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Synergistic combinations of signaling pathway inhibitors: Mechanisms for improved cancer therapy

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Abstract

Cancer cells contain multiple signal transduction pathways whose activities are frequently elevated due to their transformation, and that are often activated following exposure to established cytotoxic therapies including ionizing radiation and chemical DNA damaging agents. Many pathways activated in response to transformation or toxic stresses promote cell growth and invasion and counteract the processes of cell death. As a result of these findings many drugs, predominantly protein and lipid kinase inhibitors, of varying specificities, have been developed to block signaling by cell survival pathways in the hope of killing tumor cells and sensitizing them to toxic therapies. Unfortunately, due to the plasticity of signaling processes within a tumor cell, inhibition of any one growth factor receptor or signaling pathway frequently has only modest long term effects on cancer cell viability, tumor growth, and patient survival. As a result of this realization, a greater emphasis has begun to be placed on rational combinations of drugs that simultaneously inhibit multiple inter-linked signal transduction/survival pathways. This, it is hoped, will limit the ability of tumor cells to adapt and survive because the activity within *multiple* parallel survival signaling pathways has been reduced. This review will discuss some of the approaches that have been taken to combine signal transduction modulatory agents to achieve enhanced tumor cell killing.

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1. Introduction on growth factor receptors, signal transduction pathways and small molecule inhibitors

Cancer cells have elevated activities within multiple signal transduction pathways due to (reviewed in Valerie et al., 2007; Dent et al, 2003):

- **i.** the increased expression of paracrine ligands acting on growth factor receptors;
- **ii.** receptor over-expression;
- **iii.** oncogenic activating mutations of receptors and downstream RAS GTP binding proteins;
- **iv.** inactivation of tumor suppressor lipid and protein phosphatases;
- **v.** oncogenic activation of downstream protein and lipid kinases.

As a result of these modifications, in comparison to non-transformed cells, cancer cells divide more rapidly, are more migratory and invasive and have a greater capacity to survive exposure to a variety of toxic stresses, including those from anticancer therapeutics.

An overview of signal transduction pathway structure is provided to assist with understanding some of the concepts in this review, with emphasis on the original “mitogen activated protein (MAP) kinase” pathway. The p42 (ERK2) and p44 (ERK1) MAPKs are activated by dual tyrosine and threonine phosphorylation; they activate another protein kinase ($p90^{rsk}$) by catalyzing its serine/threonine phosphorylation **(**Figure 1). When activated, ERK1/2 and $p90^{rsk}$ can migrate to the nucleus where they regulate transcription factors such as CREB, Elk-1 and Ets1 (Sturgill, 2008;Dent et al., 2003). ERK1/2 are phosphorylated and activated by MAPK kinases termed MEK1/2 (MAP2K). MEK1 and MEK2 are dual specificity tyrosine/threonine kinases and are activated by dual serine phosphorylation. The protein kinase responsible for catalyzing MEK1/2 activation was initially described as Raf-1 (C-Raf). Raf-1 is a member of a family of serine-threonine protein kinases termed Raf-1, B-Raf, and A-Raf; all “Raf” family members to varying extents phosphorylate and activate MEK1/2. Thus, the “Raf” protein kinases act at the level of a MAPK kinase kinase (MAP3K). The NH_2 domain of Raf-1 reversibly interacts with RAS proteins in the plasma membrane, and the ability of Raf-1 to associate with RAS proteins is dependent on the RAS molecule being in the GTP-bound state (Ramos, 2008;McCubrey et al, 2008). Growth factor receptors, such as the epidermal growth factor receptor (EGFR) family (ERBB1–4), stimulate GTP for GDP exchange in RAS. Raf-1 activation is dependent on Raf-1 translocation to the plasma membrane followed by phosphorylation of Raf-1 S338 and Y341, and dephosphorylation of Raf-1 S259. Thus, a signaling pathway can be delineated from receptor tyrosine kinases through the activation of RAS proteins, translocation of Raf proteins to the plasma membrane, and the feeding of signals into MEK1/2-ERK1/2-$p90^{rsk}$ with resultant regulation of gene expression and modulation of apoptosis regulatory proteins. The activity of this pathway is elevated in many tumor cells, compared to their non-transformed counterparts, and as a result it was one of the first to be therapeutically targeted by small molecule kinase inhibitors at the level of the growth factor receptors (ERBB1, ERBB2), at the level of RAS proteins and at the level of MEK1/2 (Wong, 2009;Anjum and Blenis, 2008; Sebolt-Leopold and Herrera, 2008).

Additional comparative cloning studies in mammalian and yeast cells showed that the original "MAPK" pathway was in fact one of many MAPK pathways. Thus, the ERK1/2-MAPK pathway has served as a template around which other MAPK family pathways have been described: the c-Jun NH_2-terminal kinase (JNK) 1/2/3, the p38 MAPK, and the "big MAPK" MEK5-ERK5 pathways. In each pathway, a MAPK, a MAP2K, and a MAP3K have been shown to exist and behave phenomenologically in a similar manner to proteins described in the original ERK1/2 cascade (reviewed in Dent et al., 2003; Raman et al., 2007).

