Short Title: Reversal of Resistance by RKIP

RKIP-Mediated Chemo-Immunosensitization of Resistant Cancer Cells via Disruption of the NF-κB/Snail/YY1/RKIP Resistance-Driver Loop

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ABSTRACT: Cancer remains one of the most dreadful diseases. Whereas most treatment regimens for various cancers have resulted in improved clinical responses and sometimes cures, unfortunately, subsets of cancer patients are either pre-treatment resistant or develop resistance following therapy. These subsets of patients develop cross-resistance to unrelated therapeutics and usually succumb to death. Thus, delineating the underlying molecular mechanisms of resistance of various cancers and identifying molecular targets for intervention are the current main focus of research investigations. One approach to investigate cancer resistance has been to identify pathways that regulate resistance and develop means to disrupt these pathways in order to override resistance and sensitize the resistant cells to cell death. Hence, we have identified one pathway that is dysregulated in cancer, namely, the NF-KB/Snail/YY1/RKIP loop, that has been shown to regulate, in large part, tumor cell resistance to apoptosis by chemotherapeutic and immunotherapeutic cytotoxic drugs. The dysregulated resistant loop is manifested by the overexpression of NF-KB, Snail and YY1 activities and the underexpression of RKIP. The induction of RKIP expression results in the downregulation of NF-KB, Snail and YY1 and the sensitization of resistant cells to drug-induced apoptosis. These findings identified RKIP, in addition to its anti-proliferative and metastatic suppressor functions, as an anti-resistance factor. This brief review describes the role of RKIP in the regulation of drug sensitivity via disruption of the NF-κB/Snail/YY1/RKIP loop that regulates resistance in cancer cells.

KEYWORDS: chemotherapy, immunotherapy, NF-κB, resistance, RKIP, Snail, stem cells, YY1

ABBREVIATIONS

CPT: carboplatin; **CRC:** colorectal cancer; **CSC:** cancer stem cell; **CTL:** cytotoxic T lymphocyte; **DETANONOate:** diethylenetriamine NONOate; **DcR1:** decoy receptor 1; **DcR2:** decoy receptor 2; **DR4:** death receptor 4; **DR5:** death receptor 5; **EGFR:** epidermal growth factor receptor; **EMT:** epithelial mesenchymal transition; **EZH2:** enhancer of zeste homolog 2; **FasL:** Fas ligand; **GPCR:** G-protein coupled receptor; **HSP:** heat shock protein; **KEAP-1:** Kelch-like ECH-associated protein 1; **MDR:** multiple drug resistance; **NK:** natural killer; **NPI-0052:** proteasome inhibitor; **NRF2:** NF-E2 related nuclear factor 2; **OXP:** oxiplatin; **PEBP:** phosphatidyl ethylenolamine binding protein; **PDT:** photodynamic therapy; **Rituximab:** chimeric antiCD20 mAb; **RKIP:** Raf kinase inhibitor protein; **YY1:** Yin-Yang 1

I. INTRODUCTION

An aspect in the pathogenesis of cancer is the recognition that the majority of cancers arises and persists as a consequence of the activity of oncogenes and tumor suppressor genes and that such oncogenes dysregulate anti-apoptotic pathways involved in the resistance. The genomic characterization of tumors has highlighted the pivotal role of "driver" somatic mutations and tumor dependency called "oncogenic addiction".[1] Further, "non-oncogenic addiction" was also caused to define dependences that are not manifested by somatic cancer gene modifications.[2] Other tumor dependencies include hormone dependency, lineage dependency and metabolic dependency.[2,3] Clinically, several drugs were developed to target tumor dependency factors.[4]

We have experienced three waves of drug development, namely, a wave of drugs targeting DNA replication and cell division, a wave of drugs targeting signaling intermediates that contribute to cancer growth, and a wave of drugs that target cellular mechanisms essential for tumor growth and survival.[5] The first wave of drugs, namely, chemotherapeutic drugs, still represents the vast majority of clinically used chemotherapeutic drugs today in several cancers (examples platinum derivatives, topoisomerase inhibitors, nucleoside analogs, vinca alkaloids, and taxanes). The second wave of drugs are developed and targets genetic alterations in cancer cells and ascribed for tumor survival and "oncogene addiction" and other targets that are not genetically altered "non-oncogenic addiction". Many of the drugs have been used for the treatment of variety of cancers. In addition, another form of a second wave drugs is monoclonal antibodies targeting cell surface receptors. The third wave consists of drugs that have developed to target factors that regulate tumor growth and survival and are not directly involved in DNA replication or cell division.[5]

Although the above therapies resulted in significant clinical responses, however, many patients experience drug resistance. The phenomenon of drug resistance dates back for several decades. For instance, Brockman reported in a long review chapter the mechanisms of resistance

that were known at that time, primarily emphasizing the biochemical basis of resistance, that the resistant cancer cells differed biochemically from the sensitive cancer cells in their response to chemotherapeutics.[6] However, the insights made by Brockman were not validated in clinical practice. The malignant variants that emerged after the initial therapy developed cross-resistance against the drugs used and several unrelated classes of drugs.[7,8] This phenomenon of multiple drug resistance (MDR) was analyzed by the use of various inhibitors to reverse the MDR phenotype, however, such analyses were not translated in the clinic.[9] In addition, the drugs used were under the rationale that once inside the tumor cells they should inhibit cell proliferation and kill the cells; however, it is clear nowadays that drug resistance is a complex and a multi-faceted system consisting of biochemical, molecular and genetic alterations that the tumor cells exploit to avoid cytotoxic drugs.

