

Original Article

Network modeling of CDF treated pancreatic cancer cells reveals a novel c-myc-p73 dependent apoptotic mechanism

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Abstract: Systems biology and molecular network modeling are important tools that are finding application in anti-cancer drug discovery. These technologies can be utilized to map and evaluate the entire set of pathways modulated by drugs in cancer cells without losing key details. Such integrated approaches are especially useful in understanding the mechanism of action of agents that do not have a defined target. Our novel compound CDF (a synthetic analogue of curcumin), is one such multi-targeted agent with proven anti-cancer activity *in vitro* and *in vivo*. However, its mechanism of action is not fully understood, and thus a thorough analysis of key pathways targeted by CDF would be important for developing targeted and tailored therapy in the future. Applying Ingenuity Pathway Analysis (IPA), we have mapped the pathways altered by CDF treatment of BxPC-3 pancreatic cancer (PC) cells. Illumina HT-12 microarrays were performed on RNA extracted from CDF treated cells. IPA analysis of gene expression at early time point (24 hrs) revealed deregulation of genes in the c-Myc hub. Western blot analysis validated the activation of c-Myc, p73 and its downstream pro-apoptotic effector Bax with simultaneous down-regulation of Bcl-2 in two distinct pancreatic cancer cell lines (BxPC-3 and Colo-357). In order to further delineate the role of c-Myc in inducing apoptosis, siRNA silencing technology was used. As expected, c-Myc siRNA knockdown resulted in abrogation of the growth inhibitory and apoptotic potential of CDF. In conclusion, our results demonstrate a novel c-Myc driven apoptotic network activated by CDF in PC cells that is independent of wild-type p53, and thus warrants further investigation on the clinical utility of CDF.

Keywords: Systems Biology; Network Modeling; Curcumin; CDF; c-Myc; p73; p53; Bax; Bcl-2; Apoptosis; Pancreatic Cancer

Introduction

Integrated systems biology and molecular network modeling are highly powerful and new tools that are making inroads in the area of anti-cancer drug discovery [1]. These technologies are increasingly being utilized by investigators to map the pathways modulated in response to a single agent or combination drug treatments in virtual cancer models, cell lines and actual tumors [2]. Systems science becomes useful in instances where older techniques fail to deliver desirable molecular information. For example, traditional molecular biology tools, although partially successful, lack the power to delineate the mechanisms of action of pleiotropic drugs (i.e. drugs having multiple targets) [3]. It is in-

creasingly being realized that in order to understand the mechanisms of such multi-targeted drugs requires departure from protein-centric to a more robust integrated pathway-centric approach [4]. This is because network modeling has the power to mine the entire set of drug response pathways without losing key details [5]. To this end our laboratory was among the first to demonstrate in a "proof-of-concept" study that systems biology and network modeling can be utilized to understand the mechanism of action of novel drug combinations [6].

CDF (curcumin difluorinated) is a difluoro analogue of a well recognized chemopreventive agent curcumin [7,8,9]. It is a multi-targeted agent with proven anti-cancer effects *in vitro* and *in*

vivo [7,8,9]. Over the last several years our laboratory has extensively characterized the growth inhibitory and apoptotic potential of CDF against PC [7,8], colon [10] and prostate tumors [11]. Many different mechanisms have been attributed to its efficacy against a wide variety of tumors. In PC, CDF induced growth inhibition and apoptosis, which was found to be mediated through down-regulation of microRNA (miR)-21 (miR-21), PTEN and up-regulation of miR-200 [8]. In a colon cancer stem-like cell model with functional p53 (wt-p53), CDF-induced apoptosis was associated with down-regulation of the membrane transporter ABCG2 and attenuation of EGFR, IGF-1R, and NF- κ B signaling, which was also consistent with inactivation of β -catenin, COX-2, Bcl-xL and activation of the pro-apoptotic Bax [9]. Additionally, in a castrate resistant aggressive prostate cancer cell line model, CDF inhibited the signal transduction in the androgen receptor (AR) TMPRSS2-ERG fusion gene wnt signaling (AR/TMPRSS2-ERG/Wnt) network, leading to the inactivation of Wnt signaling, which was consistent with inhibition of cell invasion [11]. Taken together, the above multiple studies, although provide incremental knowledge, yet fail to offer a concrete molecular mechanism of action of this potent agent; i.e., which driver oncogene is the legitimate target of CDF.

