Journal of Cellular Biochemistry 92:646–650 (2004)

PROSPECTS

# Blocking Anti-Apoptosis as a Strategy for Cancer Chemotherapy: NF-κB as a Target

**N.R. Monks,[1] D.K. Biswas,[1,2] and A.B. Pardee[1]***

[1]Dana-Farber Cancer Institute, Department of Medical Oncology, Harvard Medical School, Boston, Massachusetts 02115

[2]Department of Cancer Biology, Harvard Medical School, Boston, Massachusetts 02115

**Abstract** Critical processes underlying cancers must be better understood to develop strategies for treatment and prevention. A chemotherapeutic strategy is proposed that is based upon re-establishment, with a drug, of nullified programmed cell death (apoptosis) in cancer cells, which to survive have mutated to block apoptosis. A chemotherapy that is specific against tumors implanted in mice demonstrated the feasibility of this principle. This therapy is specific because it affects a process unique to cancer cells. It also has the advantage of killing these cells, in contrast to reversibly blocking their proliferation. The anti-apoptotic transcription factor NF-κB provides a potential therapeutic target in estrogen receptor negative (ER−) breast cancers that over-express the epidermal growth factor family of receptors (EGFR). Further investigations of the pathways utilize dominant negative protein inhibitory peptide, and small inhibitory RNAs (siRNAs) to block the production of relevant enzymes. J. Cell. Biochem. 92: 646–650, 2004. 

**Key words:** apoptosis; NF-κB; inhibitors; Go6976; siRNA

## Cell Biology

Both cell proliferation and apoptosis are important for cancer growth [Lin and Karin, 2003]. These processes are intertwined; aberrant proliferation activates tumor surveillance and apoptosis. Growth factors such as excess heregulin, transcription factor E2F1, oncogenic ras, and compounds such as retinoids can increase or decrease both proliferation and apoptosis. Cells die under conditions of stress, based upon pathways linked to the cell cycle [Vermeulen et al., 2003]. In cancers, the cells are metabolically unbalanced, and initially many die; survivors are mutants that developed anti-apoptotic properties [Weinstein, 2000]. An example is the very frequent loss of apoptotic p53; others are cancer-specific increases in Bcl-2, the inhibitor-of-apoptosis protein survivin [Altieri, 2003], and increases of heat shock proteins [Nylandsted et al., 2000]. Overcoming anti-apoptosis can thus be the basis of therapy [Kim et al., 2002; Orlowski and Baldwin, 2002; Cusack, 2003a]. Compounds such as β-lapachone, geldanamycin, and Go6976 that selectively block anti-apoptosis (or reinstate apoptosis) selectively cause cancer cell death.

An initial demonstration of this principle of a drug which extirpates tumors in vivo is as follows. Mouse mammary epithelial carcinoma cells (CSMLO) subcutaneously implanted in syngeneic mice grew into large tumors which shrank dramatically after i.v. injection with the protein kinase C (PKC-α/β) inhibitor Go6976, a nonglycosidic indolcarbazol analog of staurosporine. The tumors did not reappear during extended treatment, and the action was tumor-specific because the mice exhibited no deleterious effects [Biswas et al., 2001, 2003]. As confirmation, CSMLO cells stably expressing a dominant negative mutant protein (dnIKKβ) showed reduced and delayed tumor formation as compared to parental cells or cells plus vector alone.

The anti-apoptotic phenotype of breast cancer cells correlates with interaction of epidermal growth factor (EGF) with its over expressed

Grant sponsor: Department of Defense; Grant number: DAMD17-02-1-0692.

*Correspondence to: Prof. A.B. Pardee, Dana-Farber Cancer Institute, 1 Jimmy Fund Way, Boston, MA 02115. E-mail: Arthur_Pardee@dfci.harvard.edu

Received 29 January 2004; Accepted 4 February 2004

DOI 10.1002/jcb.20080

receptor (EGFR+) in estrogen receptor negative (ER−) human and mouse adenocarcinoma cells in culture [Sovak et al., 1997; Biswas et al., 2000]. The DNA binding of anti-apoptotic NF-κB is activated by EGF [Biswas et al., 2000]. Go6976 decreased NF-κB activation, and the cells subsequently underwent apoptosis. Gene expression profiling using microarray analysis confirmed that inhibition of NF-κB activation by Go6976 decreases the expression of anti-apoptotic genes and increases expression of genes associated with the apoptotic phenotype [Biswas et al., 2003]. Go6976 and dominant negative PKC-α block transformation of EGFR expressing cells [Hornia et al., 1999]. These experiments demonstrate the feasibility of rationally targeted therapies with the aim of activating apoptosis via the negation of an anti-apoptotic signaling pathway.