Drug resistance represents a major barrier in the eradication of cancer and its cure by all available therapeutic regimens. The failure to recognize the underlying mechanisms of drug resistance in relapsed patients and their treatments with drugs that are not targeted to these mechanisms results in dismal response rates. Our understanding on the mechanisms of resistance remains incomplete. Hence, emphasis nowadays is directed at the development of targeted agents at factors that regulate resistance that may differ for each type of cancer.

Several cancer non-specific conventional and more specific targeted therapies are currently being used in the treatment of a variety of cancers. A common denominator for all of these strategies is that there is an excellent initial clinical response for primary tumors and seldom with metastatic tumors with improvement of progress-free survival and seldom cures. Another common denominator is that a subset of patients does not respond to these treatments and another subset of responding patients develops cross-resistance to further treatments. The former is called “intrinsic resistance” and the latter “acquired resistance.” Clearly, tumor cells thrive to survive, proliferate, and metastasize and, thus, develop several mechanisms to escape

the cytotoxic effects of the various therapeutics that are currently being used.

The intrinsic resistance signifies that prior to receiving therapy, a subset of tumor cells and their microenvironment are regulated by resistant factors that make the tumor unresponsive. In contrast, acquired resistance may develop during treatment of the tumor cells that were initially sensitive. However, it may also be considered that due to tumor heterogeneity, that intrinsic resistance may be in a subset, such as cancer stem/initiated cells, that is highly drug resistant. Also, acquired resistance may consist of selective pressure to novel drug-induced oncogenic mutations.An example of acquired resistance is the mutation within the BCR-ABL oncoprotein in CML treated with the ABL inhibitor imatinib in which a residual subset was selected by the treatment and exhibited drug resistance.[10] An example of intrinsic resistance is found in approximately 10% of the $BRAF^{v600E}$ mutation in melanoma following treatment with the BRAF inhibitor vemarufenib.[11]

II. MECHANISMS OF CANCER RESISTANCE TO CYTOTOXIC DRUGS

Mechanisms of drug resistance include, but not as yet unknown other mechanisms, increased rates of drug efflux, DNA repair, alteration in drug metabolism, mutations of drug targets, activation of survival anti-apoptotic pathways, epigenetic changes, the tumor microenvironment, the presence of cancer stem cells and the molecular and genetic hallmarks of this tumor population. These above various mechanisms have been reviewed extensively recently.[12]

The mechanisms of resistance regulated by downstream factors are due to constitutively activated cell survival/anti-apoptotic pathways. Since the main objective of cancer treatment is to kill the tumor cells, clearly, the tumor cells, in turn, exhibit intrinsic adaptive responses that counteract death signals and promote cell survival. During the transformation process, pathways that regulate apoptosis are dysregulated and often malfunctional.[13] Examples of a few gene products that regulate apoptosis and their role in certain cancers[14] are briefly summarized below:

1) the anti-apoptotic members of the Bcl-2 family are, in large part, regulated by constitutively activated NF-κB and STAT3 transcription factors. For instance, overexpression of Bcl-2 correlates with resistance of leukemic cells to drug therapy.[15] Since several members regulate the mitochondrial membrane permeability and subsequent activation of the type II apoptotic pathway, the relative activity of pro-and anti-apoptotic gene products of this family dictates the ultimate fate of the tumor cells.[16] A correlation between a mitochondrial response to chemotherapy and a clinical response was reported.[17] Similar findings of the implication of Bcl-2 family members in resistance to targeted therapy have been reported. The BH3-only protein BIM was reported to play a central role in imatinib-induced apoptosis in CML and gefitinib and erlotinib-induced apoptosis in EGFR-mutated NSCLC.[18-20]

Several mechanisms of drug resistance to small molecule inhibitors have been reported including MDR1.[21-23] Monoclonal antibodies are also not immune to the development of resistance.[5,24] The cellular machines that were targets by the first wave chemotherapeutics included DNA replication, DNA repair and cell division. Analysis revealed that these machines are required for tumor cell proliferation and survival. The existence of chronic stress conditions in tumors and the ensuing non-oncogene addiction of cancer cells are now recognized as "hallmarks of cancer".[2,13] Examples are heat shock proteins (HSP90).[25] Targeting the proteasome function increases the proteotoxic stress in tumors. Also, targeting chromatin modification has been investigated.[5]

III. ROLE OF RKIP IN THE REGULATION AND REVERSAL OF RESISTANCE

The Raf kinase inhibitor protein (RKIP) is a member of the phophatidylethylnolamine-binding protein (PEBP) family, which was shown, initially, to play a role in lipid metabolism and phospholipid membrane biogenesis.[26] Yeung et al. cloned the RKIP gene and reported its inhibitory activity on both the Raf/MEK/ERK pathway (through which RKIP's name was derived) and the NF-κB survival pathways.[27,28] RKIP was shown to be inactivated via phosphorylation at Serine153 by

PKC.[29] Lorenz et al. reported that phosphorylated RKIP inhibits the G-protein coupled receptor (GPCR) kinase (GRK-2) and facilitates the crosstalk between the EGF and GPCR signaling pathways.[30] Ectopic expression of RKIP suppresses invasion and metastasis of human prostate cancer cells in mice[31] and, in contrast, the downregulation of RKIP led to invasiveness of tumor cells *in vitro*.[32]