In order to delineate the molecular mechanism of action of CDF, we performed systems and integrated pathway network analysis using CDF-treated BxPC-3 PC cells (wild-type p53). Our early gene expression profiling reveals the modulation of a unique c-Myc hub. Our results also demonstrated that CDF induced perturbations in c-Myc hub orchestrates a unique set of events that eventually led to the induction of apoptosis. These studies highlight the tumor suppressive role of c-Myc that is in contrast to its well documented role as a tumor promoter.

Materials and methods

Cell culture, experimental reagents and chemicals

BxPC-3 and Colo-357 PC cells were purchased from American Type Culture Collection (ATCC). These cell lines have been tested and authenticated in our core facility "Applied Genomics Technology Center" at Wayne State University, as late as March 13, 2009, and multiple aliquots were frozen in liquid nitrogen. The

method used for testing was short tandem repeat (STR) profiling using the PowerPlex® 16 System from Promega (Madison, WI). The cells were put in culture fresh for all experiments. Primary antibodies for c-Myc, Bax and p21 were purchased from Cell Signaling (Beverly, MA). All secondary antibodies were obtained from Sigma (St. Louis, MO).

Microarrays and expression analysis

Expression networks from BxPC-3 cells were examined from an analysis of global expression time series data. Cells were plated so that they reached 75% confluence after 3 days. At this point cultures were treated with CDF and RNA was isolated from triplicate plates after 24 hours of treatment. Media was changed the day after plating and at the start of treatment. Quantitative measurement and the high quality of all mRNA samples were assured by analysis with the NanoDrop 1000, Agilent Bioanalyzer and the Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany). Expression levels were determined by microarray analyses using the Illumina human HT12 array. Data were processed for quality control and normalized across compared arrays by quantile normalization. Genes with 1.7 or greater expression fold-change were included in Ingenuity Pathways Analyses. Cluster analysis of expression profiles was performed with Bayesian analysis using CAGED software. Canonical pathway analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the data set. Molecules from the data set that met the 1.7 fold-change cut-off and were associated with a canonical pathway in Ingenuity's Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways: 1) A ratio of the number of molecules from the data-set that map to the pathway divided by the total number of molecules that map to the canonical pathway. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the data-set and the canonical pathway is explained by chance alone.

Western blot analysis

BxPC-3 and Colo-357 cells were grown in 100 mm petri plates over night to 60-70% confluence. The next day, cells were exposed to indi-

cated concentrations of CDF for 24 hrs followed by extraction of protein for western blot analysis. Preparation of cellular lysates, and protein concentration determination and SDS-PAGE analysis was done following previously described procedures [7,8].

siRNA and transfections

To study the effect of c-Myc silencing on the activity of CDF, we utilized siRNA silencing technology. C-Myc siRNA and control siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with either control siRNA or c-Myc siRNA for 5 hrs using LipofectAMINE 2000 according to the manufacturer's protocol (Cell Signaling, Danvers MA). After the siRNA treatment period cells were further treated CDF in 96 well plates for MTT and 6 well plates for Annexin V FITC assays respectively as described below.

Cell growth MTT assay

Colo-357 and BxPC-3 cells were seeded in a 96-well culture plate (at a density of 3×10^3 cells per well) and treated with CDF in the presence or absence of siRNAs, and MTT assay was performed as described earlier [7,8]. The results were plotted as means \pm SD of three separate experiments using six determinations per experiment for each experimental condition.

Quantification of apoptosis by histone DNA ELISA and annexin V FITC flow cytometry

Apoptosis in BxPC-3 and Colo-357 cells was determined using Histone DNA ELISA (Roche, NY) and Annexin V FITC apoptosis kit (Biovision Research Products) according to manufacturer's protocol. Briefly, 50,000 cells were grown in six well plates overnight and exposed to different treatments in the absence or presence of c-Myc siRNA and control siRNA. After 72 hrs treatment, cells were trypsinized, collected and counted for further processing using Histone DNA ELISA kit or Annexin V FITC flow cytometry analysis as described previously [7,8].

