mg/kg per day or greater inhibited tumor growth. The relative hydrophobicity of GX015-000 (log P = 6.4) suggested that adequate tumor cell coverage in xenograft models might be difficult to achieve for optimal tumor regression. Accordingly, GX015-000 was found to be 99% bound to plasma protein. To address this issue, less lipophilic GX015 compounds were identified. GX015-003 has a Log P of 3.2 and only 1% of this compound is bound to plasma proteins. GX015-003 can effectively (IC_{50} in C33A cells of 40 nM) and selectively induce apoptosis in cancer cells as measured by caspase activation. GX015-003 is being tested for anti-cancer activity in nude mice bearing C33A subcutaneous tumors. The pharmacokinetic profile of GX015-003 in comparison to GX015-000 is also being determined.

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Discovery of gambogic acid and derivatives as apoptosis-inducing natural products with novel mechanism of action and potent *in vivo* anti-tumor activity

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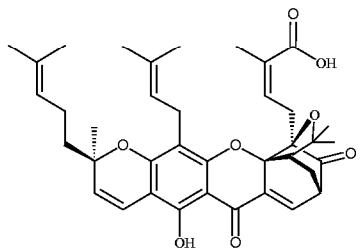
Apoptosis is a physiological form of cell death controlled by a family of cysteine proteases called caspases. Aberrant apoptosis is not only the major cause for tumor development and progression, but also plays a significant role in drug resistance to conventional treatments. Therefore, discovery of novel inducers of apoptosis provides a new therapeutic approach to anti-cancer agents. Here we report the discovery of gambogic acid (MX2060), a natural product isolated from gamboge, as a novel inducer of apoptosis in different cancer cell lines, including breast, prostate, and other cell lines. Treated cell lines also exhibited a lack of clonogenic survival. Caspase activation (EC50), ranges from 0.5 μM to 1.5 μM for different cell lines. The signaling pathway includes activation of the upstream caspase 8, as well as involvement of mitochondria and release of cytochrome c, thus engaging both the intrinsic and extrinsic pathways of cell death. Mitochondria play an important role in the regulation of apoptosis and an early event in this pathway is the release of apoptogenic cytochrome c from the mitochondria into the cytosol that is inhibited by the anti-apoptotic Bcl2 family members. MX2060-induced cell death is delayed, but not inhibited by, Bcl2 suggesting its effectiveness in tumors overexpressing Bcl2. Cells treated with MX2060 undergo apoptosis with cleavage of all key caspase substrates and rapid cell death independent of the cell cycle stage, which may offer an advantage over some of the current chemotherapeutic drugs. MX2060 also exhibited a pharmaceutically acceptable pharmacokinetic profile following i.v. administration. In order to understand the Structure Activity Relationship (SAR) and improve the chemical and pharmacological properties of MX2060, we have designed and synthesized many derivatives. We have found that MX2060 and its derivatives have good *in vivo* efficacy in mouse xenograft models. We will report the key SAR, *in vitro* and *in vivo* characterization of MX2060 and its derivatives.



In summary, MX2060 represents a new chemotype whose apoptosis-inducing antitumor activity appears to be mediated by a molecular mechanism different from the action of traditional cancer drugs. The therapeutic implications of MX2060 will be discussed.