In a similar conceptual manner to MAP kinase pathways, the PI3K signaling pathways, PI3K-phosphoinositide-dependent kinase-1 (PDK1)-AKT-glycogen synthase kinase 3 and PI3K-phosphoinositide-dependent kinase-1-AKT-mammalian target of rapamycin (mTOR)-$p70^{S6K}$, have also been delineated (Ihle and Powis, 2009; Memmott and Dennis, 2009). There are three AKT genes with AKT1 being most closely linked to proliferation and apoptosis resistance and AKT2 linked to insulin signaling, and AKT3 has been shown to act as a transforming kinase in melanoma (Steelman et al, 2008; Di Cosimo and Baselga, 2008; Stahl et al., 2004). The ability of PI3K to signal downstream to PDK1 is negatively regulated by the proto-oncogene phosphatase and tensin homologue on chromosome ten (PTEN); PTEN is frequently mutated, deleted or epigenetically silenced in prostate cancer, melanoma and glioblastoma (e.g. Carnero et al., 2008). PI3K p110 catalytic subunits are also noted in some tumors to be mutated to a partially active conformation (Gonzalez and De Groot, 2008; Knobbe et al., 2008). The activities of multiple MAPK pathways and of the PI3K pathway are variably elevated and/or suppressed in a variety of transformed cells compared to non-transformed cells, and studies have linked enhanced basal levels of ERK1/2, PI3K-AKT-mTOR, and ERK5 pathway activity in tumor cells to increased rates of proliferation and protection of cells from toxic stresses. Conceptually this heightened signaling activity could provide a therapeutic window for kinase inhibitors to selectively impact on tumor cell, but not non-transformed cell, growth and viability. Similarly, reduced basal activity levels or the abilities of stresses to activate the JNK1/2 and p38 MAPK pathways correlate with enhanced tumor cell viability.

Thus, based on data from several decades of research, a wide variety of signaling proteins and signaling pathways have been causatively linked in many tumor types to cell growth, invasion and survival that has in turn prompted multiple drug companies and synthetic chemists to generate therapeutically active inhibitors, predominantly of kinases, and in particular agents that bind within the ATP binding site(s) within the kinase domain.

Important examples are (see Table 1): small molecule inhibitors of growth factor receptors such as ERBB1–4 (erlotinib, canertinib, gefitinib, lapatinib) as well as inhibitory antibodies; PDGFR (nolitinib, sorafenib; ABT869), c-Met (PHA665752), and the IGF1R (BMS536924; and inhibitory antibodies); FLT3 (ABT869) (Chu and Small, 2009); Abl and c-Kit tyrosine kinases (imatinib, nolitinib); Src family tyrosine kinases (dasatinib, AZD0530); Checkpoint/Chk1 regulatory kinase (UCN-01, AZD7762); vascular endothelial growth factor receptors (sorafenib, sunitinib; AG13726 and inhibitory antibodies) (Broxterman and Georgopapadakou, 2005); Heat shock protein 90 (17AAG, 17DMAG); Aurora kinases (VE465, MK0457) (see Figure 2); cyclin dependent kinases (flavopiridol, R-roscovitine (CYC202)); phosphatidyl inositol 3 kinase (PX866, BEZ235, BGT226, XL147); mTOR (Rapamycin (sirolimus), RAD001 (everolimus) AP23573 (deforlimus), CI779 (temsirolimus), BEZ235, PI103) (Jiang and Liu, 2008); IκB kinase (IKI-1, AS602868); AKT (perifosine, GSK690693); MEK1/2 (AZD6244, PD184352, PD0325901); Raf kinases (sorafenib, PLX4032), Ras farnesylation/geranylgeranylation (lonafarnib, tipifarnib).

In addition to agents that target kinase activities, other therapeutic drugs that have been developed recently to modify the biology and/or kill tumor cells include those that: modify protein acetylation (histone deacetylase inhibitors, HDACIs: vorinostat, LBH589, MS275,

sodium valproate); modify the activity of protective Bcl-2 family proteins in the mitochondrion (ABT-737, GX15-070, gossypol) and agents that inhibit proteasome degradative activity (bortezomib, carfilzomib). (Grant 2008a; Tímár and Döme, 2008; Ihle and Powis, 2009; Memmott and Dennis, 2009; Sebolt-Leopold and Herrera, 2008; McCubrey et al., 2008; Steelman et al., 2008; Grant, 2008b; Grant and Dent, 2007; McConkey and Zhu, 2008). Some of the above agents have already been combined in vitro and in animal models to achieve a synergistic increase in tumor cell killing e.g. (Table 2).

2. Oncogene addiction

In vitro, numerous tumor cell types have been shown to exhibit growth reduction following inhibition of growth factor receptors, e.g. ERBB1 or inhibition of (MEK1/2) signaling pathways. However, in many such studies the primary effect of a *single* kinase inhibitory agent at low "target specific" doses on tumor cells was cyto-*static*, rather than cyto-*toxic* (e.g. Carter et al., 1998; Benavente et al., 2009; Tsai et al., 2008; Smalley et al., 2008; Yacoub et al., 2006; Yacoub et al., 2006a; Martin et al., 2008). In contrast to the relatively encouraging findings from preclinical *in vitro* work, clinical studies using many of the above mentioned inhibitors as single agents frequently did not demonstrate any form of tumor growth control (e.g. Hida et al., 2009). As a result of the patient findings with kinase inhibitors as single agents, a large body of literature has developed demonstrating in preclinical models that inhibition of growth factor receptors and/or downstream signaling molecules can promote cell death induced by a wide variety of established cyto*toxic* therapies including ionizing radiation, microtubule targeted agents, and topoisomerase inhibitors and other DNA damaging agents (e.g. Harari et al., 2007; Yacoub et al., 2006a; Takigawa et al., 2007). Thus when combined with established cyto*toxic* therapies, some of the kinase inhibitors can enhance their toxicity and have shown tumor control in patients, with subsequent FDA approval for their use, for example with ionizing radiation and cisplatin, and with capecitabine (Ryan et al., 2008; Loeffler-Ragg et al., 2008).