Results

Ingenuity network analysis of early gene changes by CDF

In order to comprehensively delineate the

mechanism of apoptosis by CDF against PC cells, a systems approach was undertaken. Microarrays were performed using RNA isolated from CDF treated BxPC-3 cells followed by network modeling of global genes. Differentially expressed genes that met the 1.7 cutoff criteria were mapped using IPA software under defined conditions as described previously [12]. Interestingly, among the different hubs activated, c-Myc and its related network was found to be deregulated upon CDF treatment (**Figure 1**). Additionally, the network consisted of a number of activated and repressed c-Myc dependent microRNAs, although not the focus of this article, were also evaluated. Building on the IPA derived network, we evaluated the expression level of c-Myc and its consequence on CDF mediated growth inhibition and apoptosis as discussed below.

CDF activates c-Myc pathway in PC cell lines

Based on network information showing the activation of c-Myc hub in PC cell treated with CDF, we sought to explore the protein expression level of c-Myc in two distinct PC cell lines BxPC-3 and Colo-357. As can be seen from results presented in **Figure 2**, CDF treatment resulted in the up-regulation of c-Myc protein in both the cell lines tested. Additionally, we also explored the consequence of c-Myc activation on related pathway that leads to apoptosis. Of importance is the observed activation of p73 and its downstream effector proteins Bax. In addition, we found that the levels of Bcl-2 were significantly decreased. These results point to a previously unrecognized and novel c-Myc driven p73 pathway activation in PC cells treated with CDF.

c-Myc siRNA abrogates CDF mediated growth inhibition and apoptosis

Once the activation of c-Myc was verified using IPA and western blot analysis, we explored the consequence of siRNA silencing of c-Myc on the growth inhibitory and apoptotic potential of CDF. As can be seen from the results presented in **Figure 3**, CDF mediated growth inhibition of Colo-357 and BxPC-3 cells were lost in the presence of c-Myc siRNA while no significant effect was observed in cells transfected with control siRNA. Moreover, we tested whether c-Myc silencing could also abolish CDF-induced apoptosis or not. As can be seen from the results presented in **Figure 4A** that in the presence of control