## Molecular Mechanisms

Apoptosis and its several signaling pathways are often decreased in cancers [Zhivotovsky and Orrenius, 2003]. Of particular interest here is the increased NF-κB found in cancer cells, which is reported to block apoptosis. This is especially influential for cancers that over express EGFR (ErbB1) or ErbB2, as compared to those that over express the ER. Serum primarily activates the MAPK pathway in ErbB1 over expressing cells, and the Akt pathway in ErbB2 over expressing cells [Tari and Lopez-Berestein, 2000]. These receptors and their downstream kinases are often over expressed in ER− breast tumors [Romieu-Mourez et al., 2002].

We demonstrated that DNA binding activity and transactivation of NF-κB responsive genes can be stimulated by EGF-EGFR interaction in ER− human and mouse mammary epithelial adenocarcinoma cells in culture. Interaction of EGF with its receptor ErbB1 activates cell proliferation and also blocks death signals [Navolanic et al., 2003]. The signaling cascade includes PKC which is upstream of and activates IκB kinase (IKK), a complex composed of three subunits IKKα, IKKβ, and IKKγ/Nemo. NF-κB is complexed with the inhibitory protein IκB and maintained in an inactive state in most cell types. IKK phosphorylates IκB which is then ubiquitinated and hydrolyzed by proteasomes. NF-κB is then released to enter the nucleus, where it is further regulated by phosphorylation, acetylation, and interactions with coactivators and corepressors to transcribe both anti-apoptotic and proliferative genes [Nakshatri and Goulet, 2002].

The NF-κB/Rel transcription factor family regulate proliferation, survival, and transformation. They are heterodimeric complexes, of which the most predominantly detected is the p50/p65 (RelA) complex. NF-κB is ubiquitously expressed and its activity is tightly controlled in normal cells. Its basal level can be activated by several signaling mechanisms, a variety of extracellular signals such as chemotherapeutic drugs, inflammatory cytokines, mitogens, growth factors, bacterial and viral infections, irradiation, and oxidative stress. It can transactivate over 200 target genes, involved in cellular processes that include anti-apoptosis (Bcl-2 and survivin), proliferation (cyclin D1), immune response, and adhesion/metastasis (uPA, integrin) [Lin and Karin, 2003].

Heterodimerized ErbB2/ErbB3 (Her2/neu), another EGF family receptor, is activated by heregulin [Stoica et al., 2003]. Over expression of ErbB2 is detected in about 30% of ER− human breast cancers. The prognosis of these patients is poor, although current therapies with Herceptin, an anti-Her2/neu antibody, in combination with other chemotherapeutic agents show some if limited success. ErbB2 activates NF-κB via signaling that includes PI3-kinase, PDK1, Akt, protein kinase 2 (CK2) [Litchfield, 2003] and CKBBP1. Akt generates anti-apoptosis [Hill and Hemmings, 2002]. These pathways interconnect, in that PKC inhibits the Akt survival pathway [Tanaka et al., 2003], as do the ERα and Akt pathways [Stoica et al., 2003].

## Tests of the Model

Our original hypothesis is that elevated NF-κB in tumors provides a difference specific to cancer cells that suggests it as a target for drug therapy. NF-κB is an attractive target because it often prevents apoptosis. Go6976 might nullify this anti-apoptosis by inhibiting PKC and thereby IKK activity; then IκB is not phosphorylated nor degraded, NF-κB activation is blocked and decreases because of its short half life. NF-κBs transcription of anti-apoptotic genes is thereby prevented, increasing apoptosis.

Consistent with our results, Go6976 inhibits PKC-α/β and enhances apoptosis, as do GF109203X and Ro318425 which are also selective inhibitors of PKC-α. They also enhanced

heregulin-induced apoptosis of SkBr3 cells that over express Her2. This action is via down regulation of Bcl-2 and activation of caspases-7 and -9, and poly-ADP-ribose polymerase cleavage [Le et al., 2002]. Unlike Go6976, several other PKC inhibitors did not protect neurons from cytotoxicity, suggesting Go6976 might alternatively block other enzymes [Jeohn et al., 2000].

We are currently exploring the effects of specific inhibition of NF-κB activation in cancer cells by small inhibitory RNA (siRNA) targeted at the IKK complex in ER− human breast cancer cells in culture. This approach is in principle like the stable expression of dnIKKβ mutant protein. Both are believed to block IKKβ activity and should thereby down-regulate NF-κB activation and permit apoptosis.