RKIP has been classified as a tumor metastatic suppressor gene and was found to be downregulated during the metastatic process.[31,33-36] In contrast to cervival cancer, in many other tumors the loss of RKIP was of poor prognostic significance.[33,35,37-42]

A. Role of RKIP in Inhibiting Resistance to Chemotherapeutic Drugs

Chatterjee et al.[43] were the first to report on the role of RKIP in the regulation of drug resistance-mediated apoptosis. The induction or overexpression of RKIP in drug-resistant human cancer cell lines sensitized the cells to various chemotherapeutic drugs-induced apoptosis. Due to the original findings by Yeung et al.[27.28] on the role of RKIP in the inhibition Raf/MEK/ERK and NF-κB pathways, clearly, several upregulated anti-apoptotic gene products and downregulated pro-apoptotic products that are transcriptionally regulated by these pathways will result in opposing effects by overexpression of RKIP. Hence, the tumor cells will experience a lowering of the threshold of resistance and become sensitized to apoptosis by cytotoxic drugs. [43-47,48]

RKIP depletion is associated with radio and chemoresistance .Al-Mulla et al.[48] reported that depletion of RKIP in HEK-293 cells resulted in the induction of a reactive oxidative stress response. Oxidative stress-induced NRF2 (NF-E2 related nuclear factor 2) activation to the ubiquitination of KEAP-1 (Kelch-like ECH-associated protein 1), which binds and inhibits NRF2 nuclear translocation.[49] NRF2 activation confers cell survival and its expression is upregulated in greater than 90% of head and neck cancer patients[50] and determines chemoresistance.[51] Al-Mulla et al.[52] reported that KEAP-1 expression in colorectal cancer is associated with RKIP stability and that KEAP-1-NFR2 is a novel target in RKIP-induced resistance. The authors suggested that in the presence of RKIP, NRF2 is kept in the cytoplasm by

KEAP-1 binding. In the absence of RKIP, inactivation/ubiquitination of KEAP-1 takes place and NRF2 is stabilized and active in the nucleus where its transcripts of anti-oxidants correlated with drug resistance.

A previously reported study analysis by microarray revealed that RKIP was one of the genes that is differentially expressed between tumor samples of cervical cancer patients.[53] And in a large series of patients, RKIP protein expression was markedly downregulated in cervical cancer and lymph nodes metastasis.[54] Martenko et al.[33] investigated the expression levels of RKIP protein in nonmalignant tumor cervical samples and clinical outcome and the role of RKIP in chemotherapy response. RKIP was highly expressed in the cytoplasm of benign tissues, but significantly reduced in cervical cancer tissues as examined by IHC. Further, using human cervical cancer cell lines, there was a correlation between the resistance to cisplatinum and low RKIP expression. In drug sensitive lines, downregulation of RKIP resulted in the resistance to several chemotherapeutic drugs.

There was a correlation between phosphorylated RKIP and STAT3 activity in chemo-resistant colon cancer. Reports in the literature have examined the clinical resistance in colon cancer patients.[55,56] In Duke's B CRC patients, it was reported that RKIP may be a biomarker identifying patients with high resistant aggressive CRC.[57,58] Cross-Knoll et al.[59] demonstrated that treatment of cell lines with IL-6 resulted in the activation of STAT3 concomitant with phosphorylation of RKIP *in vitro*. The drugs OXP and CPT (used in the clinic for CRC) inhibited IL-6-induced STAT3 activation and pRKIP through the inhibition of STAT3 interaction with the IL-6 receptor ß subunit, namely, gp130. Stage II colon cancer patients with low levels of nuclear pRKIP experienced longer occurrence free survival compared to patients with high levels of pRKIP.

The cell survival signaling triggered by IL-6 and STAT3 activation is abolished by treatment with OXP or CPT and this treatment also resulted in the inhibition of pRKIP and pSTAT3. JAK-1 and JAK-2

overexpression resulted in the increase of STAT3 transcription and was associated with increased pRKIP.[60] The above findings implied the role of RKIP in the sensitization of drug-resistant tumor cells via drug-induced inhibition of phosphorylation of STAT3 and RKIP.

The enhancer of zeste homolog 2 (EZH2) is overexpressed in breast and prostate cancers.[61,62] EZH2 exerts oncogenic activity and treatment of prostate cancer cells with shRNA EZH2 inhibited proliferation of cancer cells *in vitro*.[61] Overexpression of EZH2 supports tumor invasiveness and metastasis and underlies the suppression of various genes involved, such as PRC1 proteins,[63] Kruppel-like factor 2,[64] and the EMT suppressor gene CHH1 that encodes E-cadherin.[65] Noteworthy, the suppression of E-cadherin by the repressor Snail requires the participation of EZH2.[66,67] E-cadherin shares its metastatic suppressor activity with the metastatic suppressor RKIP.[26] Loss of RKIP is associated with EMT induction, enhanced invasion, as well as chemoresistance. The repressor activity of EZH2 on RKIP transcription requires the presence of EMT-inducer Snail. Snail is one of the recruiters of EZH2 to the RKIP promoter and links the induction of RKIP expression.[68]