CDF treatment and c-myc-p73 dependent apoptosis

BxPC-3 CDF 24h

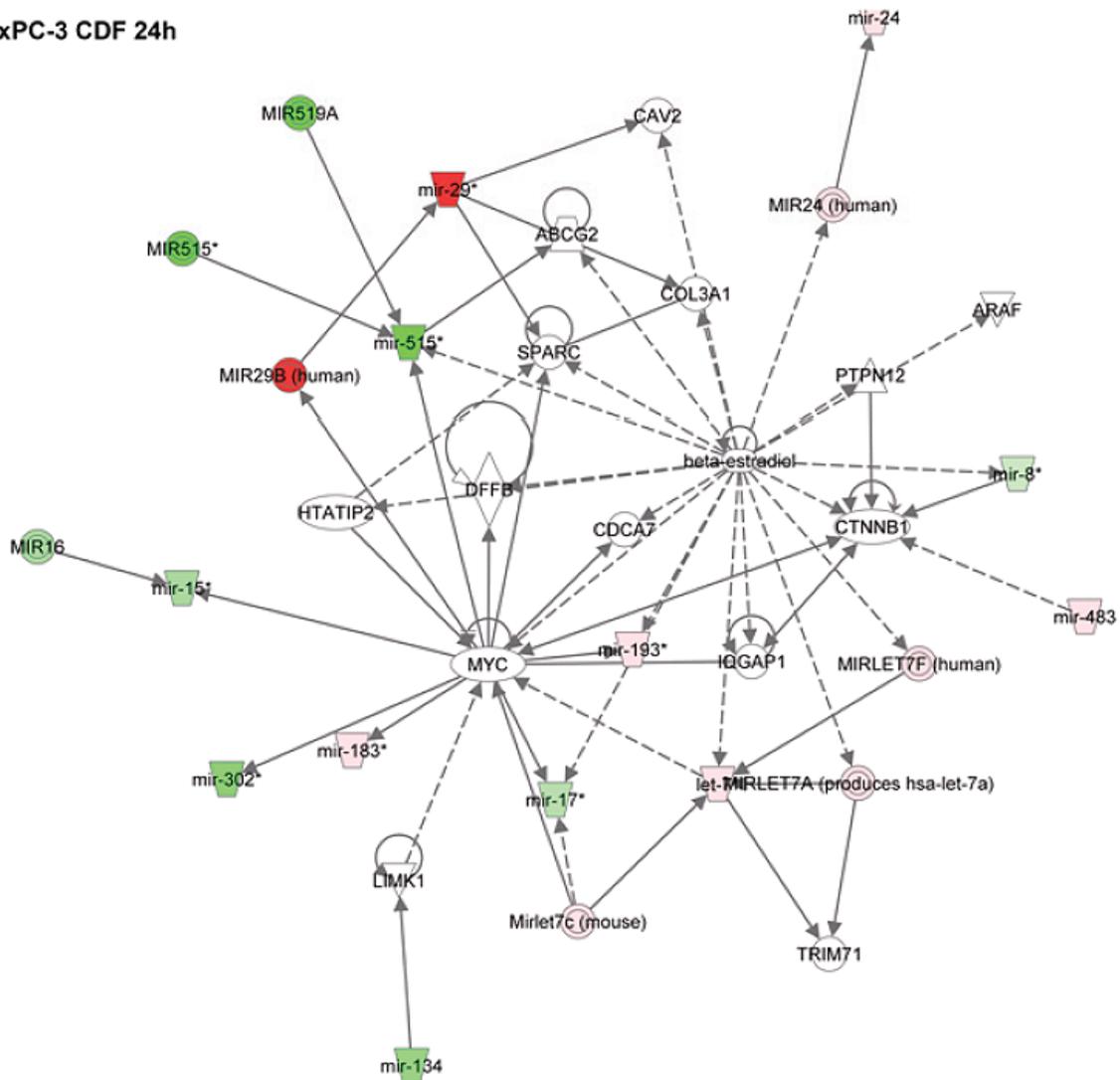


Figure 1. Ingenuity pathway analysis (IPA) of CDF driven pathway changes in BxPC-3 cells. BxPc-3 cells were seeded in triplicate 100 mm plates and treated with CDF (500 nM) for 24 hrs. RNA was isolated using our previously published reports [7,8]. Microarrays were run using the Human HT-12 microarrays. The microarray data was fed into the IPA software and networks were mapped under defined conditions as described elsewhere [6,12]. Note: activation of c-Myc hub.

siRNA there was no effect on apoptosis by CDF; however, CDF was ineffective in inducing apoptosis in the presence of c-Myc siRNA as detected by histone DNA ELISA assay. We further evaluated the apoptotic induction using Annexin V FITC which showed similar trend with almost complete loss of apoptosis by CDF in the presence of c-Myc siRNA (**Figure 4B**). These siRNA results provide irrevocable proof of the role of c-Myc in CDF mediated growth inhibition and apoptosis. The summary of our overall observa-

tions are depicted in **Figure 5**.

Discussion

In this article, utilizing a systems approach and supported by molecular validation studies, we demonstrate for the first time the key role of c-Myc in CDF mediated cell growth inhibition and induction of apoptosis. The results of our investigation revealed a tumor suppressive role of c-Myc-p73 apoptotic network induced by CDF,

CDF treatment and c-myc-p73 dependent apoptosis

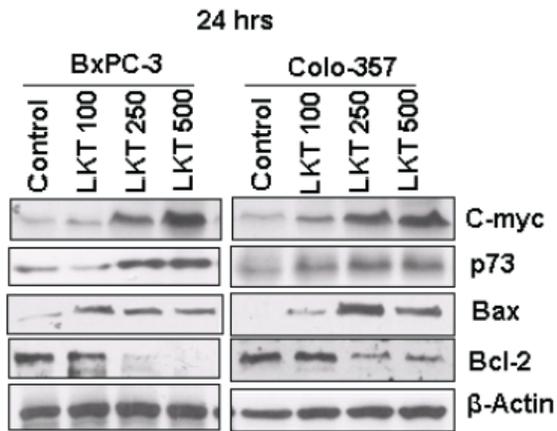


Figure 2. Protein expression analysis of CDF treatment. BxPC-3 and Colo-357 cells were exposed to increasing concentrations of CDF (0-500 nM) for 24 hrs. Protein was isolated according to previously published methods [7,8]. The lysates were resolved through 10% SDS-gel. Western blot was performed according to our previous publications [7,8]. The blots were probed using antibodies for c-Myc, p73, Bax and Bcl-2, and β -actin was used as protein loading control. Note: activation of c-Myc, p73 and Bax and down-regulation of Bcl-2. Blots are representative of two independent experiments.

which appears to be independent of wild-type p53 functional, suggesting that CDF could be useful in tumors with or without function p53.

