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Targeting the RAF/MEK/ERK, PI3K/AKT and P53 pathways in hematopoietic drug resistance

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Introduction

Biology and Unanswered Questions Regarding Acute Myeloid Leukemia (AML)

Cancer remains the second leading cause of death in the USA despite recent advances in treatment of patients with anti-neoplastic drugs. Approximately 42,000 people in the USA die each year from leukemias and lymphomas which represent 10% of all cancer deaths. Approximately 11,000 Americans will be diagnosed with AML this year, and about 75% will eventually die from this disease. While improvements in the outcomes have been observed with young patients with AML over the past 40 years, progress in the treatment of older AML patients has not been as significant (Tallmann *et al*., 2005). Fifty to 75% of adults with AML achieve complete remission with a combination chemotherapy which consists of combination of the deoxycytidine analogue cytarabine and an anthracycline antibiotic (doxorubicin, daunorubicin, idarubicin or the anthracenedione mitoxantrone, which inhibit the enzyme topoisomerase IIa). However, this treatment is not always effective as only approximately 25% of these patients enjoy long term survival (Tallmann *et al*., 2005). The incidence of AML increase with age, 1.2 cases per 100,000 at age 30 and greater than 20 cases per 100,000 at age 80 (Tallmann *et al*., 2005). Unfortunately the outcome decreases with age. As the average life span of Americans increases due to improvements in health care and life styles, AML will be an increasing problem in American health care.

While approximately 50% of AML cases have genetic aberrations which can be identified (*e.g.*, deletions such as 5q-, translocations such as t(8;21) AML-ETO, or duplications such as

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Flt-3 internal tandem duplication [ITD]), the other 50% do not have currently identifiable genetic mutations (Tallmann *et al*., 2004). Unlike chronic myelogenous leukemia (CML) where the BCR-ABL translocation is present in virtually all patients and the majority of the patients are sensitive to Imatinib, treatment with a targeted "upstream" inhibitor (*e.g*., Flt-3 inhibitor) would be ineffective in many AML cases. In summary, AML remains a difficult disease to treat due in part to its genetic diversity.

Upregulation of the Ras>Raf>MEK>ERK and PI3K>Akt pathways and phosphorylation of the downstream target Bad are observed frequently in AML specimens and associated with a poorer prognosis than patients lacking these changes (Kornblau *et al*., 2006; Martelli *et al*., 2206). Aberrant expression of a single pathway is associated with a poor prognosis and abnormal expression of multiple signaling pathways is associated with an even worse prognosis (Kornblau *et al*., 2006). Flt-3 ITD mutations have been detected in 20% of AMLs and these patients have a poorer prognosis than patients lacking these mutations (Stone *et al*., 2004). Dysregulation of the Ras>Raf>MEK>ERK and PI3K>Akt pathways in some AMLs may result from constitutive activation of Flt-3 (Birkenkamp *et al*., 2004; Stirewalt *et al*., 2003; Meshinchi *et al*., 2003). Thus these two signaling pathways provide important clues regarding the mechanisms responsible for autonomous AML growth (Yokota *et al*., 1998; Hoelzer *et al*., 2000; Pui *et al*., 1999; Attwell *et al*., 2003; McKearn *et al*., 1985). Targeting these "downstream" pathways may prove effective for AML therapy, especially in those cases where the precise mutation responsible for malignant transformation is unknown.

Drug Resistance and AML

A frequent side effect of treatment of AML patients with chemotherapeutic drugs is the development of drug resistance. After chemotherapeutic drug treatment, drug resistant cells arise which exhibit enhanced efflux of chemotherapeutic drugs (Tallmann *et al*., 2005Tallmann *et al*., 2006;Burnett *et al*., 2006) Furthermore, the drug resistant cells often exhibit multi-drug resistance as they are resistant to multiple chemotherapeutic drugs which are structurally unrelated. In some cases, this phenomenon has been shown to be due to the increased expression of membrane transporters (Gottesman *et al*., 2002; Norgaard *et al*., 2004; van den Heuvel-Eibrink *et al*., 2000; Ross 2000; Kruh *et al*., 2003). These transporters belong to a large family of proteins which contains an ATP binding cassette (ABC) domain. Multi-drug resistance protein (Mdr-1 *a.k.a*., P glycoprotein, Pgp) was one of the first of these molecules to be identified to have a role in drug resistance. Subsequently, additional proteins with this ABC domain were identified and determined to have a role in drug resistance. This family includes: breast cancer resistance protein (BCRP-1), multi drug resistant protein (MRP), MRP1, MRP2, MRP3, MRP4, MRP5, MRP6, MRP7, MRP8 as well as some other proteins. Inhibitors to some of these membrane transporters have been developed and evaluated in clinical trials. Unfortunately, these clinical trials have not yet yielded support for inclusion of these inhibitors in drug resistance therapy (Teodori *et al*., 2006; Polgar *et al*., 2005; Ross 2004; Mahadevan *et al*., 2004). An alternative approach could be to target the growth and survival pathways which become activated in the drug resistant cells. Two pathways frequently implicated in drug resistance are Raf>MEK>ERK and PI3K>Akt (Steelman *et al*., 2004; Lee *et al*., 2002; Osaki *et al*, 2004; Tsuro *et al*., 2003, Kim *et al*., 2005). The proposed studies will investigate the roles these pathways play in AML growth, drug resistance and sensitivity to targeted therapy.

The Ras>Raf>MEK>ERK Pathway

The Ras>Raf>MEK>ERK pathway is activated by many cytokines which are important in driving the proliferation and promoting the survival of myeloid cells (Steelman *et al*., 2004). After receptor ligation, Shc, Src homology (SH)-2, a SH2-domain containing protein, becomes associated with the c-terminus of the cytokine receptor (Matsuguchi *et al*., 1994; Inhorn *et al*., 1995; Okuda *et al*., 1999). Shc recruits the GTP-exchange complex Grb2/Sos resulting in

the loading of membrane bound Ras with GTP (Tauchi *et al*., 1994; Lanfrancone *et al*., 1995). Ras:GTP then recruits Raf to the membrane where it becomes activated, likely via a Src-family tyrosine kinase (Karin *et al*., 1994; Lange-Carter *et al*., 1994; Marais *et al*., 1995). Raf is responsible for phosphorylation of the mitogen associated/extracellular regulated kinase-1 (MEK1) (Marais *et al*., 1997; Mason *et al*., 1999; Xu *et al*., 1995). MEK1 phosphorylates extracellular regulated kinases 1 and 2 (ERKs 1 and 2) on specific threonine and tyrosine residues (Marais *et al*., 1997; Mason *et al*., 1999; Xu *et al*., 1995). Activated ERK1 and ERK2 serine/threonine kinases phosphorylate and activate a variety of substrates including $p90^{Rsk1}$ (Cardone *et al*., 1998; Allan *et al*., 2003; Davis *et al*., 1995; Xing *et al*., 1996; Coutant *et al*., 2002; Iijima *et al*., 2002; Blalock *et al*., 2003). $p90^{Rsk1}$ can activate the cyclic-AMP response element binding protein (CREB) transcription factor (Xing *et al*., 1996). Moreover, ERK can translocate to the nucleus and phosphorylate additional transcription factors such as Elk1, CREB and Fos which bind promoters of many genes, including IL-3, a cytokine important in stimulating the growth and survival of early myeloid progenitor cells (Deng *et al*., 1994; Davis 1995; Robinson *et al*.,, 1998; Aplin *et al*., 2001; McCubrey *et al*., 2000; Tresini *et al*., 2001; Eblen *et al*., 2001; Adachi *et al*., 2002; Wang *et al*., 1994; Thomas *et al*., 1997; Ponti *et al*., 2002; Fry *et al*., 2002). The Raf>MEK>ERK pathway can also modulate the activity of many proteins involved in apoptosis including: Bcl-2, Bad, Bim, Mcl-1, caspase 9, and Survivin (Deng *et al*., 2001; Carter *et al*., 2003; Steelman *et al*., 2004; Jia *et al*., 2003; Troppmair *et al*., 2003; Harada *et al*., 2004; Marani *et al*., 2004; Ley *et al*., 2003; Weston *et al*., 2003; Domina *et al*., 2004; Gelinas *et al*., 2006).

B. 2.1 Roles of the Ras>Raf>MEK>ERK Pathway in Neoplasia

Ras is one of the most frequently mutated oncogenes in human cancer. Ten-50% of individuals diagnozed with myelodysplastic syndrome or AML have Ras mutations (Janssen *et al*., 1987; Padua *et al*., 1988; Needleman *et al*., 1988; Nakagawa *et al*., 1992; Lubbert *et al*., 1992; Kubo *et al*., 1993; Aurer *et al*., 1994; Vasioukhin *et al*., 1994; Neubauer *et al*., 1994; Gougopoulou *et al*., 1996; Gallagher *et al*., 1997; Parry 1997; Constantinidou *et al*., 1997; de Souza Fernandez *et al*., 1998; Kiyoi *et al*., 1999; Flotho *et al*., 1999; Stirewalt *et al*., 2001; Nakamura *et al*., 2004; Zebisch *et al*., 2006; Zebisch *et al*., 2006; Wellbrock *et al*., 2004; Garnett *et al*., 2004). These are often point mutations which alter key residues that affect Ras activity. Mutations which alter Ras activity also perturb the Raf>MEK>ERK kinase cascade. Mutation of B-Raf is frequently observed in melanomas and most thyroid cancers (~70%) but rarely in hematopoietic cancers (<4% in AML & NHL) (Davies *et al*., 2002; Brose *et al*., 2002; Lee *et al*., 2003; Chan *et al*., 2003; Xu *et al*., 2003; Mercer *et al*., 2003; Lilleberg *et al*., 2004; Kambara *et al*., 2004; Daniotti *et al*., 2004; Puxeddu *et al*., 2004; Wan *et al*., 2004; Kim *et al*., 2004; Reifenberger *et al*., 2004; Fransen *et al*., 2004; Lee *et al*., 2004). Activating mutations have been detected at Raf-1 in therapy-induced AML in certain families in Austria (Zebisch *et al*., 2006). These preexisting Raf-1 mutations are genetically transmitted. MEK and ERK are not thought to be frequently mutated in human cancer; however, the actual published studies which document this are few, although they are listed at the Catalogue of Somatic Mutations in Cancer, COSMIC, http://www.sanger.ac.uk/genetics/CGP/cosmic). Mutations in upstream receptors such as Flt-3 (20 to 30%), Kit (7 to 17% of AMLs), Fms (12% of MDS) and granulocyte colony stimulating factor receptor (G-CSF-R) have been documented in AML and will cause the activation of the Ras>Raf>MEK>ERK pathway (Kiyoi et., 1998; Shimada *et al*., 2006; Christiansen *et al*., 2005; Padua *et al*., 1998, Dong *et al*., 1997, Dong *et al*., 1999). Furthermore, over expression of VEGF-R receptors has been observed in AML which could result in activation of this pathway (Hiramatsu *et al*., 2006). Constitutive activation of the Raf>MEK>ERK pathway has been implicated in invasion (Silberman *et al*., 1997), metastases (Canman *et al*., 1995; Keller *et al*., 2005), angiogenesis (Canman *et al*., 1995; Simon *et al*., 1996; Loda *et al*., 1996; Magi-Galluzzi *et al*., 1997) and radioresistance (Pirollo *et al*., 1997). Aberrant activation of the Raf>MEK>ERK cascade has been associated with Bcl-2 and

multi-drug resistance gene expression (Kim *et al*., 1996; Weinstein-Oppenheimer *et al*., 2001; Arcinas *et al*., 2001; Wilson *et al*., 1996; Ji *et al*., 1996; Nunez *et al*., 1996; Davis *et al*., 2003). A diagram of the mutations which can result in activation of the Raf>MEK>ERK cascade is presented in Figure 1.

The PI3K>Akt Pathway

Cytokine receptor ligation also leads to rapid activation of phosphatidylinositol 3 kinase (PI3K) (Drexler 1996; Rao *et al*., 1995; Chang *et al*., 2003; Steelman *et al*., 2004). Only Class IA PI3K consists of an 85-kDa regulatory subunit, which contains SH3 Src-homology 2 (SH) and SH3 domains, and a 110-kDa catalytic subunit (Rao *et al*., 1995; Chang *et al*., 2003; Steelman *et al*., 2004). Cytokine stimulation often creates a PI3K binding site on the cytokine receptor. The p85 subunit SH2 domain associates with this site (Rao *et al*., 1995; Chang *et al*., 2003; Steelman *et al*., 2004). The p85 subunit is then phosphorylated, which leads to activation of the p110 catalytic subunit. Activated PI3K phosphorylates the membrane lipid phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] to phosphatidylinositol (3,4,5)-tri-phosphate [PtdIns(3,4,5)P_3] which activates PI3K-dependent kinase (PDK1). PDK1 then phosphorylates Akt at threonine 308 (T308) (Steelman *et al*., 2004). A second kinase phosphorylates Akt on serine 473 (S473) (Cardone *et al*., 1998; Allan *et al*., 2003; Troussard *et al*., 2003; Xu *et al*., 2003; Persad *et al*., 2003; Kumar *et al*., 2004; Songyang *et al*., 1997).

Akt can transduce an anti-apoptotic signal by phosphorylating downstream target proteins involved in the regulation of cell growth [*e.g.*, glycogen synthase kinase-3β (GSK-3β), Bim, Bad, MDM-2, $p21^{Cip1}$, X-linked inhibitor of apoptosis (XIAP) and the Foxo3a transcription factor](Songyang *et al*., 1997; Scheid *et al*., 1998; del Peso *et al*., 1997; Nakae *et al*., 1999; Brunet *et al*., 1999; Medema *et al*., 2000; Dijkers *et al*., 2000; Qi *et al*., 2006; Mayo *et al*., 2001; Gottlieb *et al*., 2002; Zhou *et al*., 2002, Dan *et al*., 2004). Phosphorylated Foxo3a loses its ability to induce Fas, $p27^{Kip1}$, Bim, Noxa, and Puma gene transcription (Nakae *et al*., 1999; Brunet *et al*., 1999; Medema *et al*., 2000; Dijkers *et al*., 2000; You *et al*., 2006; Obexer *et al*., 2006). Akt also phosphorylates I-κK, which subsequently phosphorylates I-κB, resulting in its ubiquitination and subsequent degradation in proteosomes (Ozes *et al*., 1999; Romashkova *et al*., 1999; Madrid *et al*., 20000; Howe *et al*., 2002, 2004; Hu *et al*., 2004; Mayo *et al*., 2000; Shishodia *et al*., 2004; Du *et al*., 1998; Arcinas *et al*., 2001). Disassociation of I-κB from NF-κB enables NF-κB to translocate into the nucleus to promote gene expression that, under certain circumstances, stimulates growth and prevents apoptosis (Du *et al*., 1998; Arcinas *et al*., 2001). The PI3K>Akt pathway can also phosphorylate and activate CREB which regulates anti-apoptotic genes including Mcl-1 and Bcl-2 (Du k *et al*., 1998;Wang *et al*., 1999).