Where *single* receptor-targeted agent-induced anticancer responses were particularly pronounced in patients, such as for imatinib in the treatment of Bcr-Abl+ CML, it was hypothesized and proven that the tumor control effect was due to CML cells being exquisitely "addicted" to the kinase activity of the Bcr-Abl fusion protein for growth and survival (Druker, 2008). Similar findings were made for imatinib in gastro-intestinal tumors that express a mutated active form of c-Kit (Antonescu, 2008). On the contrary, in non-small cell lung cancer (NSCLC), despite the tumors of ~70% of patients are overexpressing ERBB1, only a small subpopulation of patients (~10%) responded to ERBB1 inhibitors and these individuals statistically tended to be non-smokers and with an Asian/female genetic background (Ladanyi and Pao, 2008). Subsequently it was shown in responsive NSCLC patients, in a conceptually parallel manner to data from Bcr-Abl+ cells, that ERBB1 was mutated to become a constitutively active kinase, with such NSCLC cells being addicted to the survival signals emanating from the mutated receptor (Johnson and Janne, 2006; Le Tourneau et al., 2008).

Thus only a minority of tumor cell types appear to present with a relatively simple *single oncogene* activating mutation/survival signaling addiction that would predict for effectiveness of a *single* kinase inhibitory drug. These findings make clear that the rational development of approaches which simultaneously target *multiple* signal transduction pathways to kill tumor cells will more likely have broad therapeutic usefulness. Despite this growing body of knowledge, the testing of combinations of multiple kinase inhibitory agents has only within the last 5 years truly begun to be explored. In part, such approaches have often been hampered by the lack of (commercial) availability of clinically relevant drugs for testing in academic institutions and by intellectual property issues that preclude the combination of proprietary agents from different pharmaceutical companies.

3. Redundant survival signaling pathways and the combinations of multiple kinase inhibitors

3.1. Receptors

Exposure of tumor cells expressing a mutated active form of ERBB1, but generally not an over-expressed wild type ERBB1, to kinase domain inhibitors results in growth arrest and tumor cell death (Pao et al., 2004; Sordella et al., 2004). Over the course of many months' kinase inhibitor exposure, secondary mutations in the receptor kinase domain develop which render the receptor resistant to the kinase inhibitor. A more rapid mechanism of resistance to ERBB receptor inhibitors as single agents, prior to the development of secondary mutations, is the compensatory activation of growth factor receptors such as c-MET (+c-Src), and the IGF1R which can act in parallel to provide survival signaling **(**Figure 1) (Jin and Esteva, 2008; Mueller et al., 2008; Bean et al., 2007). These receptors can provide a survival signal in their own right as receptor tyrosine kinases as well as causing trans-phosphorylation of inhibited ERBB receptors, thereby permitting the ERBB receptors to act as docking sites for e.g. RAS GTP exchange factors. Combinations of ERBB receptor inhibitors with inhibitors of c-Met or of the IGF1R have proven efficacious in promoting cell death and reverting significantly the ERBB inhibitor resistant phenotype (Huang et al, 2009; Arteaga, 2007). Others have noted lower levels of the pro-apoptotic protein Bim in ERBB1 inhibitor resistant cells (Deng et al., 2008). In cells that are oncogene addicted to mutated active forms of ERBB1, the use of Bcl-2 family inhibitors such as ABT-737 can promote drug toxicity demonstrating that these receptors, in part, regulate cell survival through maintaining mitochondrial stability (Cragg et al., 2007). We have noted that resistance to the ERBB1/ERBB2 inhibitor lapatinib can be mediated by increased expression of protective Mcl-1 and Bcl-XL with reduced expression of pro-apoptotic Bax (Martin et al., 2008). The Bcl-2 family antagonist gossypol can also partially circumvent this resistance mechanism (unpublished findings).

One profound problem in many cancer cell types, for the use of single agent or combinations of multiple receptor tyrosine kinase inhibitors is that oncogenic mutations downstream of growth factor receptors have the potential to abrogate any long-term anti-proliferative effect of receptor inhibition, e.g. mutations of RAS proteins, B-Raf, p110 PI3K or of PTEN. Thus tumor cell types which have a high penetrance of such downstream oncogenic mutations e.g. K-Ras in pancreatic cancers, PTEN in glioblastoma, PI3K p110 mutation in breast and colorectal cancers, will be *a priori* predicted to be relatively refractory to toxic and/or cyto-static effects caused by inhibition of one or multiple growth factor receptor function(s) such as ERBB1 (Koliopanos et al., 2008; Gonzalez and De Groot, 2008; Zhao and Vogt, 2008; Sartore-Bianchi et al, 2009). Hence, inhibitors of multiple signaling pathways downstream of the growth factor receptors, and in all likelihood at or below the level of RAS and PTEN, will need to be rationally combined.

3.2. RAS proteins

RAS proteins were initially envisaged as a prime target for therapeutic intervention and drugs that blocked farnesylation of RAS proteins were developed. However, possibly due to a redundancy of lipid modification where RAS proteins can be geranylgeranylated and still maintain plasma membrane localization and activity, the direct targeting of RAS by farnesyltransferase inhibitors has not significantly progressed as a single agent therapeutic in the clinic. In the laboratory, inhibitors of RAS farnesylation have been shown to interact with cytotoxic drugs such as cisplatin, ionizing radiation and with anti-estrogens to kill breast cancer cells (Rengan et al., 2008; Sizemore et al., 1999; Martin et al., 2007). We have noted in both carcinomas and hematological malignancies that inhibitors of RAS farnesylation synergistically interact with Chk1 inhibitors *in vitro* and *in vivo* to cause cell killing by blocking

Chk1 inhibitor –induced activation of the ERK1/2 pathway (see below) (Dai et al., 2008; Hamed et al., 2008).