We have targeted two of the three proteins in the IKK complex. IKKβ (IKK2) is a kinase which activates NF-κB by phosphorylating the inhibitory protein IkBα at serine residues 32 and 36, causing its dissociation from the p65/p50 complex (the primary NF-κB heterodimer), and subsequent degradation by the 26S proteasome. IKKγ (Nemo) is the regulatory protein that is considered to control the kinase activities of both IKKα and β. In initial studies with the MDA-MB-231 ER− human mammary adenocarcinoma cell line we have successfully knocked down both IKK-β and IKK-γ with siRNAs. They could be reduced by greater than 85 and 98%, respectively. Surprisingly, we observed no significant changes in the level of active nuclear NF-κB, growth characteristics, or apoptosis.

These observations suggests that other pathways are involved in the activation of NF-κB. Other kinases can also phosphorylate IκB [Bennett et al., 1996]. Highly purified PKC preparations did not phosphorylate IκBα, whereas a crude preparation that contains contaminating CK2 did so [Janosch et al., 1996]. CK2 is constitutively active and it constitutively phosphorylates IκBα in murine tumor cells. Inhibitors of CK2 induce apoptosis of Jurkat cells [Meggio and Pinna, 2003]. PI3-Kinase also directly interacts with IκBα and contributes to the phosphorylation of p65, thereby by-passing the IKK complex [Kang et al., 2003]. Decreased NF-κB does not always cause apoptosis, perhaps because of other survival mechanisms in some tumor cells [Orlowski and Baldwin, 2002]. We are currently investigating these pathways with respect to the constitutive activity of NF-κB in MDA-MB-231 and other cells, either alone or in synergy/conjunction with the IKK complex.

## DISCUSSION

The primary goal of this study is to define a target-directed drug therapy for ER− human breast cancer patients, by blocking activation of NF-κB. The rationale for choosing NF-κB activation as a potential therapeutic target for ER− human breast cancers is based on its over expression in many cancers. Our results identified the importance of rationally targeted therapies with the aim of activating apoptosis via the inactivation of an anti-apoptotic signaling pathway, in particular leading to activation of NF-κB.

More than 100 compounds including glucocorticoids, proteasome inhibitors, and PPARγ antagonists decrease NF-κB activity [Nakshatri and Goulet, 2002]. It should be mentioned that some inhibitors of NF-κB are toxic due to effects independent of NF-κB [Pozarowski et al., 2003]. Some of them might prove to be therapeutic or preventive [Lin and Karin, 2003]. For example, genistein, an isoflavone, inhibits the Akt pathway and the activation of NF-κB; it is apoptotic [Sarkar and Li, 2002]. Many anticancer drugs cause apoptosis, but the increased NF-κB which they also produce might prevent death. Synergies could exist between apoptotic agents and inactivators of NF-κB [Weaver et al., 2003].

With this in mind, studies are in progress on cancer therapy with proteasome inhibitors [Kisselev and Goldberg, 2001] such as PS-341 (Velcade, Bortezomid), alone or in combination with other drugs [Voorhees et al., 2003; Cusack, 2003b]. One of the proposed mechanisms of action is based upon inhibition of IκB proteolysis, with consequent decrease of active NF-κB and activation of apoptosis. This principle has been demonstrated using a combination of lactacystin, a natural proteasome inhibitor, and doxorubicin which together demonstrated increased levels of apoptosis than when used alone [Tergaonkar et al., 2003]. These studies support the investigation of NF-κB as a target for chemotherapy.

An important question that has arisen from these investigations suggests caution in applying siRNA in the discovery of new drug targets and it's role in deciphering the mechanisms

underlying the activity of new, supposedly specific, small molecule inhibitors. Go6976 can effectively block the stimulation of NF-κB by EGF in the ER− setting, and more importantly demonstrated striking anti-tumor activity in vivo [Biswas et al., 2001, 2003]. Contrarily, specific inhibition of IKKβ and γ by siRNA, without induction of cell death suggests either that the inhibition of PKC-α and -β by Go6976 has an effect on other pathways that culminate in NF-κB inhibition, or alternatively also inhibits other kinases that, in conjunction with PKC inhibition, result in the observed anti-tumor effects. The complexity of drug effects and their low specificity [Orlowski and Baldwin, 2002] versus the high specificity of siRNA might be involved. Completeness of the effect depends on siRNA stability, time of exposure, variability of inhibition of IKK and NF-κB between cell lines, extent of apoptosis signaling mechanisms, etc. Also, depleting a protein with siRNA may produce effects differing from inhibiting its catalytic activity. Apoptosis created from blocking NF-κB activation may also depend on activating a pro-apoptotic signal. In conclusion, one needs to be cautious with siRNA with regard to predictions regarding the molecular pathways of drug actions.