The PI3K pathway also results in activation of ribosomal protein kinases such as p70S6K (an S6 ribosomal protein kinase) (Mahalingam *et al*., 1996; Dufner *et al*., 1999; Romanelli *et al*., 1999; Harada *et al*., 2001; Edinger *et al*., 2004; Panwalkar *et al*., 2004; Jonassen *et al*., 2004). p70S6K enhances translation of certain mRNAs, is needed for the early events of cell cycle progression and suppresses apoptosis by phosphorylating Bad (Mahalingam *et al*., 1996; Dufner *et al*., 1999; Romanelli *et al*., 1999; Harada *et al*., 2001; Edinger *et al*., 2004). p70S6K is regulated by the mammalian Target of Rapamycin (mTOR) (Ma *et al*., 2005; Shaw *et al*., 2006).

The PI3K pathway is negatively regulated by phosphatases. PTEN (phosphatase and tensin homologue deleted on chromosome 10) is considered a tumor suppressor gene (Chang *et al*., 2003; Steck *et al*., 1997; Li *et al*., 1997; Steelman *et al*., 2004). PTEN is primarily a lipid phosphatase that removes the 3-phosphate from the PI3K lipid product PtdIns (3,4,5)P_3 to produce PtdIns (4,5)P_2 which prevents Akt activation. PTEN is also reported to be a protein phosphatase, although there is some controversy over the precise protein substrates (Steelman

et al., 2004; Mahimainathan *et al*., 2004; Raftopoulou *et al*., 2004). Two other phosphatases, SHIP-1 and SHIP-2, remove the 5-phosphate from PtdIns(3,4,5)P_3 to produce PtdIns(3,4)P_2 (Damen *et al*., 1996; Kavanaugh *et al*., 1996; Lioubin *et al*., 1996; Taylor *et al*., 2000; Muraille *et al*., 1999).

Roles of the PI3K>Akt Pathway in Neoplasia

This pathway provides proliferative and anti-apoptotic signals and its dysregulation have often been linked with malignant transformation and drug resistance (Kubota *et al*., 2004; Cuni *et al*., 2004). Ras can activate PI3K and some Ras mutations result in deregulated PI3K and downstream Akt activation (Rodriguez-Viciana *et al*., 1994; Hu *et al*., 2003; Gire *et al*., 2000; Sun *et al*., 2000; Ninomiya *et al*., 2004). Mutations at the p85 subunit of PI3K have been detected in Hodgkin's lymphoma cells (Jucker *et al*., 2002). Recently it was shown that the p110 subunit of PI3K is frequently mutated (~25%) in breast and some other cancers but it has not been reported to be frequently mutated in leukemia (Engelman *et al*., 2006; Vogt *et al*., 2006; Bader *et al*., 2005; Kang *et al*., 2005; Muller *et al*., 2006). PTEN negatively regulates Akt activity; hence mutations which result in PTEN loss may lead to persistent elevated Akt levels (Leslie *et al*., 2000; Dahia *et al*., 1999; Sakai *et al*., 1998). Mutations and hemizygous deletions of PTEN have been detected in some primary acute leukemias and non-Hodgkin's lymphomas (Sakai *et al*., 1998; Aggerholm *et al*., 2000; Herranz *et al*., 2000; Nakahara *et al*., 1998; Butler *et al*., 1999). Some hematopoietic cell lines lack or have low PTEN protein expression (Sakai *et al*., 1998; Aggerholm *et al*., 2000; Herranz *et al*., 2000; Nakahara *et al*., 1998; Butler *et al*., 1999). Increased Akt expression has also been linked with tumor progression; the Akt-related Akt-2 gene is amplified in some cervical, ovarian, pancreatic cancers and non-Hodgkin's lymphomas (Graff *et al*., 2000; Staal 1987; Cheng *et al*., 1992, 1996). SHIP-1 may also affect Akt activity by controlling the levels of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. SHIP mutations have been detected in certain leukemias including AML (22%). One study reported 22% of AML samples were mutated at SHIP1 (Luo *et al*., 2003, 2004). Thus the PI3K>Akt pathway is intricately regulated and there are many possible mechanisms which can lead to elevated Akt levels. Hence targeting the PI3K>Akt pathway may prove effective in leukemia therapy.

Interactions Between PI3K>Akt and Raf>MEK>ERK Pathways which Regulate Apoptosis

Akt can phosphorylate Raf-1 on S259 and lead to its inactivation in certain cell types (Rommel *et al*., 1999; Zimmermann *et al*., 1999). Akt and serum/glucocorticoid regulated kinase (SGK) can phosphorylate B-Raf which results in its inactivation in certain cell types (Guan *et al*., 2000; Zhang *et al*., 2001). Studies in 32D myeloid hematopoietic cells have shown that Akt can activate Raf-1 through a Ras-independent but protein kinase C (PKC)-dependent mechanism which results in the prevention of apoptosis (Majewski *et al*., 1999). Thus Akt and related proteins phosphorylate Raf family members and either inhibit or enhance their activity and these effects may depend on the cell lineage or environmental cues. Suppression of apoptosis in some cells by Raf and MEK requires PI3K dependent signals (McCubrey *et al*., 2001; Gelfanov *et al*., 2001; von Gise *et al*., 2001; Shelton *et al*., 2003, 2004).

Both PI3K>Akt and Raf>MEK>ERK pathways contribute to the transcriptional and post-translational regulation of Bcl-2 family members as they can regulate CREB phosphorylation and CREB binds the Mcl-1 and Bcl-2 promoter region (Yang *et al*., 1995; Pugazhenthi *et al*., 2000, 1999; Bonni *et al*., 1999). Moreover, both pathways phosphorylate pro-apoptotic Bcl2 homology (BH)-3 only domain protein Bad which abolishes its apoptotic effects as it is complexed with 14-3-3 proteins and is cytoplasmically localized (Cardone *et al*., 1998; Allan *et al*., 2003; Datta *et al*., 1997; Harada *et al*., 1999). Another MAPK, Jun N-terminal kinase (JNK) can phosphorylate 14-3-3 proteins which results in their disassociation with phosphorylated Bad proteins and the Bad proteins translocate to the mitochondrion (Sunayama

et al., 2005). When Bad associates with Bcl-2 or Bcl-X_L, Bad promotes apoptosis by preventing Bcl-2 or Bcl-X_L from interacting with Bax (She *et al*., 2005). Bad is phosphorylated in most AML specimens examined suggesting that inhibition of this molecule is important in AML (Zhao *et al*., 2004). Interestingly, the anti-apoptotic Mcl-1 protein which is expressed in myeloid cells, is not reported to interact with Bad (Chen *et al*., 2005).

Both the Raf>MEK>ERK and PI3K>Akt pathways can phosphorylate the BH3 only domain protein Bim (Harada *et al*., 2004; Qi *et al*., 2006). When Bim is phosphorylated by ERK and Akt it is targeted for ubiquitination and degradation in the proteosome (Gelinas *et al*., 2006). ERK also can phosphorylate Mcl-1 which results in its stabilization. Mcl-1 can bind Bim which prevents the activation and mitochondrial translocation of Bak and Bax and it can bind Bim and is able to prevent the activation and mitochondrial translocation of Bak and Bax (Domina *et al*., 2004). In contrast, JNK can phosphorylate Bim at S65 which enhances its ability to induce Bax activation and hence stimulates apoptosis (Putcha *et al*., 2003). Mcl-1 can also bind pro-apoptotic Bak (Chen *et al*., 2005). The Mcl-1:Bax interaction can be disrupted by the binding of the BH3 domain Noxa protein which results in Mcl-1 being ubiquitinated and degraded in the proteosome (Willis *et al*., 2005). Bak can then form active dimers and induce apoptosis. The stability of Mcl-1 is influenced by both transcriptional (PI3K>Akt) and post-transcriptional (Raf>MEK>ERK) mechanism (Gelinas *et al*., 2006; Wang *et al*., 1999).

Cytokines such as IL-3 also induce the Jak/STAT pathway which regulates the transcription of Bcl-X_L (Nosaka *et al*., 1999). Bcl-X_L can prevent the formation of Bax:Bax homodimers (Wang *et al*., 1998). Furthermore JNK can antagonize some of the effects of Raf>MEK>ERK and PI3K>Akt pathways by phosphorylating 14-3-3 proteins which result in released Bad that can translocate to the mitochondrion (Sunayama *et al*., 2005) or JNK can phosphorylate Bim at different residues than ERK and Akt which results in Bim stabilization. Hence it is clear that the Raf>MEK>ERK, PI3K>Akt, Jak>STAT and JNK pathways regulate many molecules involved in prevention of apoptosis. Dysregulation of these pathways may lead to drug resistance. A diagram of these interactions is presented in Figure 1.

Chemotherapeutic Drugs and Induction of Reactive Oxygen Species (ROS)

Doxorubicin exerts its chemotherapeutic effects through multiple mechanisms. One mechanism is through its interactions with DNA and inhibition of topoisomerase II (Fornari *et al*., 1994). The other mechanism of action is due to the generation of ROS that occurs via the interaction of doxorubicin with iron (Myers *et al*., 1986). It is reported that doxorubicin treatment results in the intracellular generation of superoxide anion, hydrogen peroxide, and the hydroxyl radical (Myers *et al*., 1986; Liu and Tan, 2003). ROS appear to be important for some of the therapeutic effects of doxorubicin as scavenging oxygen radicals using anti-oxidants decreases the ability of doxorubicin to induce apoptosis (Friesen *et al*., 1999; Gewirtz, 1999; Singal *et al*., 2000). While ROS are important for some of the activities of doxorubicin they are also are the cause of some of the undesirable side effects of this drug (Hoke *et al*., 2005).

ROS are known to induce the activation of ERK, JNK, p38 and Big MAP Kinase (BMK)/ERK5 signaling pathways. Oxidative stress-induced ERK1/2 activation is reported in a variety of cell types (Jimenez *et al*., 1997; Tournier *et al*., 1997; Griffith *et al*., 1998; Buder-Hoffmann *et al*., 2001; Kim *et al*., 2001; Xiao *et al*., 2002; Blanc *et al*., 2003; Usatyuk *et al*., 2003; Conde de la Rosa *et al*., 2005). In some cases ROS can act directly on receptors, such as the EGFR, and induce the ERK1/2 signaling pathway (Knebel *et al*., 1996). Triggering of the EGFR is well known to result in the activation of Ras and the subsequent activation of the Raf>MEK>ERK module. ROS can induce the ligand-independent activation of the PDGF receptor and a subsequent increase in Ras and ERK1/2 activity (Knebel *et al*., 1996). Ligand-independent receptor activation is not the only mechanism by which oxygen radicals activate

the ERK1/2 signaling pathway. ROS not only act via growth factor receptors, but also appear to mediate activation of Ras independently of reactive oxygen intermediate-induced receptor activation (Lander et al., 1996). Nor is Ras expression is an absolute requirement for reactive oxygen intermediate activation of the ERK1/2 signaling pathway. ROS will induce the activation of the ERK1/2 signaling pathway in Ras negative cells (Zou *et al*., 1996). The non-receptor tyrosine kinase, Src, is sensitive to cellular redox and can phosphorylate and activate PLC-γ (Wang *et al*., 2001). This results in the generation of DAG and increases in intracellular calcium which in turn induce activation of several forms of PKC. Although PKC can lead to Ras activation, it has also been shown to directly activate Raf (Buhl *et al*., 1995). ROS are also known to inhibit protein phosphatases (Whisler *et al*., 1995; Rao and Clayton, 2002) and inhibition of phosphatase activity results in activation of the ERK1/2 signaling pathways (Lee and Esselman, 2002). Thus, it would appear that the ERK1/2 kinase signaling cascade can be activated at multiple points by ROS. However, the MEK1 and 2 inhibitors U0126 and PD98059 both block oxidative stress-induced ERK1/2 activation (Lee *et al*., 2005a;Lee *et al*., 2005b), indicating that activating actions of oxidative stress on ERK are not direct but instead upstream of ERK. Hydrogen peroxide is able to stimulate ERK5/BMK1 activation in human skin fibroblasts, human vascular smooth muscle cells, and human umbilical vein endothelial cells (Abe *et al*., 1996). In PC12 cells, hydrogen peroxide-induced ERK5/BMK1 activation requires the activation of a Src kinase (Suzaki *et al*., 2002). Superoxide anion may play a role in BMK1 activation as superoxide scavengers prevented Angiotensin II- and endothelin-1-induced BMK1 phosphorylation. Since doxorubicin induces ROS, and ROS may induce the ERK signaling pathway. Understanding this pathway may be important in determining how AML cells develop drug resistance. A diagram of the effects of signaling pathways, p53 and ROS and how they may result in drug resistance is presented in Figure 2.

Targeted Therapy in AML

While treatment of some subsets of AML, such as acute promyelocytic leukemia (APL) have shown great success with retinoids and arsenic tri-oxide, a significant problem in the remainder of AML patients is that most chemotherapy does not ultimately work and eventually the patients relapse and succumb to the disease (Tallman *et al*., 2005). Also another nagging problem in AML therapy is the emergence of drug resistance (Teodori *et al*., 2006; Polgar *et al*., 2005; Ross 2004; Mahadevan *et al*., 2004). Unlike the success stories observed with Gleevec (Imatinib) and Dasatinib in treatment of CML, similar successes have not been observed in AML due in part to the genetic heterogeneity of the disease (Talpaz *et al*., 2006). Flt-3 inhibitors have been developed, but only approximately 20% of AMLs have mutations at Flt-3 which render them somewhat sensitive to Flt-3 inhibitor monotherapy (Traxier 2003; Markovic *et al*., 2005; Stone *et al*., 2005). There have been some combination clinical trials to evaluate the sensitivity of Flt-3 positive AML to chemotherapy and Flt-3 inhibitors.