B. RKIP Disrupts the Dysregulated NF-κB/Snail/YY1/RKIP Loop Resulting in the Reversal of Drug/Immune Resistance

We have reported the existence of a dysregulated NF-KB/Snail/YY1/RKIP loop in cancer cells that is intimately involved in cell survival and resistance to cytotoxic stimuli. The resistant cancer cells exhibit primarily a constitutively hyperactivated NF-KB pathway and downstream targets, Snail, and YY1 and underexpression of RKIP Snail-induced repression. The inhibition of NF-κB, for example, results in the inhibition of Snail and YY1 and induction of RKIP through the inhibition of the RKIP repressor, Snail. These various modifications result in overriding resistance and the cells become sensitive to both chemo- and immuno-therapeutic drugs.[69]

The hyperactivation of NF-κB in cancer cells results downstream in the transcription of

gene products involved in cell growth and proliferation, apoptotic pathways, EMT and metastasis.[70] NF-κB regulates the transcription of Snail, a repressor of RKIP, as well as it regulates YY1 that in turn regulates Snail.[71] Snail, in turn, represses both RKIP and PTEN.[72] Hence, inhibition of NF-κB results in the inhibition of Snail and YY1 expressions and the induction of both RKIP and PTEN. The upregulation of RKIP accentuates the inhibition of NF-κB and downstream both Snail and YY1 and, therefore, it acts in a feedback mechanism. Likewise, the repression of PTEN results in the inhibition of the PI3K-AKT pathway and this also results in the inhibition of NF-KB via a crosstalk between the two pathways.[73]

1. Reversal of chemoresistance through the RKIP-disruption of the loop

YY1 is a multi-functional DNA-binding protein that can activate, repress, or initiate transcription depending on the context in which it binds (directly or indirectly) through the formation of a complex with other DNA-binding proteins.[74] There is an inverse relationship between YY1 and RKIP. This is the result of both direct and indirect underlying mechanisms. Indirectly, since YY1 regulates positively the transcription of Snail and since Snail represses RKIP, therefore, it will also regulate negatively RKIP. Inhibition of YY1 will result in the inhibition of Snail and derepression of RKIP, leading to its upregulation. Also, transfection of cells with siRNA YY1 resulted in the upregulation of RKIP. There are also preliminary findings demonstrating that YY1 binds to the RKIP promoter as assessed by chromatin-immunoprecipitation (unpublished results). In addition, YY1 represses PTEN and also PTEN suppresses YY1 through its induction of HIF-2-α transcription activity.[75]

Inhibition of YY1 by various agents sensitizes drug-resistant tumor cells to drug-induced apoptosis.[76] It is not clear whether the inhibition of YY1 and its sensitizing activity are the direct role of YY1 or whether they are the consequence indirectly of the induction of RKIP and PTEN and their respective inhibition of the NF-κB and the PI3K-AKT anti-apoptotic pathways.

Beach et al.[77] reported that RKIP transcription was under the control of the repressor transcription factor Snail in tumor cell lines. Snail is a member of the Snail superfamily of the zinc-finger transcription factors that plays a role in embryonic development and cell survival.[78] Snail is transcriptionally regulated, in part, by NF-κB[79] and by itself.[80] To upregulate RKIP expression and sensitization of resistant tumor cells to drugs, as reported by Chatterjee et al.,[43] we examined 2 proteasomal inhibitors, Bortezomib and NPI-0052 (marizomib), which inhibit NF-κB and downstream inhibit Snail and resulting in the derepression of RKIP transcription and expression of RKIP. Treatment of human prostate cancer cell lines with these inhibitors resulted in their sensitization to chemotherapeutic drugs-induced apoptosis. The sensitization was the result of the induction of RKIP. These findings demonstrated that the dysregulated NF-KB /Snail/YY1/RKIP loop is modified by the proteasome inhibitors and resulting in the inhibition of NF-KB, Snail and YY1 and in the induction of RKIP and resulting in the reversal of chemoresistance.[45]

In addition to the proteasome inhibitors, we have also reported that treatment of tumor cells with the NO donor DETANONOate sensitized tumor cells to chemotherapeutic drugs. The NO treatment resulted in the inhibition of NF-KB and downstream inhibition of Snail and YY1 along with the induction of RKIP, findings similar to those obtained above with the proteasome inhibitors.[81]

2. Reversal of immunoresistance through the RKIP disruption in the loop

The role of RKIP expression in target cells and their response to cytotoxic lymphocytes have been examined using the cytotoxic ligands FasL and TRAIL. The innate (such as NK cells) and adaptive (such as CTL) cytotoxic immune systems have evolved to fight infections and transformed cells like cancer. The human cytotoxic lymphocytes (such as NK and CTL) mediate their killing mechanisms by both apoptotic and necrotic mechanisms. The necrotic mechanism is mediated by the perforin/granzyme system in which perforin perforates holes on the membrane resulting in changes in the osmotic pressure and lysis of the cells. The apoptotic mechanisms

consist primarily of the interaction of membrane expression of ligands on the lymphocytes (consisting primarily of TNF-α, FasL, and TRAIL) with their corresponding receptors (TNFR-1/2, Fas, DR4 and DR5), respectively, expressed on the surface of the target cells. Sensitive target cells in contact with the cytotoxic lymphocytes are triggered via ligand receptors-interactions which lead to activation of the apoptotic pathways (Type I and/or Type II) and resulting in cell death by apoptosis. However, often, a subset of infected/cancer cells is resistant to apoptotic stimuli due to a dysregulation of the apoptotic pathways in favor of anti-apoptotic mechanisms.[82,83] Several mechanisms of immune resistance have been postulated and reviewed extensively elsewhere.[69,84-87]