Drugs are often recognized to modulate several processes. 7-hydroxystaurosporine (UCN-01), an anticancer drug, potently inhibits several kinases including PKC and PDK1 [Komander et al., 2003]. Go6976 is also a potent inhibitor of DNA damage-induced cell cycle checkpoints [Kohn et al., 2003]. Inhibition of NF-κB transforms deacetylase inhibitor-induced growth arrest to apoptosis [Dai et al., 2003]. CK2 that both phosphorylates IκB and activates cytoplasmic NF-κB has more than 300 substrates [Meggio and Pinna, 2003]. Additional drug effects are usually deleterious, e.g., causing host toxicity. But rarely, a drug might modulate more than one desirable target, and could thereby create a synergistic effect. Such results were observed with microarray: Go6976 caused numerous changes of apoptotic genes' expression, which differed from those produced in dnIKKβ-mutant cells [Biswas et al., 2003]. Such a "Drug Reinforcement" could be therapeutically beneficial.

## REFERENCES


Altieri DC. 2003. Survivin and apoptosis control. Adv Cancer Res 88:31–52.

Bennett BL, Lacson RG, Chen CC, Cruz R, Wheeler JS, Kletzien RF, Tomasselli AG, Heinrikson RL, Manning AM. 1996. Identification of signal-induced IkappaB-alpha kinases in human endothelial cells. J Biol Chem 271:19680–19688.

Biswas DK, Cruz AP, Gansberger E, Pardee AB. 2000. Epidermal growth factor-induced nuclear factor kappa B activation: A major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. Proc Natl Acad Sci USA 97:8542–8547.

Biswas DK, Dai SC, Cruz A, Weiser B, Graner E, Pardee AB. 2001. The nuclear factor kappa B (NF-kappa B): A potential therapeutic target for estrogen receptor negative breast cancers. Proc Natl Acad Sci USA 98:10386–10391.

Biswas DK, Martin KJ, McAllister C, Cruz AP, Graner E, Dai SC, Pardee AB. 2003. Apoptosis caused by chemotherapeutic inhibition of nuclear factor-kappa B activation. Cancer Res 63:290–295.

Cusack JC, Jr. 2003a. Overcoming antiapoptotic responses to promote chemosenstivity in metastatic colorectal cancer to the liver. Ann Surg Oncol 10:852–862.

Cusack JC. 2003b. Rationale for the treatment of solid tumours with the proteasome inhibitor bortezomib. Cancer Treat Rev 29 (Suppl 1):21–31.

Dai Y, Rahmani M, Grant S. 2003. An Intact NF-kappaB Pathway is required for histone deacetylase inhibitor-induced G1 arrest and maturation in U937 human myeloid leukemia cells. Cell Cycle 2:467–472.

Hill MM, Hemmings BA. 2002. Inhibition of protein kinase B/Akt. Implications for cancer therapy. Pharmacol Ther 93:243–251.

Hornia A, Lu Z, Sukezane T, Zhong M, Joseph T, Frankel P, Foster DA. 1999. Antagonistic effects of protein kinase C alpha and delta on both transformation and phospholipase D activity mediated by the epidermal growth factor receptor. Mol Cell Biol 19:7672–7680.

Janosch P, Schellerer M, Seitz T, Reim P, Eulitz M, Brielmeier M, Kolch W, Sedivy JM, Mischak H. 1996. Characterization of IkappaB kinases. IkappaB-alpha is not phosphoryated by Raf-1 or protein kinase C isozymes but is a casein kinase II substrate. J Biol Chem 271: 13868–13874.

Jeohn GH, Wilson B, Wetsel WC, Hong JS. 2000. The indolecarbazole Go6976 protects neurons from lipopolysacccharide/interferon-gamma-induced cytotoxicity in murine neuron/glia co-cultures. Brain Res Mol Brain Res 23:32–44.

Kang JL, Lee HS, Pack IS, Hur KC, Castranova V. 2003. Phosphoinositide 3-kinase activity leads to silica-induced NF-kappaB activation through interacting with tyrosine-phosphorylated I(kappa)B-alpha and contributing to tyrosine phosphorylation of p65 NF-kappaB. Mol Cell Biochem 248:17–24.

Kim R, Tanabe K, Uchida Y, Emi M, Inoue H, Toge T. 2002. Current status of the molecular mechanism of anticancer drug-induced apoptosis. The contribution of molecular-level analysis to cancer chemotherapy. Cancer Chemother Pharmacol 50:343–352.