Materials and methods

Cell line models for identifying signaling pathways involved in hematopoietic drug resistance

The FL5.12 cell line is an IL-3-dependent early hematopoietic progenitor cell line isolated from the fetal liver of mice (McKearn *et al*., 1985). It is strictly cytokine (interleukin-3[IL-3]) dependent and does not form tumors upon injection into immunocompromised mice. However, FL5.12 cells can be transformed to cytokine-independent and leukemic cells by oncogenes such as v-*abl* and BCR-ABL (McCubrey *et al*., 1989). It has wild type (WT) p53 genes.

The effects of the Raf>MEK>ERK and PI3K>Akt pathways on transformation and drug resistance were examined by infecting FL5.12 cells with retroviral vectors encoding activated Akt, activated Raf-1, activated and dominant negative (DN) MEK1, WT and DN p53 as

described (Shelton *et al*., 2003). FL/ΔAkt:ER*(Myr$^+$) + ΔRaf-1:AR are derivatives of FL5.12 cells which grow in response to Akt and Raf activation in the absence of exogenous IL-3 (Shelton *et al*., 2003). Activated MEK1 (ΔStuMEKLIDEMAN), DN MEK1 encoding retroviral vectors (von Gise *et al*., 2001) were generously provided by Dr. Jakob Troppmair (Daniel Swarovski Research Laboratory, Innsbruck Medical University, Innsbruck, Austria). The effects of p53 on drug resistance were examined by infecting FL5.12 cells with retroviruses encoding WT and DN p53 (Gottlieb *et al*., 1994) generously provided by Dr. Moshe Oren, (The Weizmann Institute of Science, Rehovot, Israel). The FL5.12 cells and derivative transformed lines represent models to understand normal and transformed as well as drug resistant early hematopoietic progenitor cells.

Cell lines and growth factors

Cells were maintained in a humidified 5% CO_2 incubator with RPMI-1640 [(RPMI) Invitrogen, Carlsbad, CA, USA] supplemented with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA, USA). The IL-3 dependent FL5.12 murine cell line was cultured in this medium supplemented with 10% WEHI-3B(D$^-$) conditioned medium (WCM) as a source of IL-3. Conditionally-transformed FL/ΔAkt:ER*(Myr$^+$) + ΔRaf-1:AR cells were grown in RPMI + 5% FCS + 500 nM 4 hydroxyl tamoxifen (4HT), an estrogen receptor antagonist which activates the ΔAkt:ER*(Myr$^+$) (Sigma, St. Louis, MO, USA), and 100 nM testosterone (Sigma), which activates the ΔRaf-1:AR. ΔAkt:ER*(Myr$^+$) contains a mutated ER domain (ER*) which responds to 4HT 100-fold more efficiently than β-estradiol (Shelton *et al*., 2003). Thus 4HT as apposed to β-estradiol was used to stimulate Akt activity. ΔRaf-1:AR contains the androgen receptor (AR) hormone binding domain and is activated by testosterone (Shelton *et al*., 2003).

Limiting dilution analysis in doxorubicin and paclitaxel

FL5.12 and FL/ΔAkt:ER*(Myr$^+$)+ΔRaf-1:AR cells were plated at cell concentrations ranging from 0.1 to 100,000 cells/well in 96 well plates (Corning, Corning NY). Limiting dilution analysis with the parental FL5.12 cells was performed in the presence of IL-3 in doxorubicin (1, 10, 25, 50, 100, 1000 nM) or paclitaxel (0.01, 0.1, 1, 10 and 100 nM). Limiting dilution analysis in the FL/ΔAkt:ER*(Myr$^+$)+ΔRaf-1:AR cells was performed in the presence of IL-3, 4HT, Testosterone or 4HT+Testosterone in doxorubicin (1, 10, 25, 50, 100, 1000 nM) or paclitaxel (0.01, 0.1, 1, 10 and 100 nM). Fresh medium containing the drugs was added every three days and clones isolated from the plates with the least number of colonies after approximately 1 month in culture. After isolation of the clones, they were first expanded in 1 ml cultures in 24 well plates, then subsequently expanded into 5 ml cultures in 25 cm^2 tissue culture flasks. The drug resistant cells were grown in medium containing doxorubicin (10 to 100 nM) or paclitaxel (0.1 to 1 nM) with either IL-3 or 4HT+testosterone.

Analysis of Cell Sensitivity to Anticancer Agents

Sensitivity of FL5.12 and FL/ΔAkt:ER* (Myr$^+$)+ ΔRaf-1:AR cells to doxorubicin, paclitaxel, daunorubicin, cisplatin or 5-flurouracil (all purchased from Sigma) were investigated by characterizing effects of these agents on proliferation (Lee *et al*., 2004). Proliferation assays were performed in order to measure cellular growth under various conditions over a period of 5 days. FL5.12 and FL/ΔAkt:ER*(Myr$^+$)+ΔRaf-1:AR cells were resuspended in phenol red free RPMI containing 5% FBS and either IL-3 (FL5.12 cells) or 4HT, Test, 4HT+Test or no supplement (FL/ΔAkt:ER*(Myr$^+$)+ΔRaf-1:AR cells). Cells were seeded in 96-well cell culture plates (BD Biosciences) at a density of 5,000 cells/well in 100 μL/well of cell culture medium. A 100 μl dose of treatment medium (chemotherapeutic drugs) was added to each well the day after cells were initially seeded. Treatment medium with doxorubicin consisted of 4,000 nM, 2,000 nM, 1,000 nM, 500 nM, 250 nM, 125 nM, 63 nM, 31 nM, 16 nM, 8 nM, 4 nM, or 0 nM

in cell culture medium for analysis of proliferation by spectrophotometry. Treatment medium with paclitaxel consisted of 400 nM, 200 nM, 100 nM, 50 nM, 25 nM, 13 nM, 6.3 nM, 3.1 nM, 1.6 nM, 0.8 nM, 0.34 nM, or 0 nM paclitaxel in cell culture medium for analysis of proliferation by spectrophotometry. Treatment medium with daunorubicin consisted of 4,000 nM, 2,000 nM, 1,000 nM, 500 nM, 250 nM, 125 nM, 63 nM, 31 nM, 16 nM, 8 nM, 4 nM, or 0 nM daunorubicin in cell culture medium for analysis of proliferation by spectrophotometry. Treatment medium with cisplatin consisted of 1000 nM, 500 nM, 250 nM, 125 nM, 62.5 nM, 31.3 nM, 15.6 nM, 7.8 nM, 3.9 nM, 2 nM, 1 nM, or 0 nM cisplatin in cell culture medium for analysis of proliferation by spectrophotometry. Treatment medium with 5 flurouracil (5FU) consisted of 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.1 nM, 1.6 nM, 0.8 nM, 0.4 nM, 0.2 nM, or 0 nM 5FU in cell culture medium for analysis of proliferation by spectrophotometry. Cell culture plates were incubated in a cell culture incubator at 37 °C until extent of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma) reduction in each well was quantified.

Extent of MTT reduction in each well was measured daily from the day cells were treated until 4 days after treatment. A 40 μl aliquot of MTT medium was added to each well at the end of the treatment period. MTT medium consisted of 3 mg/ml MTT in cell culture medium. MTT medium was sterilized by vacuum filtration before use. After addition of MTT medium, the final volume of medium in each well was 240 μl and the final concentration of MTT was 500 μg/ml. Cell culture plates were incubated in a cell culture incubator for 3 hours at 37 °C to permit MTT reduction by viable cells. MTT reduction produces 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan, which forms crystals that adhere to the bottom of each well because it is insoluble in aqueous solution. Cell culture media was removed after incubation by manually shaking cell culture plates in an inverted position. Crystals of reduced MTT remaining in each well were dissolved in 200 μl of DMSO (Sigma). Cell culture plates were gently shaken for 5 minutes at 37 °C to facilitate dissolution of reduced MTT crystals. Absorbance of each well was measured at 530 nm with a FL600 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT).

MTT dissolved in DMSO has a yellow color and a visible light absorbance maximum of approximately 410 nm (Plumb *et al*., 1989). In contrast, MTT reduced by viable cells then dissolved in DMSO has a purple color and a visible light absorbance maximum of approximately 560 nm. It is assumed that absorbance of each well above background at 530 nm is proportional to the number of cells present. Background absorbance at 530 nm was estimated from 56 wells in which no cells were seeded. A 200 μl aliquot of cell culture medium lacking cells was added to each of these wells. These wells were incubated in a cell culture incubator for 1 day at 37 °C before extent of MTT reduction in the absence of cells was determined. Mean absorbance of these 56 wells in which no cells were seeded was subtracted from original absorbance values for all wells containing cells to yield adjusted absorbance. Original absorbance values were adjusted in order to account for background absorbance.

Adjusted absorbance values were normalized by dividing by the mean initial adjusted absorbance. Mean initial adjusted absorbance was measured from wells containing cells incubated for 1 day after seeding. The mean and corresponding standard deviation of normalized adjusted absorbance was calculated from 8 replicate wells for each drug concentration and duration of incubation in order to investigate effects of the drugs and, in some cases, ectopic gene (Raf-1, Akt, MEK1, DN-MEK, p53, DN-p53) expression on cell proliferation rate and sensitivity to the different chemotherapeutic drugs. Relative growth rate was calculated by subtracting mean initial adjusted absorbance from adjusted absorbance after 4 days of treatment then dividing this difference by the increase in mean adjusted absorbance after 4 days of incubation in the absence of the drugs. The mean and standard deviation of

relative growth was calculated from 8 replicate wells for each drug concentration to compare effects of the chemotherapeutic drugs on proliferation rates of the different cells.

Inhibitory concentration 50% (IC_{50}) is defined in this context as the concentration of drugs that causes the cells to proliferate at a rate that is half as rapid as cells incubated in the absence of drugs. IC_{50} values were estimated by linear interpolation of the highest drug concentration yielding a mean relative growth rate greater than 0.5 and the lowest drug concentration yielding a mean relative growth rate less than 0.5.

Annexin V apoptotic assays

AnnexinV/PI binding assays were performed as previously described (Blalock *et al*., 2000; Bertrand *et al*., 2006) with a kit purchased from Roche (Indianapolis, IN, USA)

Western blot analysis

Cells were cultured and then protein lysates prepared as described (Blalock *et al*., 2000). Western blots were performed with antibodies specific for phospho and total MEK, ERK, Akt, JNK, p53, $p21^{Cip\text{-}1}$, $p27^{Kip\text{-}1}$ as we have previously described (Bertrand *et al*., 2006). The above antibodies were obtained from Cell Signaling (Beverly, MA, USA). Antibodies which recognize total Caspase 3, Bcl-2 and Bcl-X_L were obtained from Cell Signaling. An antibody which recognizes Mcl-1 was obtained from Pharmingen (San Diego, CA).

Results and discussion

To elucidate the pathways involved in hematopoietic drug resistance, FL5.12 cells were plated in limiting dilution experiments in the presence of different concentrations of doxorubicin in 96 well plates. Doxorubicin resistant cells (FL/Doxo) were isolated in the presence of IL-3 and either 10 or 100 nM doxorubicin but not 1000 nM doxorubicin (Figure 3, Panel A). Approximately 1 in 20 FL5.12 cells would form a colony in the presence of IL-3 + 10 nM doxorubicin while only 1 in 500 (frequency 2×10^{-3}) FL5.12 cells would form a colony in the presence of IL-3 + 100 nM doxorubicin. Approximately 25 different clones were isolated, expanded into 200 μl, 1 ml, 5 ml, 10 ml then 25 ml cultures. These individual clones were frozen down. Three different clones were chosen for further study: FL/Doxo-1 FL/Doxo-2, and FL/Doxo-3. These clones have been maintained continuously in 10 to 100 nM doxorubicin for the past two years. The results presented in this manuscript were obtained with FL/Doxo-1, hereafter referred to as FL/Doxo. Similar results were obtained with FL/Doxo-2 and FL/Doxo-3.

Additional limiting dilution experiments indicated that the doxorubicin resistant cells had an enhanced subcloning efficiency when they were plated in medium containing doxorubicin than the parental cells (Figure 3, Panels A & B). The doxorubicin selected cells that had been maintained in 10 nM doxorubicin had a plating efficiency of 1.6×10^{-2} as 1 out of 60 cells formed a colony when the cells were plated in 100 nM doxorubin. This represents an approximate 8.3-fold increase in cloning efficiency in 100 nM doxorubicin as compared to the unselected FL5.12 cells.

The morphologies of the doxorubicin sensitive (FL5.12) and resistant (FL/Doxo) cells were examined by light microscopy (Figure 4, Panels A & B). The parental cells grew as non adherent individual cells (Panel A). The doxorubicin resistant cells tended to grow in clusters on the bottom of the flask (Panel B). The doxorubicin resistant cells were larger and more blast like (Panel D) than the doxorubicin sensitive cells (Panel C). Furthermore upon staining the cells with acridine orange, which enables visualization of the nucleus, many of the doxorubicin

resistant cells had multiple nuclei whereas the parental cells had single nuclei (Figure 4, Panels E & F).

The sensitivities of the parental and doxorubicin resistant cells to five common chemotherapeutic drugs were examined. The doxorubicin resistant cells had increased IC_{50}s for doxorubicin, paclitaxel, daunorubicin but not 5-flurouracil (5FU) or cisplatin (Table 1).

The effects of these drugs on the induction of apoptosis were determined by the Annexin V/PI binding assay (Table 2). The parental FL5.12 cells were more sensitive to the induction of apoptosis by doxorubicin, paclitaxel and daunorubicin than the doxo resistant FL/Doxo cells. In contrast, the parental and FL/Doxo cells displayed similar sensitivities to 5FU (data not presented). Again, the greatest difference between the sensitive and resistant cells was observed with paclitaxel.