Tumor cells are inherently resistant to TRAIL apoptosis or selected for resistance by immune selection.[88] Resistance to apoptosis can be mediated by inhibitory proteins such as the Bcl-2 family of anti-apoptotic proteins[89] cFLIP short,[90] and inhibitors of apoptosis[91] all of which are targets of constitutively activated NF-KB. Also, the poor expression or functional signaling by DR5 in tumor cells also results in failure to TRAIL apoptosis.[92,93] Treatment of resistant tumor cells with subtoxic concentrations of chemotherapeutic dugs sensitized the cells to TRAIL apoptosis.[44,94] We have also reported that drug sensitization to TRAIL was accompanied by upregulation of DR5.[44]

TRAIL binds to four receptors, namely, DR4, DR5, DcR1 and DcR2. Only DR4 and DR5 signal the cells for apoptosis.[95,96] It was reported that TRAIL-resistant cancer cells can be sensitized to TRAIL apoptosis by overexpression of RKIP. We have reported that both Fas and DR5 are under the negative regulation of the transcription factor repressor YY1, a target of NF-κB.[44,97] We hypothesized that RKIP-induced sensitization to TRAIL may be due to the inhibition of YY1 and upregulation of DR5 as a consequence of RKIP-mediated inhibition of NF-κB that regulates YY1. The direct effect of RKIP-induced sensitization to TRAIL apoptosis was corroborated in cells treated with siRNA RKIP and resulting in resistance to TRAIL apoptosis.[76] Overexpression of RKIP, however, resulted in

inhibition of YY1 and upregulation of DR5. RKIP induced sensitization to TRAIL was the result of activation of both type I and type II apoptotic pathways.

Both YY1 reporter and expression were inhibited by RKIP. Inhibition of NF-κB by chemical inhibitors mimics RKIP overexpression and sensitized the cells to TRAIL apoptosis. The findings observed with TRAIL can also be extrapolated to sensitization to FasL since Fas is negatively repressed by YY1. The findings above support the role of RKIP in the immunosurveillance of cancer.

IV. INDUCITON OF RKIP BY VARIOUS AGENTS AND REVERSAL OF RESISTANCE BY SENSITIZATION TO DRUGS

A. NO-Mediated Upregulation of RKIP

We have reported that treatment of drug-immune resistant tumor cells with the NO donor, DETANONOate, sensitized the tumor cells to both chemotherapeutic drugs and immune cytotoxic ligands-mediated apoptosis.[81,97,98] Treatment with the NO donor disrupted the NF-κB/ Snail/YY1/RKIP loop and S-nitrosylated both the NF-KB subunits (p50 and p65) and Snail, and resulted in the inhibition of NF-KB, Snail and YY1 and the induction of RKIP.[69] We have reported on the role of NO-mediated sensitization to CDDP and TRAIL, as examples of chemotherapeutic drugs and immune ligands, respectively. Treatment with NO sensitized the resistant tumor cells to CDDP and TRAIL apoptosis. The combination treatment was synergistic. The inhibition by DETANONOate of NF-κB, Snail, and YY1 and induction of RKIP suggested strongly that each member of this loop is involved (directly and indirectly) in the sensitization through its participation in the loop.[69]

The direct role of NF-κB in sensitization to CDDP and TRAIL by DETANONOate was corroborated by the use of specific NF-KB inhibitors (e.g. DHMEQ) which mimicked DETANONOate in inhibiting NF-κB, Snail and YY1 and the induction of RKIP concomitantly with sensitization and apoptosis.

The direct role of Snail inhibition by DETANONOate and its role in sensitization was shown by the demonstration that DETANONOate nitrosylates Snail and thus, inhibits its transcriptional repressor activity on RKIP. In addition, using Snail silencing by siRNA resulted in upregulation of RKIP and chemo-immunosensitization of resistant tumor cells.

Treatment of tumor cells with DETANONOate inhibited YY1, a target gene of NF-κB as assessed by inhibition of its DNA-binding activity through direct S-nitrosylation.[98-100] Since YY1 transcriptionally activates Snail, its inhibition results in the inhibition of Snail transcriptional expression and derepression of RKIP and upregulation of RKIP expression. In addition, treatment of cells with YY1 siRNA sensitized the cells to CDDP and TRAIL apoptosis.[44]

Overall, treatment with DETANONOate resulted in the inhibition of NF-κB, Snail and YY1 and the induction of RKIP. The direct role of RKIP in sensitization was shown by treatment of tumor cells with an RKIP expression vector. Cells overexpressing RKIP were sensitized to CDDP and TRAIL apoptosis. Overexpression of RKIP was correlated with the inhibition of Snail and YY1 as a result, in part, of RKIP-mediated inhibition of NF-κB and downstream its targets Snail and YY1.[45]

B. NPI-0052 (Marizomib)-mediated induction of RKIP and chemo-immunosensitization

We investigated the underlying molecular mechanism by which the proteasome inhibitor NPI0052 (marizomib) sensitized drug-immune resistant tumor cell lines to apoptosis. Our hypothesis was based on the findings that proteasome inhibitors inhibit NF-κB activity on one hand and our findings on the dysregulated NF-κB/Snail/YY1/RKIP loop in cancer cells. Hence, we postulated that treatment with NPI-0052 of resistant tumor cells will result in the inhibition of NF-κB and downstream targets Snail and YY1 along with the induction of RKIP. Such modifications will correlate with the reversal of chemo-immune resistance. This hypothesis was investigated and the findings validated the hypothesis.[45] We demonstrated that treatment with NPI-0052 inhibited