3.3. MEK1/2 and PI3K-AKTmTOR signaling pathways

Based on knowledge that both the PI3K-AKT/PI3K-mTOR and MEK1/2-ERK1/2 pathways can provide survival signals downstream of RAS proteins and PTEN, recent studies and elegantly presented data in a genetically defined mouse model of lung cancer utilizing induction of a mutated K-RAS protein that inhibition of both PI3K and MEK1/2 can have a profound anti-tumor effect *in vitro* and *in vivo* (Engelman et al., 2008). Nonetheless, not all tumor cells exhibit an exquisite sensitivity to combined inhibition of both PI3K and MEK1/2, at least at concentrations where the actions of the inhibitory drugs can be considered specific (e.g. Yacoub et al., 2003). This could be due to additional survival pathways that can compensate for loss of PI3K and MEK1/2 signaling e.g. via NFκB. Inhibitors of the PI3K-related kinase mTOR (e.g. rapamycin; PI-103 CI779) have been examined as single agents in patient studies with sometimes excellent results e.g. in renal carcinoma, sometimes modest and sometimes confounding results showing increased tumor growth, such as in breast cancer (Kroog and Motzer, 2008; Carracedo et al, 2008). This latter undesirable effect may be due to feedback activation of AKT via TORC2 or due to activation of ERK1/2 caused by the mTOR inhibitor providing a survival signal through PI3K (Grant, 2008; O'Reilly et al, 2006; Carracedo et al, 2008). Inhibition of the *compensatory* activation of ERK1/2 following treatment with an mTOR inhibitor results in a synergistic induction of tumor cell killing combining mTOR and MEK1/2 inhibitors (Kinkade et al., 2008; Frost et al., 2009; Lasithiotakis et al., 2008). In NSCLC cells that express a mutant active RAS, and that are *resistant* to ERBB1 inhibitors, it has been shown that inhibition of PI3K or AKT results in tumor cell killing *in vitro* and *in vivo* (Janmaat et al., 2006; Ihle et al., 2005; Festuccia et al., 2008). However, in colon cancer cells, mutational activation of RAS predicts for *resistance* to PI3K inhibitors as single agents (Ihle et al., 2009a). Thus, at a "simplistic" level, inhibition of only two survival signaling pathways probably also will not always result in a profound enhancement of tumor cell killing.

Data from melanoma cells can also be particularly enlightening to address the concept of dual or multi-pathway inhibition in how we could develop approaches to enhance tumor cell killing by kinase inhibitors. Malignant melanoma cells frequently have oncogenic activating mutations in RAS proteins or in B-Raf (V600E), or inactivation of PTEN function. In melanoma cells expressing a mutant active B-Raf, inhibition of B-Raf or MEK1/2 generates only a cyto-static response (Haass et al., 2008). Similarly, in melanoma cells with RAS mutation or loss of PTEN, inhibition of PI3K or mTOR suppresses growth without causing profound cytotoxicity (Stahl et al., 2004; Howes et al., 2007). The combination of Raf/MEK inhibition with inhibition of PI3K and mTOR, however, causes both a growth arrest and a cyto-*toxic* response (Bedogni et al., 2006; Smalley et al., 2006). In this respect, several of the recently developed mTOR inhibitors also strongly inhibit PI3K p110 enzymes, thus the combination of a MEK1/2 inhibitor with, e.g. the dual specificity mTOR/PI3K inhibitors BEZ235 or PI-103, represents in fact a multi-pathway inhibitory approach that will circumvent possible re-activation of AKT due to mTOR/TORC2 signaling (Engelman et al., 2008; Fan et al., 2007). As inhibition of mTOR also results in a rapid reduction in the expression of short half-lived anti-apoptotic proteins such as Mcl-1 and c-FLIP-s, this drug combination may mediate cell killing through a varied and complex range of mechanisms (Adams and Cooper, 2007).

In vitro, inhibitors of mTOR such as rapamycin, PI-103 and CCI779 have been shown using a wide variety of tumor cell types to synergize with ERBB receptor inhibitors to promote tumor cell death (McCubrey et al., 2008; Azarriti et al., 2008; Wang et al., 2008; Wang et al., 2008a; Fan et al., 2007). This is likely due to the fact that inhibition of mTOR reduces protein synthesis resulting in a rapid reduction in the expression of short half-lived protective proteins

as well as potentially reducing receptor/cognate ligand expression, which will result in a lower apoptotic threshold. The combination of mTOR inhibitors with growth factor receptor inhibitors other than those which inhibit ERBB1, e.g. the PDGFR/VEGFR/FLT3 inhibitor ABT869, has also been shown to synergize to kill hepatocellular carcinoma cells *in vitro* and *in vivo* (Jasinghe et al., 2008). Several of the published studies combining ERBB1 inhibitors with mTOR inhibitors have also demonstrated excellent tumor cell responses in mouse models, and based on these findings, phase I trials are presently underway combining ERBB1 inhibitors with rapamycin in glioblastoma and NSCLC (e.g. Jimeno et al., 2007; LoPiccolo et al., 2008). Thus resistance to ERBB receptor inhibitors as well as those of other receptors can be overcome by the addition of agents that block signaling through *multiple* downstream pathways.

The use of agents which simultaneously target multiple additional pathways/regulatory protein molecules together with those of greater specificity has also been shown to have value, for example combining HSP90 inhibitors (17AAG/17DMAG) which suppress the expression/activity of multiple oncogenic signaling HSP90 client proteins with inhibitors of MEK1/2. This combination blocks signaling by the PI3K-AKT and MEK1/2-ERK1/2 pathways, as well as other survival pathways e.g. ERBB-STAT and NFκB pathways, generates reactive oxygen species, and causes cell death in hepatoma, pancreatic cancer and leukemia cells (Park et al., 2008; Nguyen et al., 2006). Similar data in myeloma cells using the AKT inhibitor perifosine when combined with 17DMAG have also been published (Huston et al., 2008). Collectively, these data on mTOR inhibitors or 17AAG demonstrates that three-pathway *or* a multi-pathway inhibitory approach of kinase inhibitors is required to kill a broad spectrum of tumor cell types.