Evidence for Raf>MEK>ERK Pathway in Drug Resistance

The roles of signal transduction, apoptotic regulatory and p53 pathways were examined in the doxorubicin sensitive and resistant cells. FL5.12 and FL/Doxo cells, which had been growing in IL-3 or IL-3 + 10 nM doxorubicin respectively, were collected, washed twice with PBS and then both cell types were cultured in IL-3 or IL-3 + 10 nM doxorubicin for 24 hrs. When the FL5.12 and FL/Doxo cells were cultured in IL-3 for 24 hrs, similar levels of phospho and total ERK, JNK, Akt and Bcl-X_L and Puma proteins were detected. Higher levels of Mcl-1 were detected in the FL/Doxo cells than in FL5.12 cells. In contrast, when the FL5.12 and FL/Doxo cells were culture in IL-3 + 10 nM doxorubicin for 24 hrs, activated MEK and ERK, and total Mcl-1 proteins, were detected at higher levels in the FL/Doxo cells than parental FL5.12 cells (Figure 5). Puma, which was detected at low levels when both cell types were cultured in IL-3, was induced when the FL5.12 cells were cultured in IL-3 + 10 nM doxorubicin, while it was not induced in the doxorubicin resistant cells when they were cultured in IL-3 + 10 nM doxorubicin suggesting that these two cell types may differ in their induction of Puma after doxorubicin treatment. When the doxorubicin sensitive and resistant cell lines were treated with doxorubicin, they both displayed activation of p53, as detected with an antibody which recognized p53 phosphorylated at S15 (data not presented). Thus the doxorubicin resistance of the FL/Doxo cells did not appear to be due to a defective p53 response.

Consequences of MEK/ERK and p53 expression on Drug Sensitivity

To further examine the effects of MEK and p53 on the chemosensitivity of the cells, DN MEK and DNp53 constructs were introduced into the cells and the doxorubicin IC_{50}s were determined by MTT analysis (Table 3). Cells were infected with retroviruses encoding DN MEK (MEK-LIDA), DN p53 (p53#661) or as controls an empty retroviral vector (pLXSN) or a WT p53. DN-MEK1 has serine 217 and 221 mutated to alanine which can not be phosphorylated and activated by Raf and is inactive and interferes with endogenous MEK1. DN p53 retrovirus encodes a p53 protein which lacks the DNA binding domain and results in the formation of inactive p53 tetramers.

Introduction of DN MEK1 reduced the IC_{50} for doxorubicin in FL5.12 cells 7.5-fold and in FL/Doxo cells 5.7-fold. Moreover, introduction of the DN MEK1 into the FL/Doxo and FL5.12 cells reduced the cloning efficiency in doxorubicin approximately 3 fold (Data not presented). In contrast, introduction of DN-p53 into FL5.12 or FL/Doxo cells increased the IC_{50} for doxorubicin approximately two to three fold compared to cells which were transduced with the empty vector or the WT-p53 gene respectively.

The effects of elevated Raf>MEK>ERK expression of the drug resistance of FL5.12 cells was examined by introduction of a constitutive MEK1 gene (ΔStuMEKLIDEMAN (Von Gise *et*

al., 2001), here after referred to MEK-Act (Act = Activated). The FL/Doxo cells with the activated MEK1 gene (FL/Doxo+MEK-Act) had an approximately 5-fold higher doxorubicin IC_{50} than the FL/Doxo infected with an empty retroviral vector (FL/Doxo+LXSN) cells demonstrating that constitutive MEK activity increased the resistance to doxorubicin.

Decreased Caspase 3 Cleavage in Doxorubicin Resistant Cells

Caspase 3 cleavage and activation is one of the last steps in the caspase cascade leading to apoptosis. The extent of caspase 3 cleavage was examined in the doxorubicin sensitive and resistant cells by western blot analysis (Figure 6). Cleavage of caspase 3 was detected in FL5.12 cells in a dose dependent fashion after treatment with 100 or 1000 nM doxorubicin for 24 hrs. In contrast, cleavage of caspase 3 in FL/Doxo cells was only detected after treated with 1000 nM doxorubicin. Furthermore, FL/Doxo+MEK-Act, no cleavage of caspase 3 was detected which correlated with the increased IC_{50} for doxorubicin in these cells.

Lack of Elevated Mdr-1/MRP-1 Expression in Doxorubicin resistant FL/Doxo Cells

FL/Doxo cells were shown to be resistant to doxorubicin, paclitaxel, and daunomycin, but not resistant to 5FU or cisplatin. Doxorubicin, paclitaxel and daunomycin can be transported by the membrane transporter proteins Mdr-1 or MRP-1, whereas 5FU and cisplatin are transported by different membrane transporters. A relatively simple means to determine if Mdr-1 or MRP-1 activity is elevated in FL/Doxo cells is to perform a functional Rhodamine 123 dye exclusion assay by FACS analysis. The drug resistant and drug sensitive FL5.12 cells displayed similar levels of drug efflux activity. This assay was performed 4 times. Thus by a functional assay the drug resistant FL/Doxo cells did not appear to have elevated drug efflux when compared to the parental cells.

The expression of these two transporters was examined by RT-PCR and western blot analysis. mRNA levels for MRP-1 were similar in the doxorubicin sensitive and resistant cells. Transcripts encoding Mdr-1 were not detected in ether cell line. Western blot analysis failed to detect the expression of Mdr-1 or MRP-1 proteins in these cells while they were detected in control cell lines. In summary, these results suggest that the drug resistance of FL/Doxo cells is not due to the increased expression of Mdr-1 or MRP-1 but they do not eliminate that possibility that some other transporter is involved in drug resistance.

Interactions between Raf>MEK>ERK and PI3K>Akt Pathways in Induction of Drug Resistance in Hematopoietic Cells

We previously developed a model of hematopoietic cells which proliferate in response to activation of both Raf and Akt (Shelton, *et al*., 2003). FL5.12 cells were infected with conditional retroviral vectors encoding ΔRaf-1:AR (testosterone–inducible Raf-1) and ΔAkt:ER*(Myr^+) (*= tamoxifen, [4HT]-inducible Akt). These cells are named FL/ΔAkt:ER+ΔRaf:AR cells. An advantage of the FL/ΔAkt:ER+ΔRaf:AR cells is the possibility to investigate the effects of Akt and Raf on signal transduction pathways and drug resistance either alone or together.

As described earlier with the FL5.12 cells, doxorubicin resistant FL/ΔAkt:ER+ΔRaf-1:AR cells were isolated by culturing the cells in medium containing 10 or 100 nM doxorubicin and 4HT and testosterone. The unselected FL/ΔAkt:ER+ΔRaf-1:AR cells had a subcloning efficiency of approximately 2×10^{-2} (1 in 50 cells would form a colony) in 10 nM doxorubicin. In contrast to the results observed with IL-3 and the parental FL5.12 cells, drug resistant clones were infrequently isolated from unselected FL/ΔAkt:ER+ΔRaf-1:AR cells when they were plated in 100 nM doxorubicin as less than 1 in 10^5 cells would form a colony. The difference in cloning efficiency in medium containing doxorubicin between in FL5.12 and FL/ΔAkt:ER+ΔRaf-1:AR cells is likely due to the difference in culture conditions, as IL-3 will induce many

signaling pathways in addition to Raf>MEK>ERK and PI3K>Akt such as Jak>STAT which can contribute to drug resistance while 4HT and testosterone only induce the Akt and Raf>MEK>ERK pathways.

Additional limiting dilution experiments indicated that the doxorubicin selected FL/ΔAkt:ER+ΔRaf-1:AR cells had an enhanced subcloning efficiency when they were plated in medium containing doxorubicin than the parental FL/ΔAkt:ER+ΔRaf-1:AR cells (Figure 7, Panels A & B). In the doxorubicin selected FL/ΔAkt:ER+ΔRaf-1:AR cells that had been maintained in 10 nM doxorubicin, they had a plating efficiency of 1.25×10^{-1} as 1 in 8 cells would form a colony in 10 nM doxorubicin, an approximate 6.3-fold increase in cloning efficiency. When the doxorubicin selected FL/ΔAkt:ER+ΔRaf-1:AR cells were plated in 100 nM doxorubicin a cloning efficiency of 1×10^{-5} as approximately 1 in 10^5 cells formed a colony.

The drug sensitivities of the doxorubicin sensitive and resistant FL/ΔAkt:ER+ΔRaf-1:AR cell lines were compared (Table 4).

Effects of Raf Activation on the Doxorubicin IC_5

The effects Raf and Akt individually on the doxorubicin IC_{50} were determined by performing the MTT analysis in medium supplement with: no supplement, 4HT, testosterone or the combination of 4HT + testosterone (Figure 8). Activation of Raf increased the IC_{50} approximately 10-fold, from approximately 3 nM with no supplement or 4HT to 30 nM with testosterone treatment (Panel A).

Likewise in the drug resistant FL/ΔAkt:ER+ΔRaf-1:AR cells, activation of Raf increased the IC_{50} for doxorubicin from approximately 3-fold from 15 to 25 nM with 4HT or no supplement to approximately 70 nM when Raf was activated. This figure also demonstrates that the drug resistant cells have retained their requirement for Raf for proliferation.

Requirement for Raf for the Prevention of Apoptosis

The effects of Raf and Akt activation on the prevention of apoptosis in response to doxorubicin treatment of doxorubicin sensitive and resistant FL/ΔAkt:ER+ΔRaf-1:AR cells were examined by annexin V/PI assays (Figure 9). The effects Raf and Akt individually on the doxorubicin IC_{50} were determined by culturing the cells in medium supplement with: no supplement, 4HT, testosterone (Figure 9). Activation of Raf increased the apoptosis IC_{50} approximately 10-fold in the unselected doxorubicin sensitive FL/ΔAkt:ER+ΔRaf-1:AR, from approximately 0.2 nM with no supplement or 4HT to 2 nM with testosterone treatment (Panel A). Likewise in the drug resistant FL/ΔAkt:ER+ΔRaf-1:AR cells, activation of Raf increased the IC_{50} for doxorubicin from approximately 80-fold from 0.2 nM with 4HT or no supplement to approximately 8 nM when Raf was activated. This figure also demonstrates that the drug resistant cells have retained their requirement for Raf for prevention of apoptosis.

Requirement for Raf and Akt Activation for Optimal Growth in the Presence of Chemotherapeutic Drugs

The requirement of Raf and Akt activation in the growth of the cells in the presence and absence of chemotherapeutic drugs was determined by culturing the cells in 4HT, Test, 4HT + Test or no supplement and then performing MTT analysis (Figure 10). When these cells were cultured in the absence of doxorubicin (Panel A), they proliferated equally well in response to either Raf activation or Raf and Akt activation in 100 μl cultures in 96 well plates as measured by MTT analysis. In contrast, in the presence of just 4HT, which activated Akt, or no supplement, the cells did not proliferate well. Thus, in the absence of drugs, Raf-1 activation was able to induce proliferation as estimated by an MTT assay. In contrast, when the cells were plated in the presence of 25 nM doxorubicin (Panel B), the cells proliferated better when both Raf and

Akt were activated as opposed to just activation of Raf-1 by itself. Similar results were observed with daunorubicin and paclitaxel.

Potential Mechanisms for Induction of Drug Resistance

In the following sections, we will briefly summarize potential mechanisms by which interactions between the Raf>MEK>ERK and PI3K>Akt pathways could result in drug resistance. Cytokines such as IL-3 induce multiple signal transduction pathways which can contribute to the prevention of apoptosis (Harada *et al*., 2004; Qi *et al*., 2006; Opferman *et al*., 2003). If their expression becomes deranged, drug resistance may occur. An overview of IL-3 and the different pathways which it induces is presented in Figure 11. Note that all these signaling pathways have roles in the regulation of apoptotic pathways.

Raf>MEK>ERK Expression Results in Altered Bim Localization

The pro-apoptotic Bim molecule can be phosphorylated by both the Raf>MEK>ERK and PI3K>Akt pathways on multiple residues (Harada *et al*., 2004; Qi *et al*., 2006; Opferman *et al*., 2003). Akt can phosphorylate Bim on S87 in IL-3 dependent cells. ERK induces the phosphorylation of Bim at S55, S65 and S100. Once Bim is phosphorylated it loses its association with Bcl-2 like antiapoptotic proteins associates with 14-3-3 proteins and is ubiquitinated and targeted for degradation in the proteosome. Upon IL-3 withdrawal, non-phosphorylated Bim associates with pro-apoptotic Bax proteins and stimulates apoptosis. JNK also phosphorylates Bim, but this results in enhanced pro-apoptotic activity (Tsuruta *et al*., 2004; Gao *et al*., 2005). JNK can also phosphorylate 14-3-3 proteins which may then release cytosolic Bim. The presence of phosphorylated Bim may be elevated in the doxorubicin resistant cells, alternatively, the subcellular localization of Bim may be different. Activation of both Raf>MEK>ERK and PI3K>Akt pathways and hyperphosphorylation of Bim may be necessary for the growth of the drug resistant cells in chemotherapeutic drugs. A diagram depicting these potential interactions is presented in Figure 12.

Raf>MEK>ERK Elevates Bad Phosphorylation in Doxorubicin-Resistant Cells

Increased phosphorylation of Bad could be one component of the drug resistance of FL/Doxo cells. Both the Raf>MEK>ERK and PI3K>Akt pathways phosphorylate Bad which results in Bad's translocation from the mitochondrion and association with 14-3-3 proteins in the cytoplasm. Bcl-2 and Bcl-X_L are able to bind Bax and prevent its activation. JNK will phosphorylate 14-3-3 proteins which then release Bad and Bad translocates to the mitochondrion. Bad is then able to bind Bcl-2 and Bcl-X_L and Bax is activated and apoptosis is induced. Increased ERK activity in FL/Doxo cells may result in higher levels of Bad phosphorylation. However, this component would be predicted not to involve Mcl-1 as Mcl-1 is not thought to bind Bad whereas, Bcl-1 and Bcl-X_L bind Bad. In some scenarios, inhibition of Mcl-1 is not thought to be totally sufficient to induce apoptosis in some cells, as there is thought to be compensatory effect by Bcl-X_L. An overview of the interactions of Raf>MEK>ERK, PI3K>Akt, Bcl-X_L, Bcl-2 and Bad is presented in Figure 13.

However, we do not think that Bad will be the target responsible for drug resistance for two reasons, Bad has been reported to be present at either very low levels or not at all in FL5.12 cells (Yamaguchi *et al*., 2001), and we did not see a difference in the levels of Bcl-X_L in the doxorubicin responsive and resistant cells.