NF-κB, Snail, and YY1 and induced the expression of RKIP and resulted in the reversal of resistance to CDDP and TRAIL.[45] This mechanism was an extension of previous reports demonstrating that several proteasome inhibitors sensitize tumor cells to apoptotic stimuli.[101-104]

Several lines of evidence support the role of NF-κB/Snail/ and YY1 inhibition and induction of RKIP by NPI-0052 in the sensitization of resistant tumor cells. The NF-κB inhibitor DHMEQ, when used for treatment of tumor cells, mimicked NPI 0052 in chemo immunosensitization and inhibited several anti-apoptotic gene products.[105] Treatment with DHMEQ inhibited Snail and induced RKIP expression. The direct role of RKIP induction in chemoimmunosensitization was shown by both overexpression of RKIP that resulted in the reversal of resistance and, in contrast, silencing RKIP induced resistance. The mechanism by which NPI-0052 sensitized the cells to apoptosis resulted from activation to the type II mitochondrial pathway of apoptosis.[45]

C. Induction of RKIP by anti-CD20 antibody and chemosensitization

Rituximab (anti-CD20, chimeric monoclonal antibody) is being used for the treatment of BNHL as mono-therapy or in combination with chemotherapy.[106] In addition to its activity by effector cells (NK-mediated ADCC) and complement mediated cytotoxicity, it has also been reported to signal the cells and also to sensitize drug-resistant tumor cells to apoptosis by chemotherapeutic drugs.[107] Treatment of B-NHL cell lines with another anti-CD20 antibody, BM-ca, resulted in the inhibition of constitutively activated NF-KB and p38 MAPK pathways. This inhibition was accompanied by the induction of RKIP and inhibition of its repressor Snail. In addition, there was inhibition downstream of several anti-apoptotic gene products such as Mcl-1 and the induction of a pro apoptotic gene product such as Bax. These various gene modifications by anti-CD20 antibodies resulted in the sensitization to drug-induced apoptosis.[108] These findings are in agreement with another report by Daniel et al.[109]

D. Induction of RKIP expression by photodynamic therapy

Photodynamic therapy (PDT) has been applied as a therapeutic approach to treat some cancers. It consists of the interplay of three components, namely, the photosensitizer, light, and oxygen. Combined, they produce ROS and/or a singlet oxygen (^{1}O2). Cell death occurs by multiple mechanisms, such as, apoptosis, necrosis and autophagy.[110,111] It has been reported that the level of NO in the tumor environment influences the response to PDT.[112,113] PDT induces iNOS in tumor cells in vivo and also activates inflammatory cells to produce NO.[114] As a result of the above findings and the role of NO in PDT, Rapozzi et al.[115] hypothesized that the efficacy of PDT inhibitory activity may be enhanced by the addition of an exogenous NO donor such as DETANONOate.[116] The findings by Rapozzi et al.,[115] using the B78-H1 amelanotic melanoma cells both *in vitro* and *in vivo*, demonstrated the effectiveness of the treatment with both PDT and DETANONOate. Treatment with the photosensitizer Pba induced iNOS and inhibited the antiapoptotic effects by interfering with the dysregulated NF-κB/Snail/RKIP loop, namely, inhibition of NF-κB and Snail and induction of RKIP. Low doses of NO resulted in opposing effects by low dose PDT and activated NF-κB and Snail and inhibited RKIP expression. The high level of NO resulted in the S-nitrosylation of p65[117] and induction of RKIP.[43] The findings by Rapozzi et al.[115] demonstrated, for the first time, the *in vivo* antitumor activity of PDT combined with DETANONOate and the implied role of the induction of RKIP in the reversal of resistance.

V. ROLE OF RKIP IN THE INHIBITION OF DRUG-RESISTANT CELLS WITH THE EMT PHENOTYPE

The evaluation of tumor specimens and in vitro studies revealed the resistance to EGFR inhibitors in lung cancer and revealed that EMT may underlie the EGFR pathway-independent mechanism of resistance.[118-120] A link between EMT and the acquisition of cancer stemness and the hallmark of CSC and drug resistance have been reported.[121]

In several examined solid tumors, metastasis has been shown to be the result of cells acquiring the EMT phenotype. EMT results from a number of constitutively activated survival pathways

such as the NF-κB[112-124] and the inhibitors of NF-κB suppress EMT.[125-127] The NF-κB's target gene product, Snail, is a metastasis inducer via its repressive activity on E-cadherin, a metastatic suppressor gene product.[127,128] Snail, in turn, represses a metastatic suppressor RKIP.[77]

We have reported that RKIP induction inhibits the EMT phenotype. Treating EMT positive tumor cells with siRNA Snail inhibited both Snail and NF-κB[43] and induced RKIP concomitantly with inhibition of EMT. Similar findings were observed following treatment with the proteasome inhibitor NPI-0052 or the NO donor DETANONOate.[129] The direct role of RKIP-induced expression in the inhibition of EMT was corroborated by several lines of evidence, namely, overexpression of RKIP, silencing Snail, and inhibition of NF-κB. In contrast, the induction of EMT was demonstrated by the inhibition of RKIP or the overexpression of Snail. In a reported study, it was demonstrated the mechanism by which RKIP regulates EMT through a signaling cascade involving the MAPK, Myc, lin28, let7, and downstream let7 targets.[130]

We have reported that treatment of EMT positive tumor cells with the proteasome inhibitor NPI-0052 or bortezomib resulted in the inhibition of NF-κB activity and downstream the metastatic inducer Snail concurrently with the induction of RKIP, originally repressed by Snail.[131]