3.3.1. The case of sorafenib—As noted above, many kinase inhibitors are not highly specific for one particular protein kinase, which is most likely due to their targeting the kinase ATP binding site. An outstanding example is sorafenib (Nexavar®), which was developed as an inhibitor of Raf-1 and B-Raf. Indeed, initial *in vitro* studies suggested that the agent would have activity against tumors that are oncogene addicted to mutant activated B-Raf, such as in some melanoma and colon cancer cells (Lee and McCubrey, 2003). Of note are some studies suggesting that low doses of sorafenib can actually activate ERK1/2 suggesting that the biological actions of this drug are much more complicated (Tochizawa et al., 2008). In agreement with this proposition, the clinical activity of sorafenib appeared to be only partially dependent on modulation of the Raf-MEK-ERK pathway, with the majority of anti-tumor effects now being thought to be mediated by inhibition of class III receptor tyrosine kinases that regulate tumor angiogenesis (Takimoto and Awada, 2008). Inhibition of these growth factor receptors would also be predicted to reduce activity within the ERK1/2, PI3K-AKT-mTOR and NFκB pathways in both endothelial and tumor cells.

More recently, sorafenib has been shown to cause an endoplasmic reticulum stress response in tumor cells that in part can account for its toxicity due to PKR-like endoplasmic reticulum kinase (PERK) – eIF2α mediated suppression of protein translation (Zhang et al., 2008; Park et al., 2008a; Rahmani et al., 2005; Rahmani et al., 2007). Thus the expression of short-lived anti-apoptosis proteins, such as Mcl-1, XIAP and c-FLIP-s, could be lowered after sorafenib exposure through both losses of ERK1/2, PI3K-AKT-mTOR and NFκB signaling by reduced transcription as well as by ER stress signaling through eIF2α through reduced translation. Sorafenib has been shown to synergize with the mTOR inhibitor rapamycin to reduce angiogenesis in vitro and in vivo in both hepatoma and melanoma models (Wang et al., 2008; Lasithiotakis et al., 2008). As discussed previously with regard to 17DMAG and mTOR inhibitors, the interactions of sorafenib with other kinase inhibitors to synergistically kill tumor cells may represent a highly complex multi-factorial induction of many pro-apoptotic signals due to reduced expression of survival proteins and survival signaling.

Data from our laboratories and from others has shown that sorafenib synergizes with histone deacetylase inhibitors to cause cell death in hepatoma, cholangiocarcinoma and leukemia cells (Park et al., 2008a; Zhang et al., 2008; Baradari et al., 2007; Dasmahapatra et al., 2007). Histone deacetylase inhibitors have a multi-factorial mode of action, including disruption of co-repressor complexes regulating transcription, for example with increased expression of death receptors and their ligands; the induction of reactive oxygen species; the generation of toxic lipids such as ceramide; inhibition of HSP90 chaperone function; and activation of NFκB (Emanuele et al., 2008). Our data argued that sorafenib and histone deacetylase inhibitors interacted to cause death through ceramide-dependent activation of the death receptor CD95 in parallel to an ER stress response that inhibited expression of multiple protective BCL-2 family proteins as well as c-FLIP-s. Clearly, the interaction of sorafenib with histone deacetylase inhibitors again represents the simultaneous inhibition and activation of multiple signal transduction pathways which ultimately facilitates increased levels of tumor cell killing.

3.4. Cdk inhibitors

The use of cyclin-dependent kinase inhibitors as anticancer therapeutics was originally founded on the belief that blocking Cdk function would block cell cycle progression, thereby slowing tumor cell growth and perhaps leading to differentiation and/or cell death (Senderowicz, 1999) (see Figure 2). A prototype Cdk inhibitor is flavopiridol, a drug that at clinically relevant concentrations with a modified infusion schedule has been shown to generate objective responses in CLL patients (Phelps et al., 2009). It has been appreciated that flavopiridol and other clinically relevant Cdk inhibitors such as R-roscovitine (CYC202) are excellent modulators of the apoptotic threshold in tumor cells, resulting in greater cell killing when combined with a variety of other agents. Flavopiridol has shown activity in refractory solid tumors when combined with established chemotherapeutic drugs such as taxanes and gemcitabine (Fornier et al., 2007; Shapiro, 2004). In part, the actions of Cdk inhibitors may be related to inhibition of Cdk7 (CAK1) and Cdk9/Cyclin T (p-TEFb) (Canduri et al., 2008). Inhibition of Cdk7 blocks regulatory phosphorylation of multiple Cdk proteins at T161. Inhibition of Cdk9 (p-TEFb) suppresses protein levels due to loss of RNA polymerase II transcription. Thus the expression of short-lived anti-apoptosis proteins, such as Mcl-1, XIAP and c-FLIP-s, Cyclin proteins, and growth factor receptors such as c-Met, can be rapidly reduced by flavopiridol (Moghul et al., 1994). Furthermore, both flavopiridol and CYC202 have been shown to inhibit IKK enzymes, thereby suppressing NFκB function (Takada and Agarwal, 2004; Gao et al., 2004). Thus the clinically relevant Cdk inhibitors have a large number of putative cellular targets by which they can modulate the apoptotic threshold and the proliferative index of a tumor cell. In this respect it is interesting to note that histone deacetylase inhibitors, which promote NFκB activation via acetylation and IκB degradation synergize with flavopiridol or roscovitine to kill breast cancer and leukemic cells (Gao et al., 2004; Mitchell et al., 2007). Similar data using CYC202 and the histone deacetylase inhibitor MS275 were also recently noted in hepatoma cells (Gahr et al., 2008). Inhibition of Cdk9 has also been shown to promote the toxicity of PI3K/AKT inhibitors that could potentially occur indirectly through inhibition of NFκB (Mohapatra et al., 2009). Prior work from our laboratories has suggested a direct connection between Cdk inhibitor toxicity in leukemic cells and modulation of PI3K pathway activity (Yu et al., 2003). Thus the inhibition of the p-TEFb pathway which has pleiotropic downstream targets and together with agents that target protein acetylation or signaling pathway activities represents a highly tractable approach to treating a wide variety of malignancies.