The diverse biological activities and functions attributed to c-Myc have provoked intense debate in the field of cancer biology [13]. Earlier work suggested c-Myc to be an oncoprotein that was found routinely activated in cancer [14,15]. Approximately 70% of all cancers have over expression c-Myc providing strong indication for its role in tumorigenesis [16]. Activation of c-Myc occurs through various mechanisms that include translocations, amplifications and enhanced translation [17]. Gain of function mutations have also been reported for some tumor types [18]. However, in the majority of cancers, c-Myc activation is governed by alterations in signaling pathways that induce or repress this oncoprotein. These observations provided strong support to the well accepted hypothesis that increasing threshold levels of c-Myc renders normal cells at high risk for transformation i.e. a hallmark of the carcinogenesis process [19].

In the past a number of oncogenes such as E2F-1 [20] and E1A [21] that were initially thought to serve only as tumor promoters were in fact later proven to be tumor suppressors and c-Myc was no exception. The first paradox in the field came with the discovery by Askew et al demonstrating that c-Myc could in fact trigger apoptosis [22]. Since then a number of mechanisms both intrinsic and extrinsic have been attributed to c-Myc driven apoptosis. For example, it was earlier shown that c-Myc can directly induce the

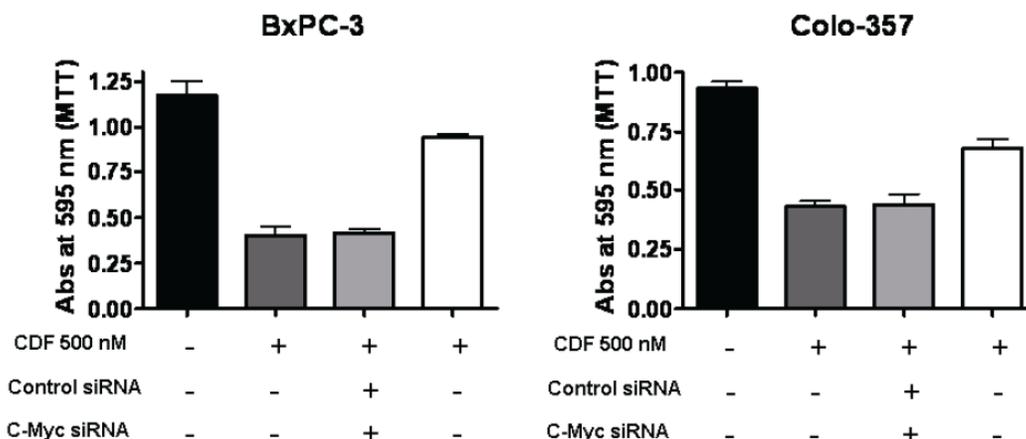


Figure 3. Effect of c-Myc siRNA on growth inhibition by CDF. BxPC-3 and Colo-357 cells, after treatment with control siRNA and c-Myc specific siRNA, and were further kept as (a) untreated control or (b) CDF 500 (nM) treatment for 72 hrs. At the end of the treatment, MTT assay was performed using previously described method [7,8]. Note: c-Myc siRNA treatment abrogates CDF mediated growth inhibition. Plots are representative of three independent experiments. * denotes $p < 0.05$ and **denotes $p < 0.01$.