Since proteasome inhibitors have been reported to inhibit NF-κB[132] and NF-κB is involved in EMT, we tested the effect of NPI-0052 on the EMT phenotype in cancer cell lines. Evidence was presented that demonstrated the treatment of the tumor cells that NPI-0052 inhibited NF-κB and downstream Snail concomitantly with induction of RKIP and E-cadherin. The suppression of Snail expression by NPI-0052 resulted in the downregulation of mesenchymal markers and the induction of epithelial markers. Treatment with NPI-0052 resulted in the upregulation of RKIP mRNA and protein levels. Also, overexpression of RKIP resulted in the inhibition of EMT-related gene products such as vimentin and fibronectin and upregulation

of epithelial gene products related to suppression of metastasis including E-cadherin and cytokeratin 18.[131] These findings supported previous findings by Fu et al.,[31] in studies done both *in vitro* and *in vivo* in mice bearing human tumor xenografts. Hence, RKIP overexpression inhibits the drug resistance of cells of the EMT phenotype.

VI. ROLE OF RKIP EXPRESSION IN DRUG RESISTANT STEM CELLS

Evidence has been reported that cancer initiated cells (CICs) also cancer stem cells (CSCs) are responsible, in part, for tumor relapse and unresponsiveness to treatments. They also contribute to tumor dormancy, metastasis, and relapse.[133,134] The ability of CSCs to exhibit highly tumorigenic properties correlated with a high degree of drug resistance.[121,135] The identification of CSCs in tumors relied primarily on phenotypic markers for certain tumors.[136,137] However, none of these have resulted as targets for therapeutic intervention to reverse drug resistance. A recent report by Sequin et al.[138] have identified α3β3 integrin, a protein associated with poor outcome and high incidence of metastasis, in a variety of epithelial tumors[139] and its expression reported in leukemic cancer stem cells.[140] Of note, α3β3 integrin can trigger anchorage-independent cell survival and metastasis in the absence of ligand binding.[139] The new finding by Sequin et al.[138] demonstrated that αvβ3 expression and its upregulation on the surface of various epithelial tumor cells that were exposed to receptor tyrosine kinases (RTK) inhibitors were also associated with drug resistance. In that study, the role of CD61 (integrin ß3) was found to be both necessary and sufficient to promote tumor stem cell properties and resistance to RTK inhibitors. They demonstrated that integrin avß3 acts in a complex with KRAS and that KRAS is necessary for resistance. By using a combination of bortezomib (inhibitor of NF-κB) and RTK inhibitors it resulted in the reversal of stemness and drug resistance. Based on the above findings on the induction of RKIP by proteasome inhibitors, these studies suggest that these inhibitors may induce RKIP expression and play a role in the reversal of resistance in CSCs.

VII. CONCLUSIONS AND FUTURE DIRECTIONS (See scheme in Figure 1)

The development of drugs to target drug resistance is based on in vitro studies that result in answering fundamental questions such as 1) Is the candidate effector molecule required to maintain a response phenotype 2) Is the candidate effector sufficient to confer resistance? 3) Does the candidate effector mediate the downstream resistant signaling pathway and 4) Is the candidate effector dysregulated in drug-resistance in patient-derived specimens.[4]

Currently, numerous studies have been developed to generate targeted drugs that inhibit cell proliferation and induce cell death. These include DNA replication, the mitotic apparatus, protein chaperones (ex. HSPS), the proteasome, the chromatin, etc. (Reviewed by Dobbles and Moll).[5] The expression level of RKIP may identify tumors requiring a specific drug for its activation to reverse resistance. It may also serve as an important prognostic biomarker. The combination of RKIP-inducing agents with other drugs that target different pathways may result in a significant synergy. Clearly, the resistant mechanisms are less likely to lead to a dominant resistant clone when two drugs are targeted against different cell pathways.

In many tumors, genomic alterations lead to dysregulation of signaling pathways. For example, resistance to MEK and Raf inhibitors have been studied. Activating mutations of MEK and NRAS are observed in tumors that progress in the presence of vemurafenib treatment in melanoma and also confers resistance to Raf inhibitors *in vitro*.[141] MEK signaling becomes uncoupled from the inhibitor B2AF oncoprotein. Erlotinib resistance to lung cancer showed dysregulated NF-κB signaling associated with patient survival.[142] It is important for any resistant mechanism that its information and validation must be relevant in cancer patients. The examination of tumor specimens from patients who relapsed upon exposure to therapy are valuable to establish correlations with the observed *in vitro* and *in vivo* pre-clinical data.

The demonstration that the metastasis suppressor and drug sensitizer RKIP is involved in the dysregulation of the NF-KB/Snail/YY1/RKIP loop is reminiscent of another loop, namely, KRAS/RalB-NF-KB, that is induced by a3ß3 expression. Seguin et al.[138] have reported that the

expression of a3ß3 and the resulting KRAS/RalB-NF-κB pathway were both necessary and sufficient for tumor metastasis, anchorage independence, self-renewal and resistance to erlotinib. Disruption of ß3 downstream signaling leads to a reversal of drug resistance. The implication of NF-κB in NF-κB/Snail/YY1/RKIP and the K-Ras/RalB-NF-κB loops suggests that these two loops may cross-talk and that RKIP may be involved in both loops. It would be interesting to demonstrate that RKIP is involved in the regulation of a3ß3-mediated metastasis and resistance. Hence, it is reasonable to assume that RKIP may be a universal gene product that is involved in the regulation of cell survival, cell growth, metastasis and resistance.