Raf>MEK>ERK Expression Results in Altered Puma/Noxa Localization

Two proteins induced by p53 are the BH3 domain only pro-apoptotic proteins Puma and Noxa (Yu *et al*., 2005). These proteins are involved in the induction of the caspase cascade by their interactions with Mcl-1 and Bcl-X_L. Two Puma proteins are generated from the Puma gene,

Puma–α and Puma-β, both are induced by p53 and bind Bcl-X_L and Mcl-1. Puma can induce the displacement of Mcl-1 from Bak and Bax Puma then induces conformational changes in Bax which results in Bax's translocation to the mitochondria, cytochrome C release and apoptosis. An overview of the interactions of Puma, Mcl-1, p53, Bak and Bax is presented in Figure 14.

The expression of Puma is under the control of the PI3K/Akt pathway as it has recently been shown that FOXO-3a regulates the expression of Puma (You *et al.*, 2006). Noxa is another BH3-domain protein which can be induced by p53. Noxa has recently been shown to interact specifically with Mcl-1 and A1 (Chen *et al*.,2005) but not with Bcl-2, Bcl-X_L or Bcl-2. The pro-apoptotic Bak molecule associates with Mcl-1 and Bcl-X_L but not Bcl-2, Bcl-w or A1 (Willis *et al.*, 2005). Upon induction of Noxa by activation of p53, Noxa binds Mcl-1 and displaces Bak. This leads to Mcl-1 degradation and Bak is free to induce apoptosis.

If the Raf>MEK>ERK pathway increases Mcl-1 protein levels and stability, that may lead to an increase in Mcl-1 associated with Noxa and Puma and a decrease in free Bak levels. Alternatively, PI3K>Akt may phosphorylate FOXO-3a which results in decreased Puma expression. Both of these effects on Noxa and Puma may be required for drug resistance.

Raf>MEK>ERK Elevates Caspase 9 Phosphorylation in Doxorubicin-Resistant Cells

Human Caspase 9 was originally thought to be phosphorylated by Akt (Cardone *et al.*, 1998), but the murine caspase 9 lacks the Akt consensus phosphorylation site (Allan *et al.*, 2003). Caspase 9 is phosphorylated by the Raf>MEK>ERK pathway at T125 which inhibits activation of the caspase cascade. Elevated phosphorylation of caspase 9 may be responsible for the decreased Caspase 3 detected in the doxorubicin resistant cells.

One of the targets of caspase 3 is Mcl-1 (Weng *et al.*, 2005). Decreased caspase 3 activation could lead to a decrease in Mcl-1 cleavage. The extent of cleavage of Mcl-1 in the doxorubicin sensitive and resistant cells could be different, resulting in the prevention of apoptosis in the doxorubicin resistant cells. An overview of the effects of the effects of Raf>MEK>ERK and PI3K>Akt pathways on the regulation of caspase activity and drug resistance is presented in Figure 15.

Raf>MEK>ERK Elevates the Phosphorylation of Other Targets Responsible for Drug Resistance

Obviously, there are other downstream targets which elevate Raf>MEK>ERK. These include: p90Rsk, p70S6K, $p21^{Cip1}$, $p27^{Kip1}$, Bcl-2 and others. However, in order to keep this discussion focused we have discussed the most direct targets of Raf>MEK>ERK which could lead to drug resistance.

Raf>MEK>ERK Activates the Expression of Membrane Transporters other than Mdr-1/MRP-1 Which Lead to Drug Resistance

A membrane transporter protein other than MDR-1 or MRP-1 may be involved in the drug resistance of the cells (BCRP-1, MRP2, MRP3, MRP4, MRP5, MRP6, MRP7, MRP8).

Summary

We have presented data which documents the importance of the Raf>MEK>ERK and PI3K>Akt pathways in the development of drug resistance in hematopoietic cells. Further understanding of how these pathways interact and induce drug resistance could result in the identification of novel approaches to treat drug resistance in leukemia. Furthermore, p53 played a role in drug resistance in these cells as introduction of a DN-p53 construct increased the

resistance of the cells to chemotherapeutic drugs. The drug sensitive and drug resistant FL/ΔAkt:ER+ΔRaf-1:AR cells will allow us the ability to determine not only which downstream components are induced by either Raf>MEK>ERK or PI3K>Akt that are necessary for proliferation and prevention of apoptosis, but also which components are important in drug resistance and how these two pathways can interact to influence drug resistance.

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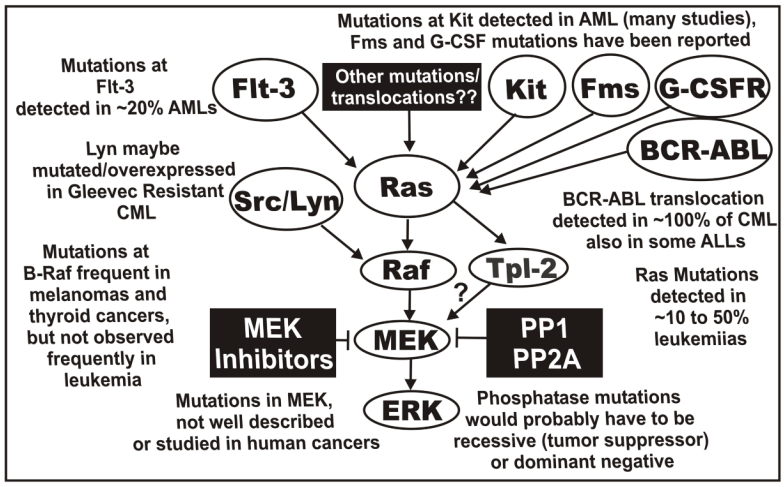

Fig. 1.

Sites of mutation which can result in activation of the Raf>MEK>ERK pathway. Mutations have been detected in Flt-3, Ras, Kit, Fms, G-CSFR, and at lower frequencies Raf-1 and B-Raf in AML. The BCR-ABL chromosomal translocation is present in virtually all CMLs and some ALLs. These mutations and chromosomal translocations could all result in activation of the Raf>MEK>ERK cascade. A ? is indicated in the connection between Tpl-2 and MEK. This is to indicate that there are other MEK activators besides Raf which can result in MEK activation and may confer sensitivity to MEK inhibitors. Mutations at phosphatase genes could also result in activation of this pathway although they would be predicted to be either tumor suppressor or dominant negative type mutations.

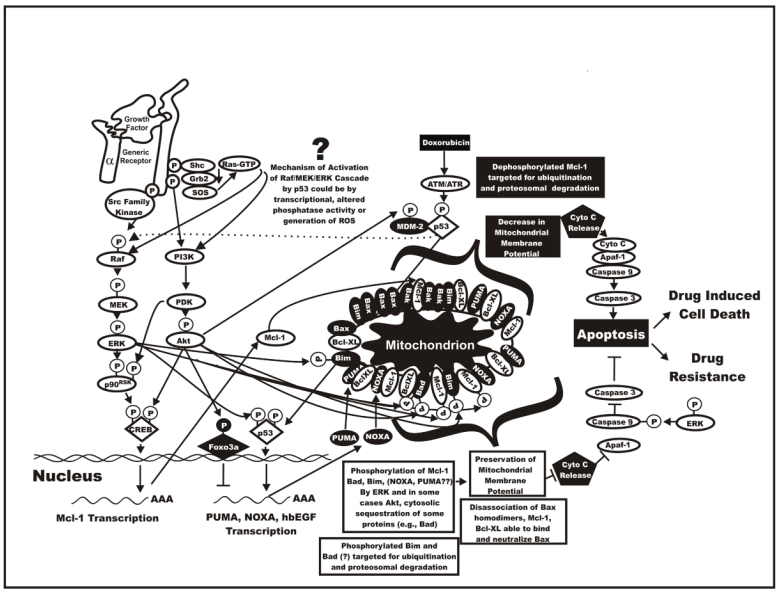

Fig. 2.

Overview of interactions between Raf>MEK>ERK, PI3K>Akt, p53 and apoptotic pathways resulting in drug resistance. The Raf>MEK>ERK and PI3K>Akt pathways can phosphorylate transcription factors which can stimulate gene transcription or apoptotic regulatory molecules which control the induction of apoptosis. Possibly through reactive oxygen species (ROS), doxorubicin can induce Raf>MEK>ERK. Doxorubicin can also activate p53 which can induce the transcription of molecules involved in the regulation of apoptosis. Heparin binding epidermal growth factor (hb-EGF) is a transcriptional target of p53 which could induce activation of the Raf>MEK>ERK cascade. Finally doxorubicin could induce p53 which alters the expression of phosphatases which could lead to prolonged ERK activation. Dysregulation of these cascades can result in the prevention of apoptosis and the induction of drug resistance.

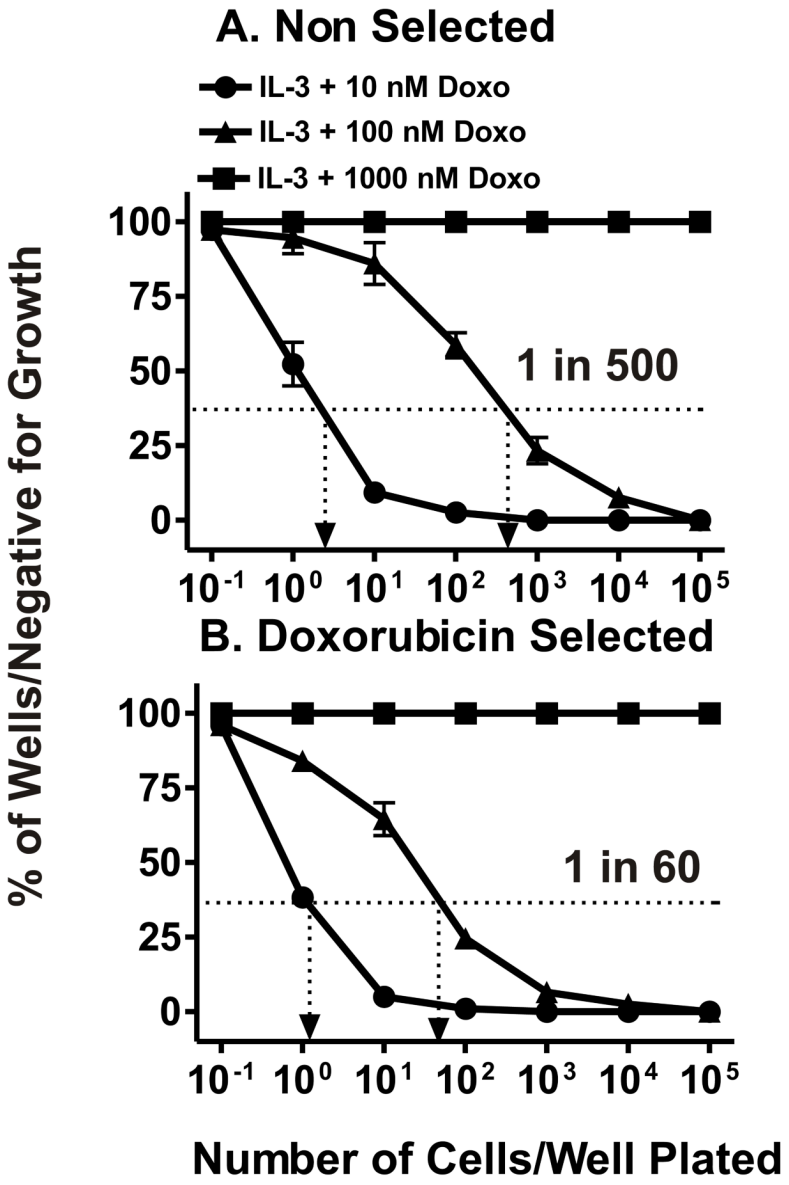

Fig. 3.

Isolation of doxorubicin resistant cells from FL5.12 and enhanced subcloning efficiency in doxorubicin. Limiting dilution analysis was performed in the presence of different concentrations of doxorubicin on the FL5.12 (Panel A) and doxorubicin resistant FL/Doxo (Panel B) cells. A dotted line is indicated at 37% of wells negative for growth from which the cloning efficiency can be estimated. These experiments have been performed 4 times and averaged together. Limiting dilution analysis with FL/Doxo-1 is presented in Panel B, similar results were observed with 2 other FL/Doxo clones.

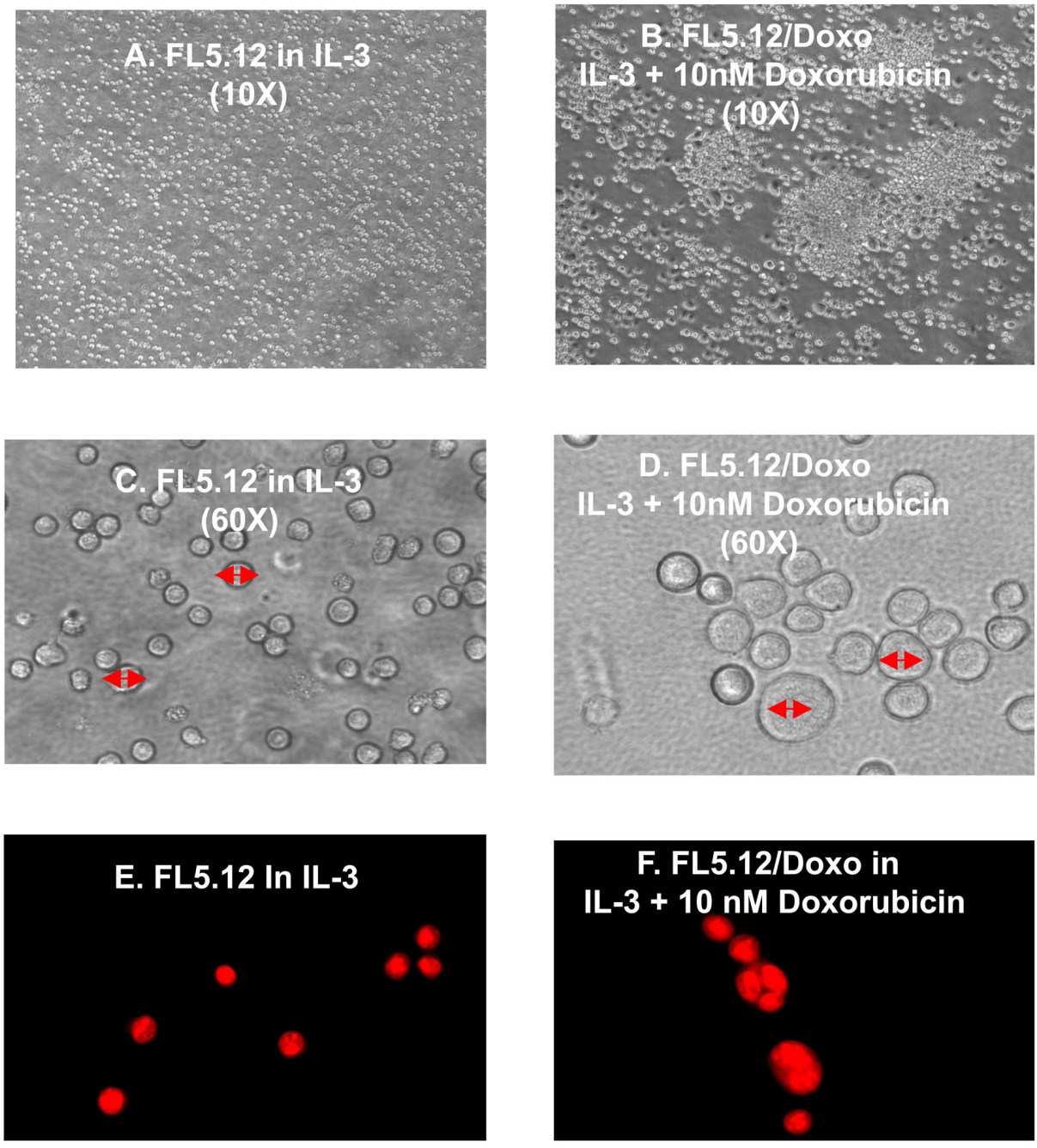

Fig. 4.