Cdk inhibitors have also been shown to interact with inhibitors of growth factor receptors. CYC202 enhances the toxicity of inhibitors of ERBB1 or ERBB2 in a synergistic fashion in some tumor cell types, though in tumor cells expressing mutant active RAS proteins or with altered PTEN function, the toxic combination effect of both drugs was additive or less than

additive (Fleming et al., 2008). We have similar synergistic drug interaction data combining the ERBB1/ERBB2 inhibitor lapatinib with flavopiridol in a variety of breast cancer cell lines (unpublished findings). Farnesyltransferase inhibitors have been shown to enhance the toxicity of roscovitine (Wesierska-Gadek et al., 2007). The less than additive effect of the CYC202/ERBB inhibitor drug combination in killing tumor cell types expressing onco-proteins downstream of the growth factor receptors to some extent provides further confirmation of the hypothesis that mutation of oncogenes downstream of growth factor receptors suppresses the ability of growth factor receptor inhibitors to have therapeutic efficacy, even in combination with other signal transduction –inhibitory agents.

4. Rapid compensatory activation of inter-linked survival signaling

In several cell systems we have noted that inhibition of one intracellular protective signaling protein/pathway rapidly results in the rapid compensatory activation of another protective signaling protein/pathway. The best example we have found to date being inhibition of the cell cycle regulatory checkpoint kinase, Chk1 (Figure 2). Deletion of Chk1 is embryonically lethal and Chk1 activation downstream of ATR/ATM plays a key role in regulating Cdc25C and Cdc2 activity, and thus cell cycle progression, after DNA damage. We discovered that in addition to its established cell cycle regulatory targets; inhibition of Chk1 rapidly lead to activation of ERK1/2 in multiple solid and blood tumor cell types, but not in their non-transformed cell counterparts (Dai et al., 2002;McKinstry et al., 2002). Activation of ERK1/2 was in part dependent on Chk1 inhibitor –induced DNA damage, as inhibition of PARP1 blocked γH2AX and ERK1/2 phosphorylation. Inhibition of MEK1/2 blocks Chk1 inhibitor –stimulated ERK1/2 activation and causes a profound increase in tumor cell killing both *in vitro* and *in vivo*. Similar data were obtained using a PARP1 inhibitor (Dent, unpublished observations). This synergistic killing effect has been noted with combinations of multiple Chk1 and MEK1/2 inhibitors (Dai et al., 2008;Hamed et al., 2008). Cell killing is mediated through mitochondrial dysfunction and the use of agents which inhibit Bcl-2/Bcl-XL, e.g. HA14-1, profoundly enhance MEK/Chk –inhibitor toxicity and circumvent the protective effect of Bcl-XL protein over-expression (Hamed et al., 2008). In myeloma cells, this drug combination appears to mediate tumor cell death through a Bim-dependent mechanism (Pei et al., 2007). Thus, this finding suggests that, unlike for earlier discussed combinations, for these two closely interrelated survival signaling pathways, a combination of only *two* specific kinase inhibitors can have a profound anti-tumor effect. The combination of MEK1/2 and Chk1 inhibitors has yet to be evaluated in the clinic.

5. Conclusions and perspective

In comparison to non-transformed cells, tumor cells over-express growth factor receptors and express mutated proteins that facilitate the oncogenic phenotype, resulting in the activation of multiple signal transduction pathways. There are profound levels of survival signaling redundancy and of signaling plasticity which permit tumor cells to adapt and overcome multiple environmental and therapeutic stresses. Collectively this implies that the use of highly specific targeted therapies, often using single inhibitory agents as was initially employed with ERBB1 inhibitors, was almost guaranteed to fail. In a few specific tumor cell types, the single agent cytotoxicity of a kinase inhibitor is evident due to very specific oncogene addiction of those cells. However, many of the most promising signal transduction modulatory drug combinations involve agents that both act to suppress survival signaling within *multiple* pathways and act to suppress expression of multiple apoptosis inhibitory proteins. Thus agents that inhibit mTOR and suppress expression of many proteins; those that destabilize multiple activated signaling proteins through HSP90 inhibition; those that block Cdk9 (p-TEFb) function and suppress expression of multiple proteins and those that inhibit *multiple* receptor tyrosine kinases appear to represent the best avenues for therapeutic efficacy in multiple malignancies.

It is also becoming apparent that simply combining more (and more) inhibitors of kinase proteins together can result in *less* tumor control in patients due to antagonistic interactions between the agents e.g. (Tol et al., 2009). In the Tol et al. study, the addition of the inhibitory ERBB1 antibody cetuximab to bevacizumab + capecitabine + oxaliplatin therapy led to shorter survival of colorectal cancer patients. We have also noted that inhibition of ERBB1 suppresses the toxicity of single agent Chk1 inhibitor treatment (McKinstry et al., 2002). One reason for these findings could be the possibility that ERBB1 signaling not only generates cyto-protective signals but has also been linked to the tyrosine phosphorylation and activation of the CD95 death receptor (Reinehr et al., 2003). Over-stimulated ERBB1 signaling can also enhance radiosensitivity (Yacoub et al., 2006). These findings emphasize the need for careful preclinical evaluations of drug combination mechanism(s) of action to provide the foundation for any subsequent clinical translation. Whether it will be possible to bring all of the most promising combinations of such kinase inhibitory agents from different pharmaceutical companies through to the clinic in a timely fashion is at present an open question (Table 2).