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Figure Legend

Figure 1. Schematic diagram demonstrating the role of RKIP in the regulation of tumor cell drug/immune resistance **(black lines).** Tumor cells exhibit a constitutively hyperactivated NF-κB pathway which regulates, in large part, the survival, growth, metastasis and resistance of tumor cells. Among many target genes that NF-KB regulates, there exists a dysregulated *NF-κB/ Snail/YY1/PTEN/RKIP* loop that is responsible, in part, for NF-κB-mediated above tumor manifestations. The hyperactivated NF-κB pathway regulates cell proliferation, anti-apoptotic pathways, EMT, metastasis and resistance. These events are the results of NF-κB activation of (1) the metastasis inducer and resistance transcription factor Snail, which regulates CSCs, EMT and anti-apoptotic pathways and (2) the drug/immune resistant transcription factor YY1, which also regulates the transcription of Snail. The overexpression of Snail results in the repression of the metastasis suppressor/drug sensitizers, RKIP and PTEN. The repression of RKIP minimally represses the NF-κB pathway directly and indirectly by the repression of PTEN, which does not inhibit the PI3K/AKT pathway that regulates NF-κB. Overall, the tumor cells exhibit a dysregulated NF-κB /Snail/YY1/PTEN/RKIP loop with the overexpression of NF-KB, Snail and

YY1 and under expression of RKIP and PTEN and resulting in tumor cell survival, metastasis and drug resistance **(red lines).** Inhibition of the dysregulated loop products, on each of the overexpressed NF-κB, YY1, Snail, PI3K/AKT, results in the derepression of RKIP and PTEN and resulting in the inhibition of cell growth, survival, EMT, metastasis, and activation of proapoptotic pathways resulting in sensitization to drug-induced apoptosis. There are many classes of inhibitors that may be used and some examples are illustrated in the scheme, for instance, the expression of the active form of RKIP in its phosphorylated form can be rendered active by the use of phosphatase inhibitors. Inhibitors of NF-κB will have both direct and indirect effects on NF-κB target genes such as Snail and YY1 and derepression of RKIP and PTEN. The direct inhibition of Snail and YY1 will have similar effects as the NF-κB inhibitors. Likewise, inhibitors of the PI3K/AKT pathway will have direct and indirect effects on the inhibition of the NF-κB pathway and its targets Snail and YY1.

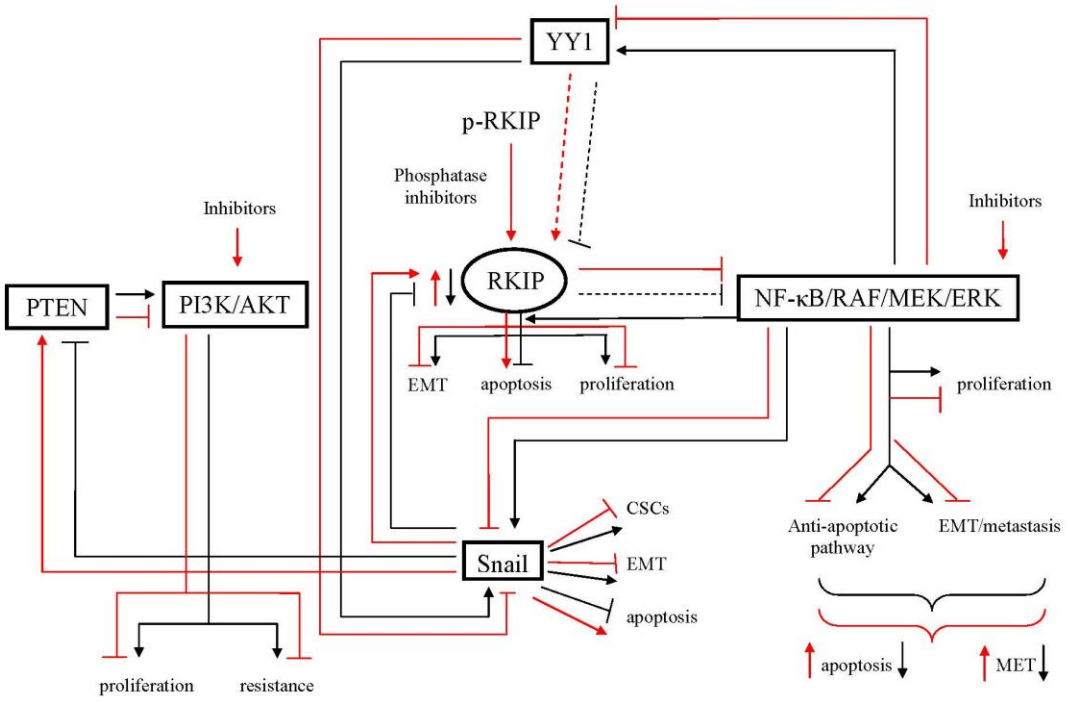
YY1
p-RKIP
Phosphatase inhibitors
Inhibitors
Inhibitors
PTEN
PI3K/AKT
RKIP
NF-κB/RAF/MEK/ERK
EMT
apoptosis
proliferation
proliferation
CSCs
Anti-apoptotic pathway
EMT/metastasis
Snail
EMT
apoptosis
proliferation
resistance
apoptosis
MET

Figure 1