Doxorubicin resistant FL5.12 cells are larger, more blast like and some are multinucleate. The morphology of FL/5.12 and FL/Doxo cells was examined by light microscopy (Panels A & B, 10X magnification), (Panels C & D, 60X magnification). The cells were also stained with acridine orange and the nuclear morphology examined (Panels E & F).

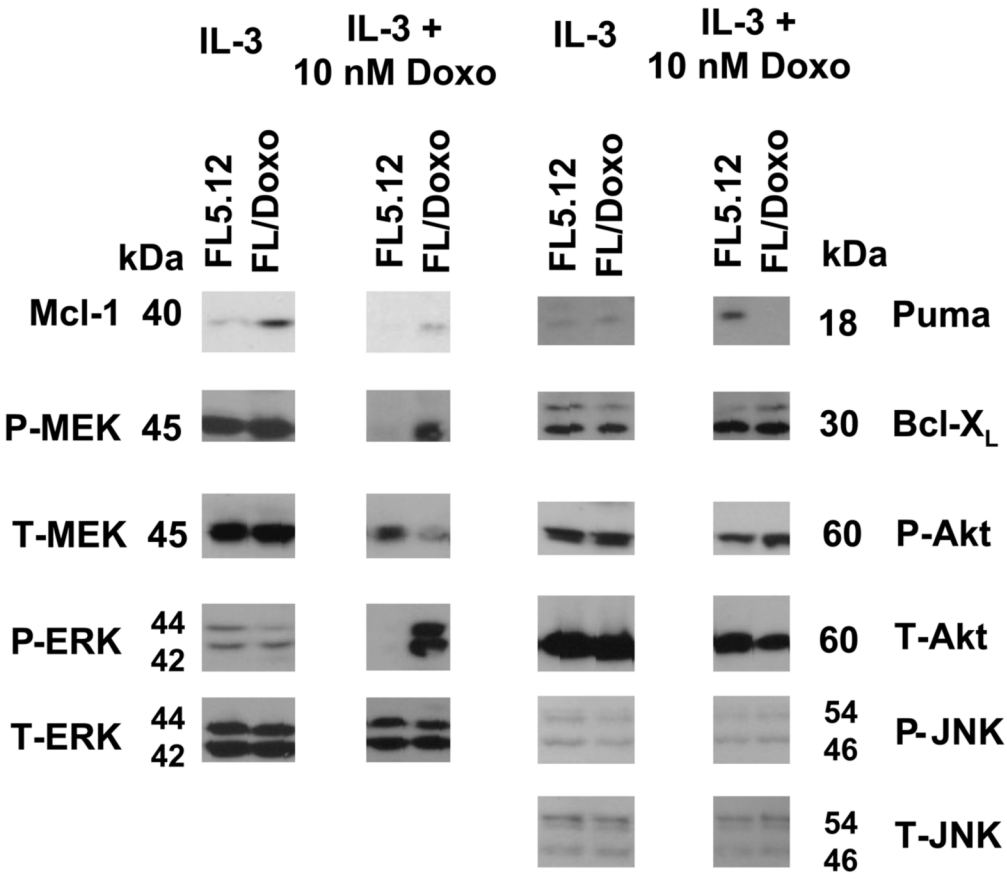

Fig. 5.

Increased activated Mcl-1, pMEK and pERK in doxorubicin resistant FL5.12 cells. FL5.12 and FL/Doxo cells were grown for 24 hours in medium containing IL-3 or IL-3 + 10 nM doxorubicin and then western blot analysis was performed with the indicated antibodies.

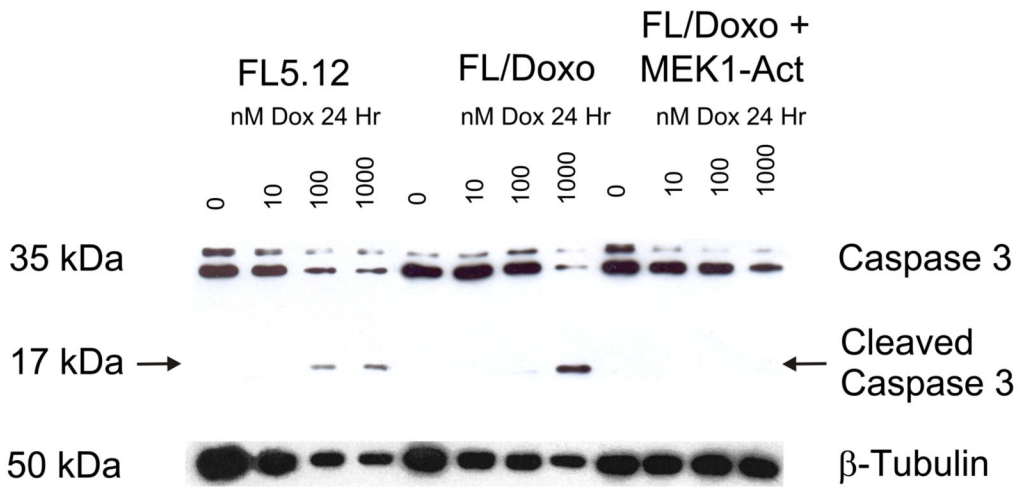

Fig. 6.

Decreased caspase 3 cleavage in doxorubicin resistant cells. The extent of cleavage of Caspase 3 was determined in doxorubicin sensitive FL5.12 cells and doxorubicin resistant FL/Doxo and FL/Doxo+MEK1-Act. The cells were incubated in the indicated concentrations of doxorubicin for 24 hours and then protein lysates isolated.

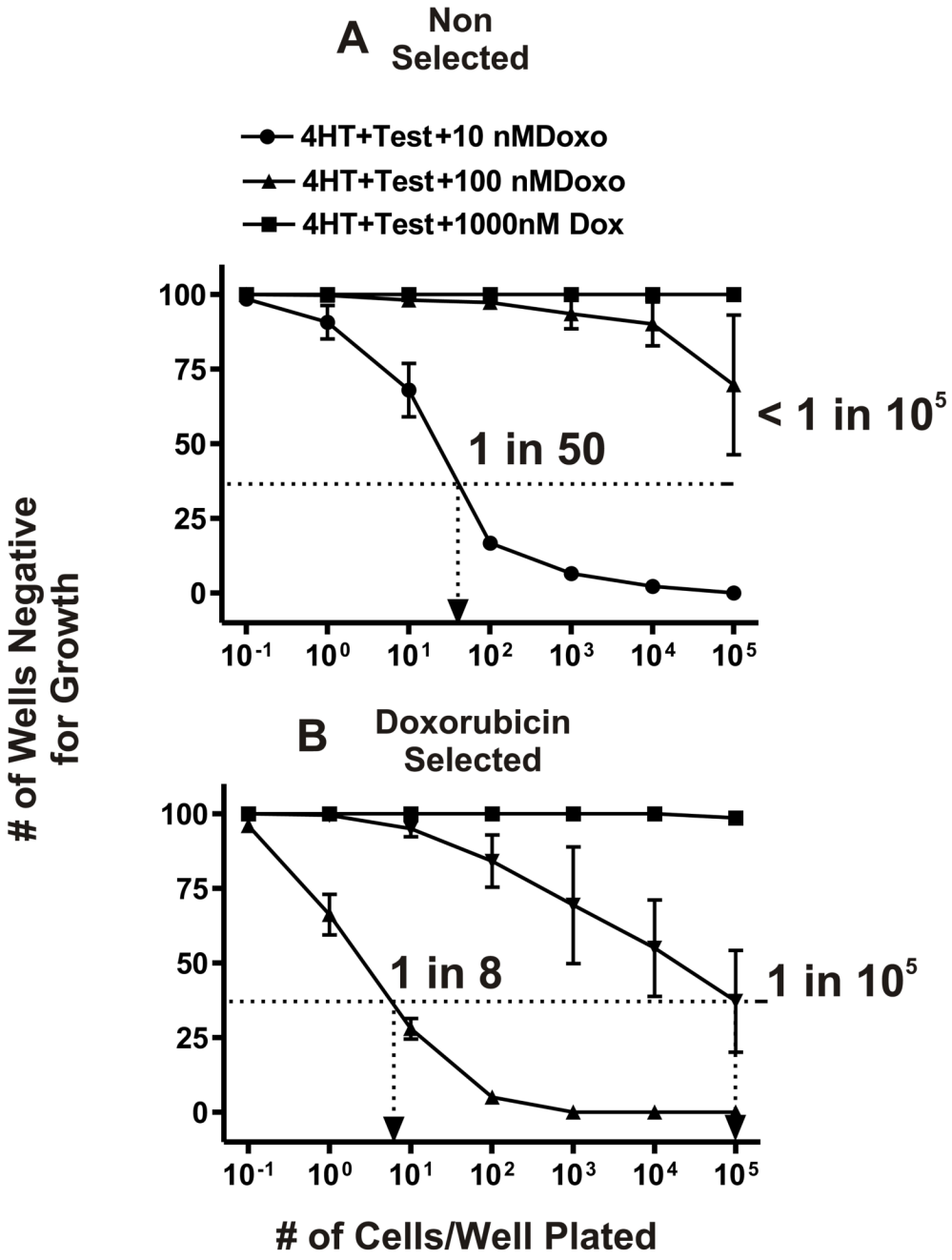

Fig. 7.

Isolation of doxorubicin resistant FL/ΔAkt:ER*+ΔRaf-1:AR cells. Limiting dilution analysis was performed in the presence of different concentrations of doxorubicin on FL/ΔAkt:ER +ΔRaf-1:AR cells. These results were obtained when the cells were cultured in medium containing 4HT + testosterone. Additional limiting dilution analyses indicated that neither 4HT nor testosterone were sufficient by themselves to result in the isolation of drug resistant cells which could be expanded into larger cultures. A dotted line is indicated at 37% of wells negative for growth from which the cloning efficiency can be estimated. These experiments have been performed 5 times and averaged together. Limiting dilution analysis with FL/ΔAkt:ER

+ΔRaf-1:AR clone 1 is presented in Panel B, similar results were observed with 2 other FL/ΔAkt:ER+ΔRaf-1:AR clones.

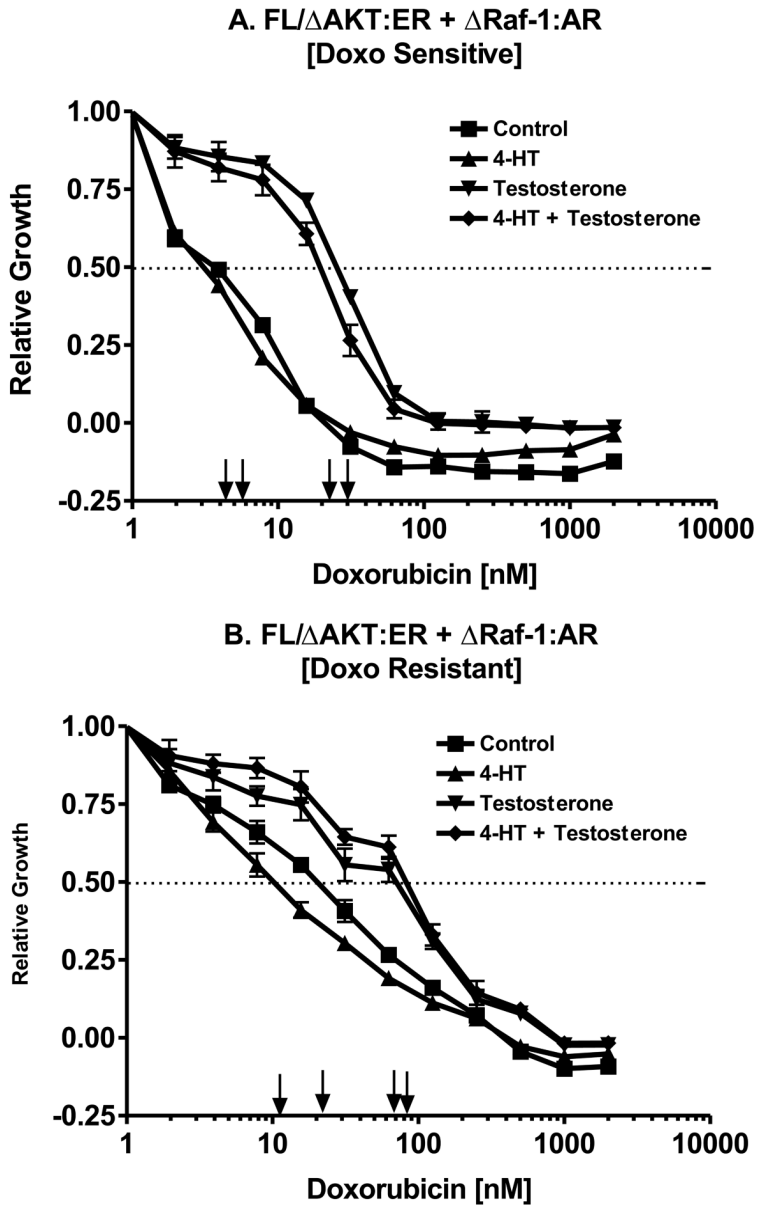

Fig. 8.