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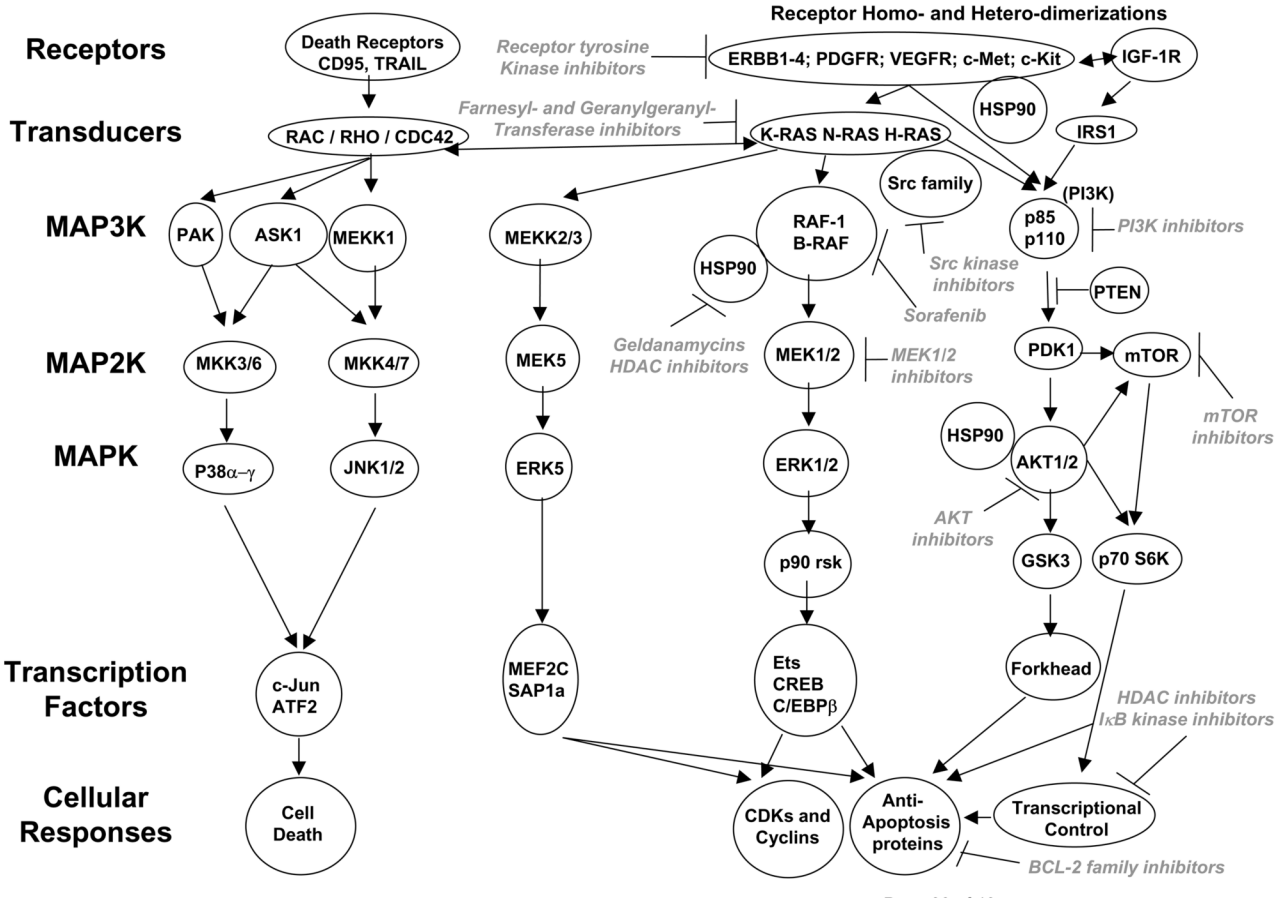

Figure 1. Signal transduction pathways in cancer cells and the enzymes which have been therapeutically targeted by drugs

Tumor cells contain multiple signal transduction pathways, some of which mediate cell survival, such as the PI3K-AKT and RAF-MEK-ERK1/2 pathways. Of note, however, at every "level" of transduction, from the plasma membrane, to transcriptional and cell cycle control in the nucleus, specific enzymes have been targeted to block growth and facilitate tumor cell death. It is the combination of these drugs which will most likely yield the greatest therapeutic benefit.