Kisselev AF, Goldberg AL. 2001. Proteasome inhibitors: From research tools to drug candidates. Chem Biol 8: 739–758.

Kohn EA, Yoo CJ, Eastman A. 2003. The protein kinase C inhibitor Go6976 is a potent inhibitor of DNA damage-

induced S and G2 cell cycle checkpoints. Cancer Res 63:31–35.

Komander D, Kular GS, Bain J, Elliott M, Alessi DR, Van Aalten DM. 2003. Structural basis for UCN-01 (7-hydroxystaurosporine) specificity and PDK1 (3-phosphoinositide-dependent protein kinase-1) inhibition. Biochem J 375:255–262.

Le XF, Varela CR, Bast RC, Jr. 2002. Heregulin-induced apoptosis. Apoptosis 7:483–491.

Lin A, Karin M. 2003. NF-kappaB in cancer: A marked target. Semin Cancer Biol 13:107–114.

Litchfield DW. 2003. Protein kinase CK2: Structure, regulation, and role in cellular decisions of life and death. Biochem J 369:1–15.

Meggio F, Pinna LA. 2003. One-thousand-and-one substrates of protein kinase CK2? FASEB J 17:349–368.

Nakshatri H, Goulet RJ, Jr. 2002. NF-kB and breast cancer. Curr Probl Cancer 26:282–309.

Navolanic PM, Steelman LS, McCubrey JA. 2003. EGFR family signaling and its association with breast cancer development and resistance to chemotherapy (Review). Int J Oncol 22:237–252.

Nylandsted J, Brand K, Jaattela M. 2000. Heat shock protein 70 is required for the survival of cancer cells. Ann NY Acad Sci 926:122–125.

Orlowski RZ, Baldwin AS, Jr. 2002. NF-kappaB as a therapeutic target in cancer. Trends Mol Med 8:385–389.

Pozarowski P, Halicka DH, Darzynkiewicz Z. 2003. Cell cycle effects and caspase-dependent and independent death of HL-60 and Jurkat cells treated with the inhibitor of NF-kappaB parthenolide. Cell Cycle 2:377–383.

Romieu-Mourez R, Landesman-Bollag E, Seldin DC, Sonenshein GE. 2002. Protein kinase CK2 promotes aberrant activation of nuclear factor-kappaB, transformed phenotype, and survival of breast cancer cells. Cancer Res 62:6770–6778.

Sarkar FH, Li Y. 2002. Mechanism of cancer prevention by soy isoflavone genistein. Cancer Metastasis Rev 21: 265–280.

Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, Traish AM, Sonenshein GE. 1997. Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. J Clin Invest 100:2952–2960.

Stoica GE, Franke TF, Wellstein A, Morgan E, Czubayko F, List HJ, Reiter R, Martin MB, Stoica A. 2003. Heregulin-beta1 regulates the estrogen receptor-alpha gene expression and activity via the ErbB2/PI 3-K/Akt pathway. Oncogene 22:2073–2087.

Tanaka Y, Gavrielides MV, Mitsuuchi Y, Fujii T, Kazanietz MG. 2003. Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. J Biol Chem 278:33753–33762.

Tari AM, Lopez-Berestein G. 2000. Serum predominantly activates MAPK and Akt kinases in EGFR- and ErbB2-over-expressing cells, respectively. Int J Cancer 86:295–297.

Tergaonkar V, Bottero V, Ikawa M, Li Q, Verma IM. 2003. IkappaB Kinase-Independent IkappaBalpha degradation pathway: Functional NF-kappaB activity and implications for cancer therapy. Mol Cell Biol 23:8070–8083.

Vermeulen K, Berneman ZN, Bockstaele DR. 2003. Cell cycle and apoptosis. Cell Prolif 36:165–175.

Voorhees PM, Dees EC, O'Neil B, Orlowski RZ. 2003. The proteasome as a target for cancer therapy. Clin Cancer Res 9:6316–6325.

Weaver KD, Yeyeodu S, Cusack JC, Jr., Baldwin AS, Jr., Ewend MG. 2003. Potentiation of chemotherapeutic agents following antagonism to nuclear factor kappa B in human gliomas. J Neurooncol 61:187–196.

Weinstein IB. 2000. Disorders in cell circuitry during multistage carcinogenesis: The role of homeostasis. Carcinogenesis 21:857–864.

Zhivotovsky B, Orrenius S. 2003. Defects in the apopototic machinery of cancer cells: Role in drug resistance. Sem Cancer Biol 13:125–134.