Dominant role of Raf in driving drug resistance. The effects of Raf activation by testosterone and Akt activation by 4HT on the doxorubicin IC_{50} of non selected and doxorubicin selected FL/ΔAkt:ER+ΔRaf-1:AR cells were examined by MTT analysis in 96 well plates. Activation of Raf increased the IC_{50} in both the non selected and doxorubicin selected cells.

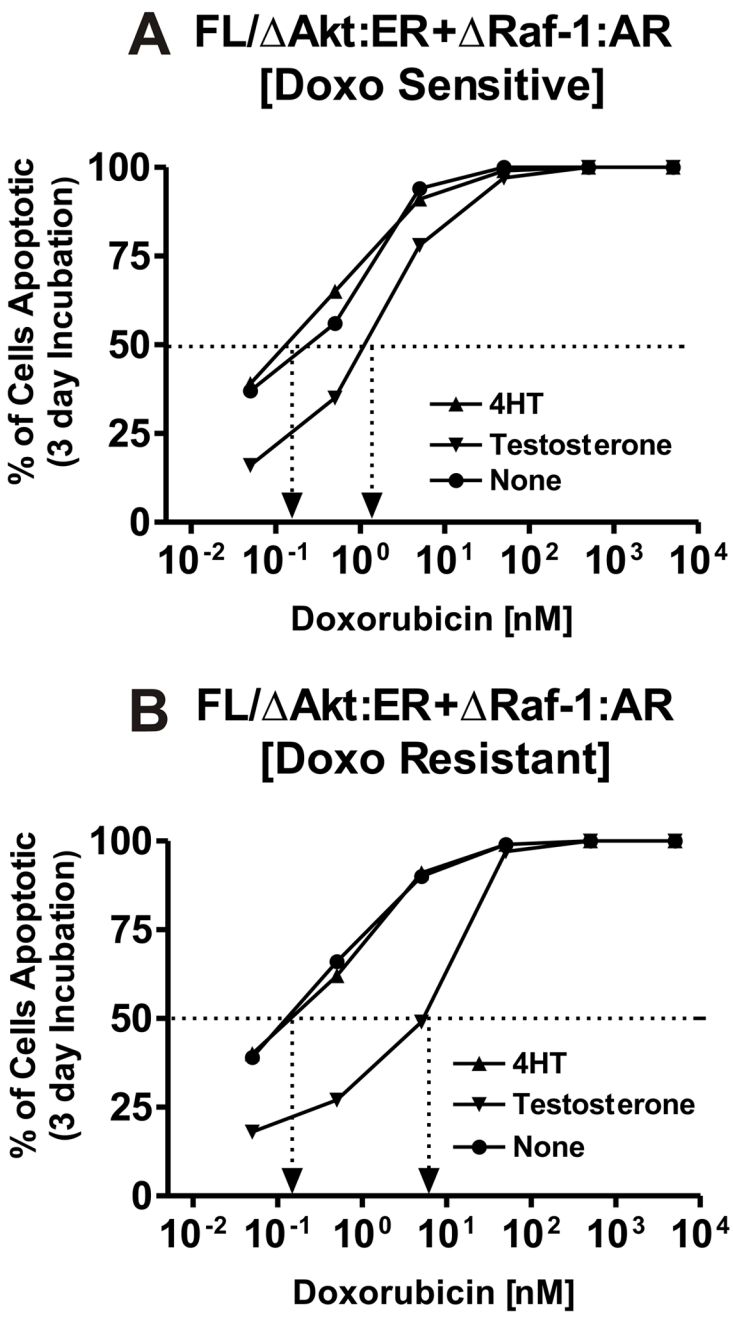

Fig. 9.

Dominant role of Raf in preventing apoptosis. The effects of Raf activation by testosterone and Akt activation by 4HT on the prevention of apoptosis induced by doxorubicin was determined in non selected and doxorubicin selected FL/ΔAkt:ER+ΔRaf-1:AR cells by the annexin V/PI technique. The extent of apoptosis was determined after incubation of the cells for 3 days in the different concentrations of doxorubicin. Cells were cultured with medium supplemented with 4HT, testosterone (test) on no supplement. Activation of Raf was dominant in the suppression of apoptosis.

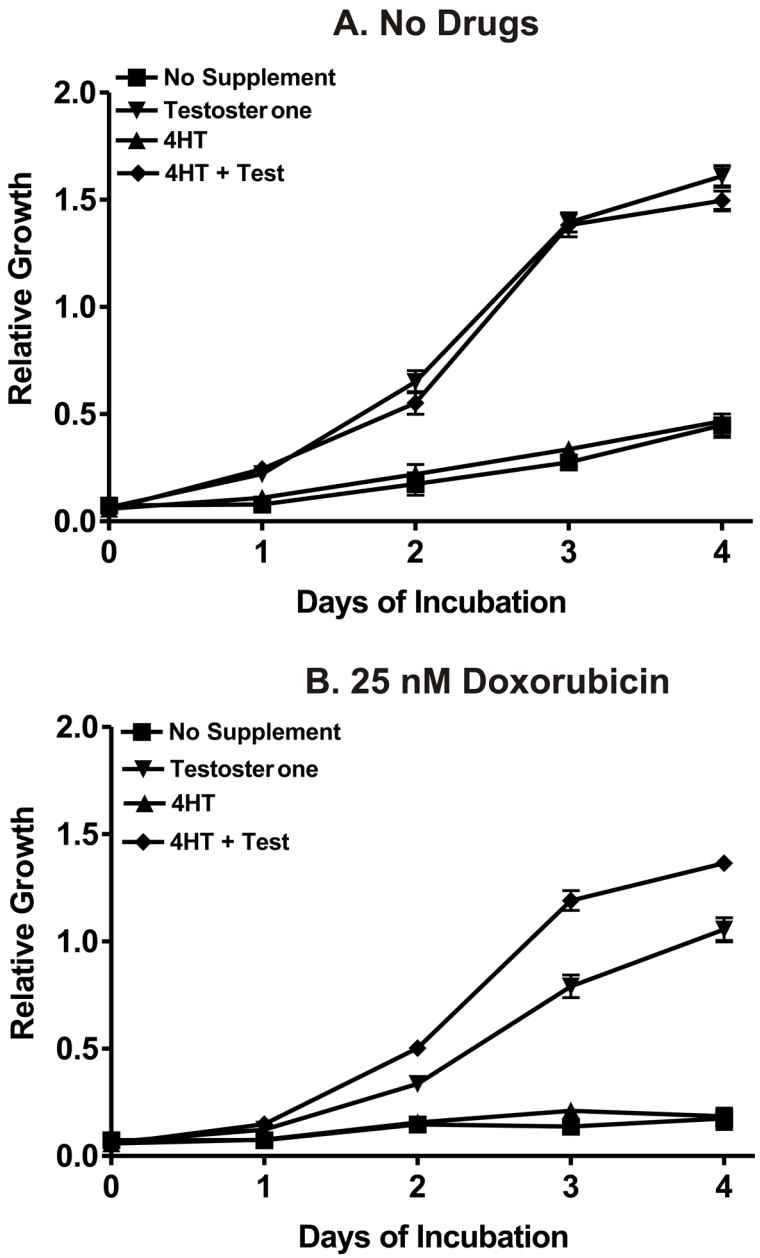

Fig. 10.

Requirement of Raf and Akt in drug resistant growth. The effects of Akt activation by 4HT, Raf activation by testosterone and their co-activation in doxorubicin selected FL/ΔAkt:ER +ΔRaf-1:AR cells was determined by MTT analysis in the presence and absence of 25 nM doxorubicin. Activation of Akt was not necessary for growth in 100 μL cultures over a 4 day period in the absence of drugs. In contrast, activation of Akt enhanced the proliferation of the cells when they were cultured in the presence of testosterone and 25 nM doxorubicin. Similar results were observed with paclitaxel and daunorubicin.

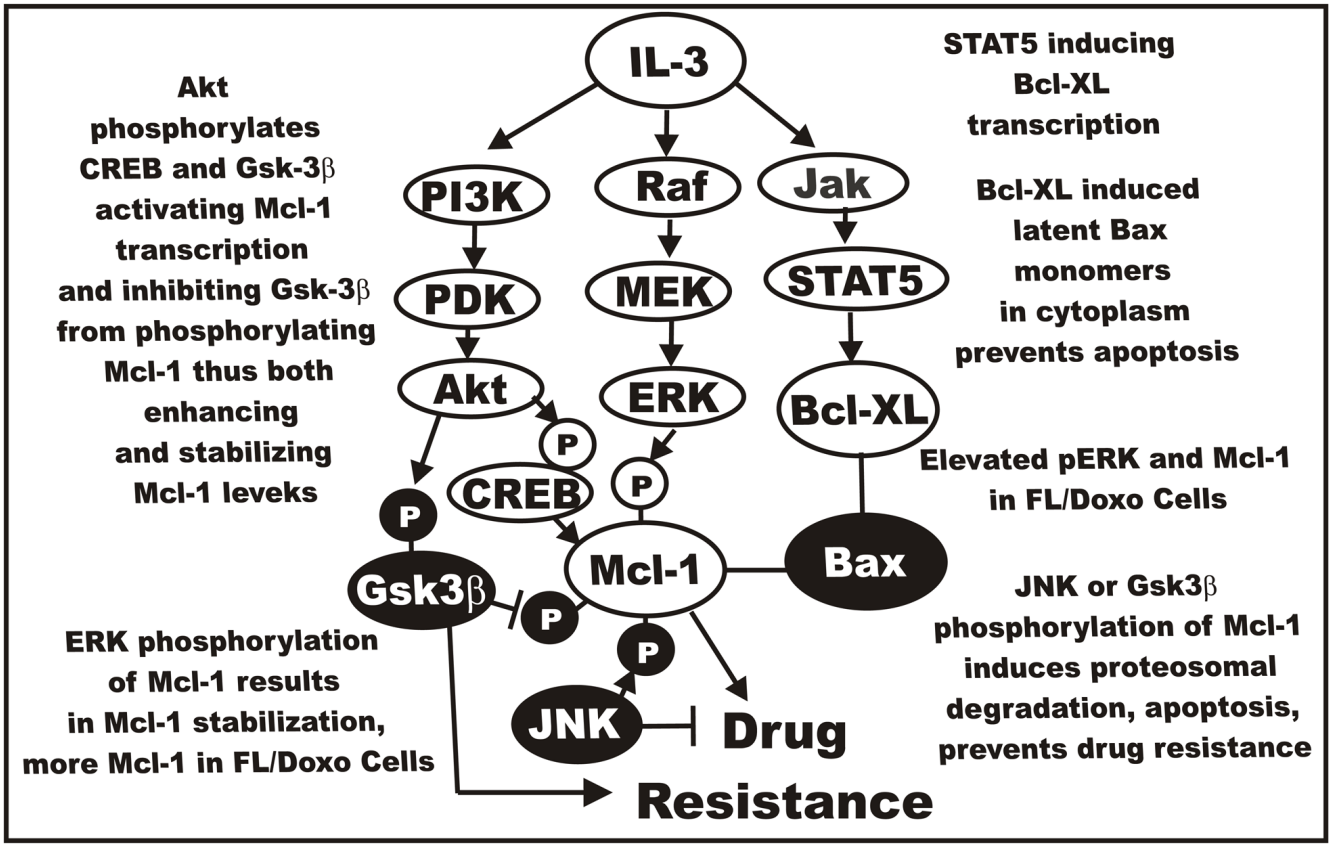

Fig. 11.

Cytokine mediated signal transduction pathways and drug resistance. Cytokines such as IL-3 can induce multiple signal transduction pathways which can effect the expression of apoptotic molecules by transcriptional and post-transcriptional mechanisms. Elevated ERK in FL/Doxo cells may phosphorylate Mcl-1 which results in its stabilization. This may result in prolonged binding to Bax, prevent activation of Bax, contribute to the prevention of apoptosis and lead to drug resistance.

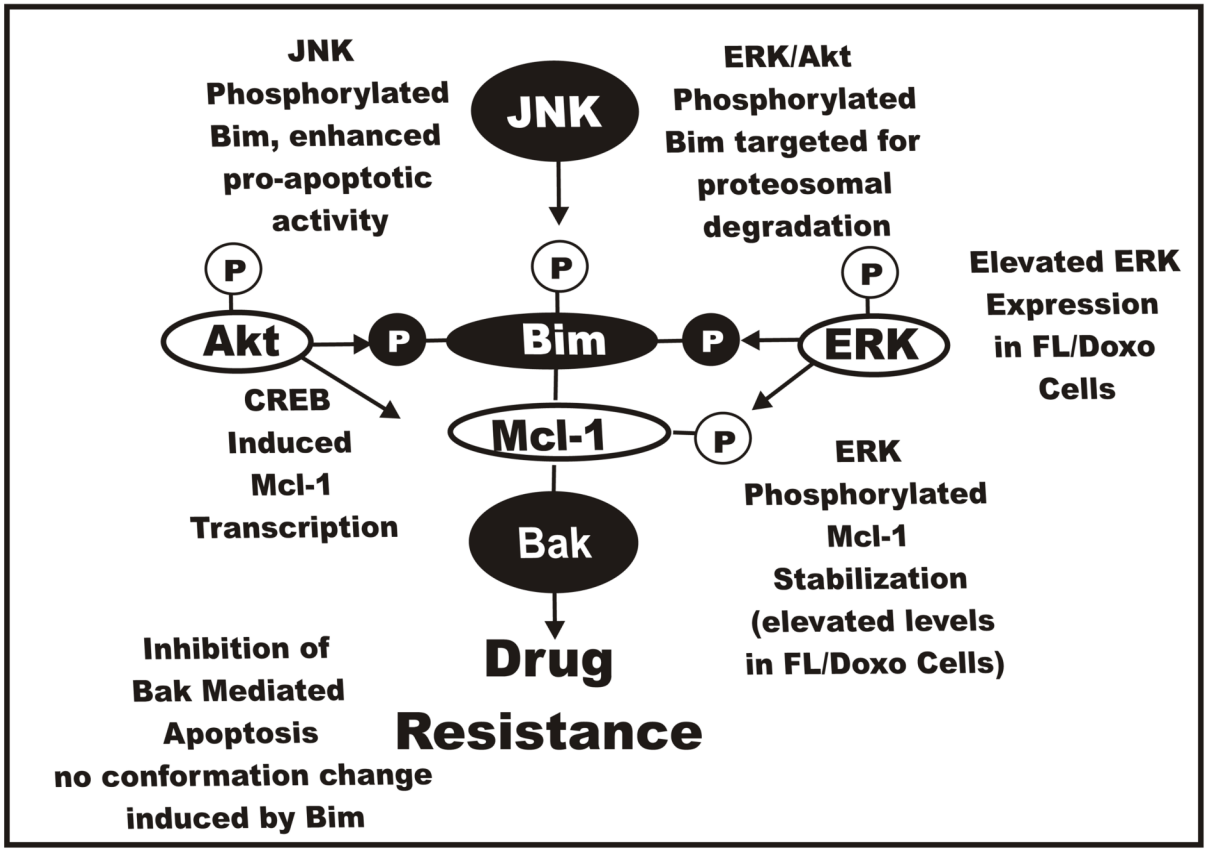

Fig. 12.