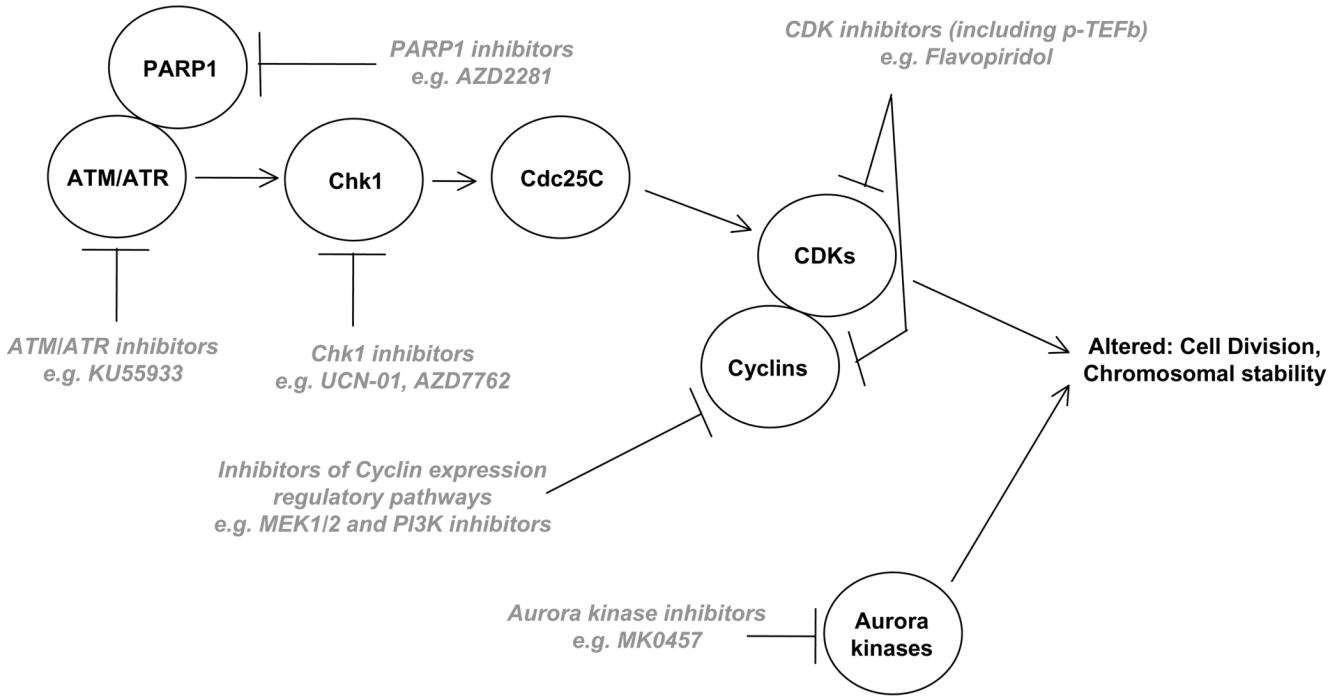

Figure 2. Therapeutic drugs that target the cell cycle

Kinase inhibitors that modulate the cell cycle have frequently been combined with toxic drugs and/or radiation which cause DNA damage. This will lead to inappropriate cell cycle progression with damage DNA and result in tumor cell death. Inhibition of PARP1 ADP ribosylation activity suppresses the ability of DNA damage to activate ATM/ATR as well as increase DNA repair processes.

Table 1

Clinically relevant therapeutic drugs and their targets

Below is a short list of some of the most widely used and novel cancer therapeutic drugs. Many of these drugs are now being combined in a rational manner in vitro and in pre-clinical animal models to achieve a synergy of drug-induced tumor cell death and growth arrest.

Growth factor Receptor Tyrosine Kinases

ERBB1–4 family: erlotinib, canertinib, gefitinib, lapatinib; and inhibitory antibodies.

PDGFR: nolitinib, sorafenib, ABT869.

c-Met: PHA665752.

IGF1R: BMS536924.

c-Kit: imatinib, nolitinib.

Vascular Endothelial Growth Factor Receptor family: sorafenib, sunitinib, AG13726, ABT869; and inhibitory antibodies.

Non-receptor Tyrosine Kinases

Abl: imatinib, nolitinib.

Src family tyrosine kinases: dasatinib, AZD0530.

Small GTPase Inhibitors

Ras family farnesylation/geranylgeranylation inhibitors: lonafarnib, tipifarnib.

Intracellular signal transduction intermediates

Phosphatidyl inositol 3 kinase: PX866, BEZ235, BGT226, XL147.

AKT: perifosine, GSK690693.

mTOR: Rapamycin (sirolimus), RAD001 (everolimus), AP23573 (deforlimus), CI779 (temsirolimus), BEZ235, PI103.

Raf family kinases: Sorafenib, PLX4032.

MEK1/2: AZD6244, PD184352, PD0325901.

Heat shock protein 90: 17AAG, 17DMAG.

Regulation of Transcription

IκB kinase: IKI-1, AS602868.

Histone deacetylase inhibitors: vorinostat, LBH589, MS275, sodium valproate.

Inhibition of proteasome degradative activity: bortezomib, carfilzomib.

Regulation of cell cycle progression and genomic stability

Aurora kinases: VE465, MK0457.

Cyclin dependent kinases: flavopiridol, R-roscovitine (CYC202).

Checkpoint/Chk1 regulatory kinase: UCN-01, AZD7762.

ATM/ATR: KU55933

PARP1: PJ34, AZD2281 (KU59436)

Regulation of mitochondrial function

Bcl-2 family protein inhibitors: ABT-737, GX15-070, Gossypol

Table 2

Clinically relevant combinations of therapeutic drugs: pre-clinical and clinical testing

Below is a short list of some of the published/tested combinations of novel cancer therapeutic drugs.

Growth factor Receptor Tyrosine Kinases
ERBB1–4 family inhibitors + mTOR inhibitors
ERBB1–4 family inhibitors + c-Met inhibitors
ERBB1–4 family inhibitors + IGF1R inhibitors
ERBB1–4 family inhibitors + Vascular Endothelial Growth Factor Receptor family inhibitors
PDGFR/VEGFR/FLT3 inhibitors + mTOR inhibitors
Non-receptor Tyrosine Kinases
Abl kinase inhibitors + Src family tyrosine kinase inhibitors
Tyrosine kinase inhibition(s) + Inhibition of proteasome degradative activity
Small GTPase Inhibitors
Ras family farnesylation/geranylgeranylation inhibitors + Checkpoint/Chk1 regulatory kinase inhibitors
Intracellular signal transduction intermediates
Phosphatidyl inositol 3 kinase inhibitors + MEK1/2 inhibitors
Phosphatidyl inositol 3 kinase inhibitors + mTOR inhibitors
AKT inhibitors + mTOR inhibitors
Inhibitors of heat shock protein 90 + MEK1/2 inhibitors
Regulation of Transcription
Histone deacetylase inhibitors + Sorafenib
Inhibition of proteasome degradative activity + Histone deacetylase inhibitors
IKK inhibitors + Histone deacetylase inhibitors
Inhibition of proteasome degradative activity + Cyclin dependent kinase inhibitors
Regulation of cell cycle progression and genomic stability
Cyclin dependent kinase inhibitors + Histone deacetylase inhibitors
Checkpoint/Chk1 regulatory kinase inhibitors + MEK1/2 inhibitors
Inhibitors of heat shock protein 90 + Cyclin dependent kinase inhibitors
Cyclin dependent kinase inhibitors + Phosphatidyl inositol 3 kinase inhibitors
Regulation of mitochondrial function
Bcl-2 family protein inhibitors + Inhibition of proteasome degradative activity