Effects of Raf>MEK>ERK and PI3K>Akt and JNK pathways on Bim phosphorylation and the induction of drug resistance. All three of these pathways can phosphorylate Bim on different residues which affect its activity and interactions with Mcl-1 and Bax and Bak. Phosphorylation events mediated by Raf>MEK>ERK and PI3K>Akt result in the prevention of Bax and Bak activation and lead to Bim being targeted to the proteosome ubiquitination and degradation. In contrast phosphorylation of Bim by JNK results in its dissociation of Bim:Mcl-1 heterodimers, Mcl-1 is targeted to the proteosome, ubiquitination, and degradation and Bim mediated activation of Bax and Bak.

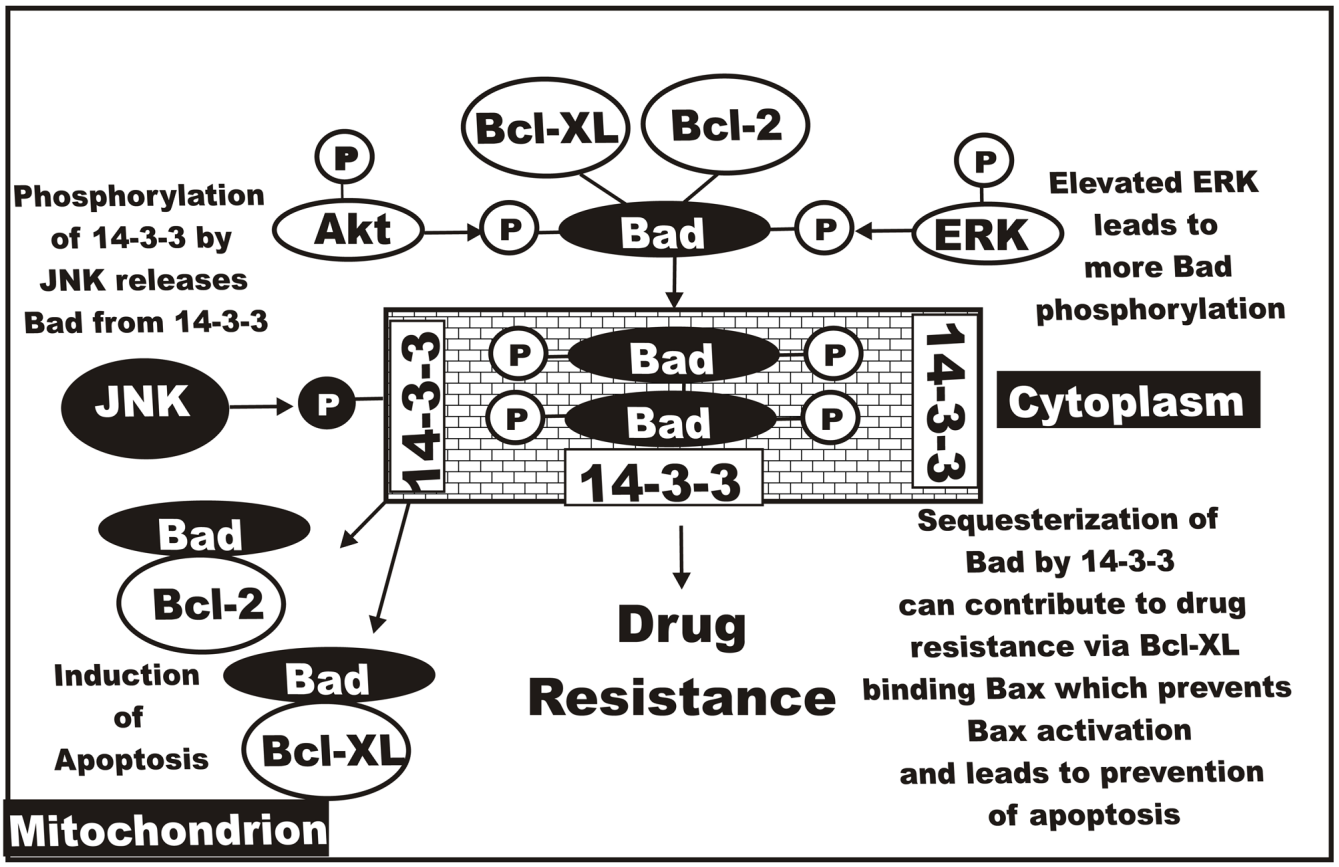

Fig. 13.

Effects of Raf>MEK>ERK and PI3K>Akt and JNK pathways on bad phosphorylation and the induction of drug resistance. All three of these pathways can phosphorylate Bad on different residues which affect its activity and interactions with Bcl-2 and Bcl-X_L. Phosphorylation events mediated by Raf>MEK>ERK and PI3K>Akt result in Bad being associated with 14-3-3 proteins and translocation from the mitochondrion to the cytoplasm. Bcl-2 and Bcl-X_L remain associated with Bax and Bak which prevent their activation and lead to suppression of apoptosis. In contrast phosphorylation of Bad by JNK results in its dissociation with 14-3-3 proteins and Bad localizes to the mitochondrion and binds Bcl-2 and Bcl-X_L. Bax and Bak are then able to induce apoptosis.

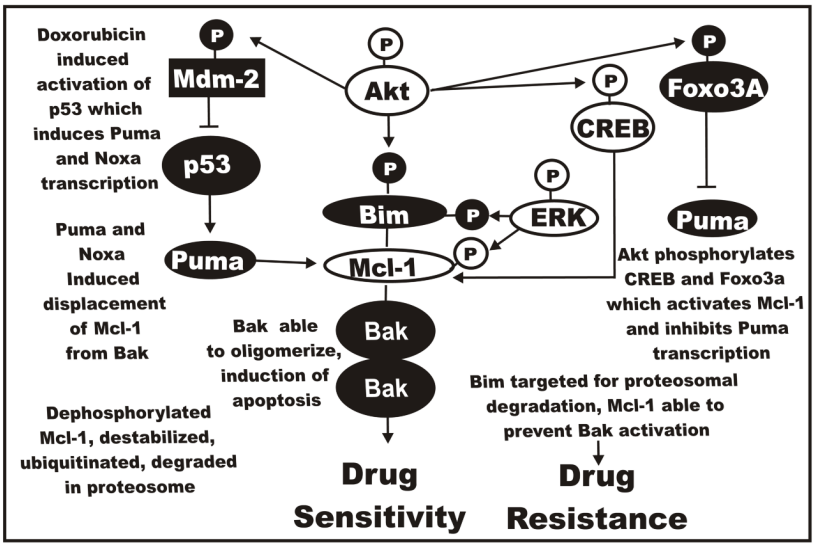

Fig. 14.

Effects of Raf>MEK>ERK, PI3K>Akt and p53 pathways on noxa and puma and the induction of drug resistance. p53 can induce the BH3 only containing Noxa and Puma proteins which interact with Mcl-1 and other anti-apoptotic proteins. When Mcl-1 is associated with Noxa and Puma that prevents their ability to interact with Bax and Bak. Increased expression of ERK in FL/Doxo cells may result in increased Mcl-1 levels which prevent Noxa and Puma abilities to activate Bax and Bak.

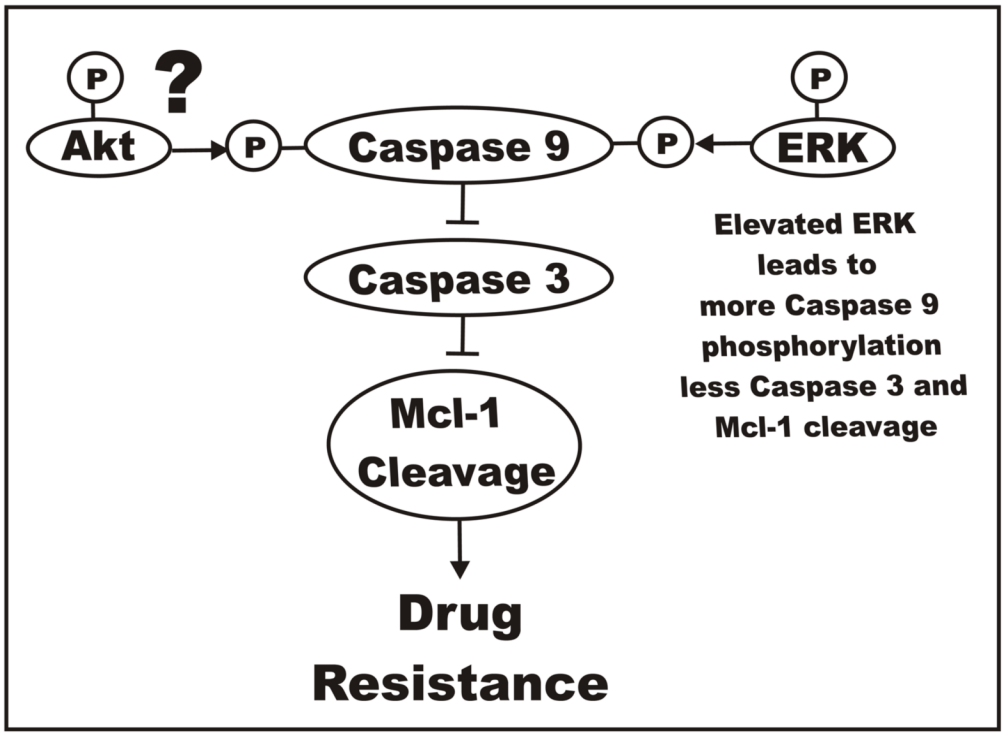

Fig. 15.

Effects of Raf>MEK>ERK and PI3K>Akt pathways on caspase 9 phosphorylation and the induction of drug resistance. The Raf>MEK>ERK pathway phosphorylates caspase 9 which prevents activation of caspase 3. The ability of Akt to phosphorylate caspase 9 is controversial as the Akt consensus phosphorylation site is present in mouse but not human caspase 9. Increased phosphorylation of caspase 9 by ERK in FL/Doxo cells could result in less caspase 9 activation, less caspase 3 activation and less Mcl-1 cleavage which could result in the prevention of apoptosis and contribute to drug resistance.

Table 1

Differences in growth IC_{50}s in doxorubicin sensitive and resistant FL5.12 cells[1]

Cell Line→	**FL5.12**	**FL/Doxo**	**Fold Difference**
Drug↓			
Doxorubicin	10 nM	90 nM	9X
Daunorubicin	4 nM	20 nM	5X
Paclitaxel	1.8 nM	130 nM	72X
5-Flurouracil	800 nM	1000 nM	1.3X
Cisplatin	65,000 nM	85,000 nM	1.3X

[1]Determined by plating 2500 cells/well in 96 well plates in phenol red free RPMI+10% FBS+IL-3 and serial 2-fold dilutions (n=12 dilutions) at 8 wells per each drug concentration. MTT analysis was performed after 4 days of incubation and results were normalized to untreated cells.

Table 2

Differences in apoptotic IC_{50}s in doxorubicin sensitive and resistant FL5.12 cells[1]

Cell Line→	**FL5.12**	**FL/Doxo**	**Fold Difference**
Drug↓			
Doxorubicin	10 nM	100 nM	10X
Daunorubicin	0.5 nM	25 nM	50X
Paclitaxel	0.1 nM	9 nM	90X
5-Flurouracil	1000 nM	1000 nM	1X

[1] Determined by plating 10^6 cells/well in 6 well plates in RPMI+10% FBS + IL-3 and serial 10-fold dilutions (n=6 dilutions) at 3 wells per each concentration of the different drugs. Annexin V/PI apoptosis analysis was performed after 3 days of incubation and results were normalized to untreated cells.

Table 3

Effects of DN MEK1 and DNp53 on doxorubicin C_{50}[1]

	Cell Line	
Gene Introduced↓	**FL5.12**	**FL/Doxo**
DN-MEK1	2 nM	15 nM
Empty Vector	15 nM	85 nM
DN-p53	30 nM	200 nM
WT-p53	10 nM	80 nM

[1] Determined by MTT analysis as described in the legend to Table 1.

Table 4

Differences in IC_{50} in doxorubicin sensitive and resistant FL/ΔAkt:ER*(Myr^+)+ΔRaf-1:AR cells[1]

Cell Line→ Drug↓	Doxo-Sensitive	Doxo-Resistant	Fold Difference
Doxorubicin	25 nM	75 nM	3X
Daunorubicin	12 nM	30 nM	2.5X
Paclitaxel	3 nM	18 nM	6X

[1] Determined by plating 2500 cells/well in 96 well plates in phenol red free RPMI+10% FBS + 500 nM 4HT +100 nM Test and serial 2-fold dilutions (n=12 dilutions) at 8 wells per each drug concentration. MTT analysis was performed after 4 days of incubation and results were normalized to untreated cells. These experiments differ from those presented in Tables 1, 2 & 3 as in those cases the cells were plated in IL-3.