CDF treatment and c-myc-p73 dependent apoptosis

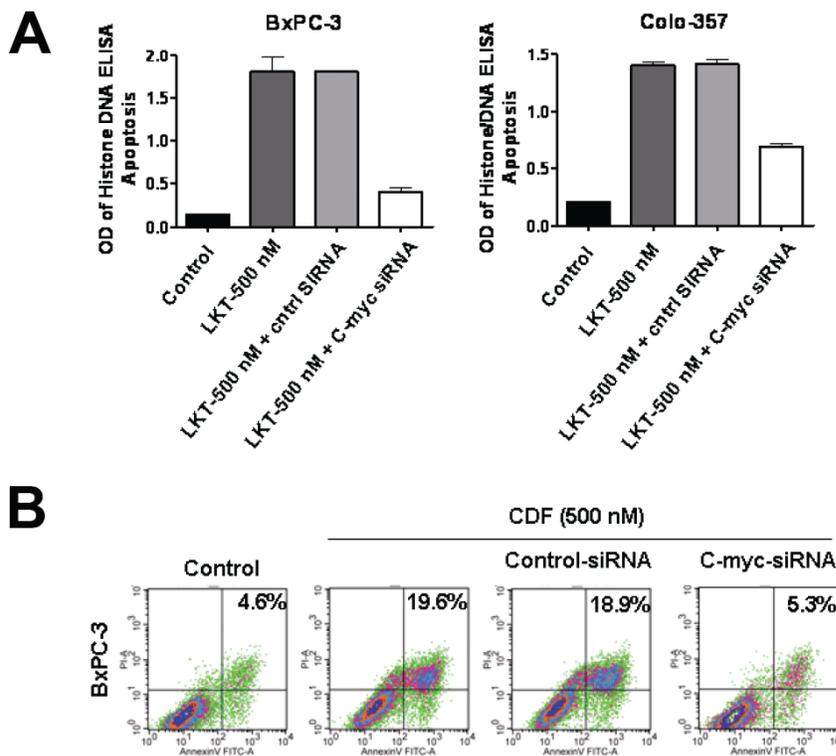


Figure 4: Effect of c-Myc siRNA on apoptosis by CDF. BxPC-3 and Colo-357 cells, after treatment with control siRNA and c-Myc specific siRNA, and were further kept as (a) untreated control or (b) CDF 500 (nM) treatment for 72 hrs. At the end of the treatment, the degree of apoptosis was analyzed using [A] Histone DNA ELISA analysis (Roche, Indianapolis, IN) and [B] Annexin V FITC assay [Biovision Inc] according to procedures described by the manufacturer. Note: c-Myc siRNA treatment abrogates CDF mediated apoptosis. Plots are representative of three independent experiments. * denotes $p < 0.05$ and ** denotes $p < 0.01$.

expression of pro-apoptotic puma in a p53 dependent manner [23,24]. Another study showed that stress activated c-Myc could, in turn, disrupt the Bcl-2-Bcl_{xL} interaction leading to mitochondrial pore permeabilization and consequent apoptosis through cytochrome c release [25]. c-Myc has also been shown to directly repress the expression of Bcl-2 indicating direct activating role of c-Myc on Bax expression [25]. Interestingly, our results showed similar trend showing substantial down-regulation in Bcl-2 expression and up-regulation in Bax in response to CDF treatment in two distinct PC cell lines.

The ARF-p53 pathway was shown to be an important mediator of Myc-induced apoptosis in cells with functional p53 [26,27]. This is supported by the observation that Myc activation in primary cells is associated with profound induction of both ARF and p53 protein levels [28]. However it should be noted that the cells tested in our study have mutant p53 and this puts forward the important question on the exact mechanism downstream of c-Myc that could be responsible for apoptosis induced by CDF. Increased evidence suggest that p73 belongs to the p53 family protein, but unlike the latter it is

rarely mutated in cancer cells [29]. Both p73 and p53 share common downstream genes such as Bax and p21 that function as effectors of apoptosis and cell cycle regulation, respectively [30,31]. Our earlier studies have shown that in the absence of functional p53, apoptosis in PC cells by MDM2 inhibitor and or cisplatin can be induced through p73-Bax pathway [32]. Additional supporting evidence comes from a number of key findings that demonstrate a strong link between c-Myc and p73 dependent apoptosis. Zaika et al. have shown that c-Myc and other oncogenes can induce and activate p73 leading to apoptosis in p53 deficient tumors [33]. Our western blot findings showing activation of p73 and Bax are consistent with these published studies, and further highlight the important link between c-Myc activation and its consequence on p73 network. Further, validation came from siRNA studies which demonstrated the critical requirement of c-Myc during CDF mediated growth inhibition and induction of apoptosis. These results provide strong evidence in support of a tumor suppressive role of c-Myc that is dependent on the p73 pathway instead of p53, which warrants future in-depth investigations.

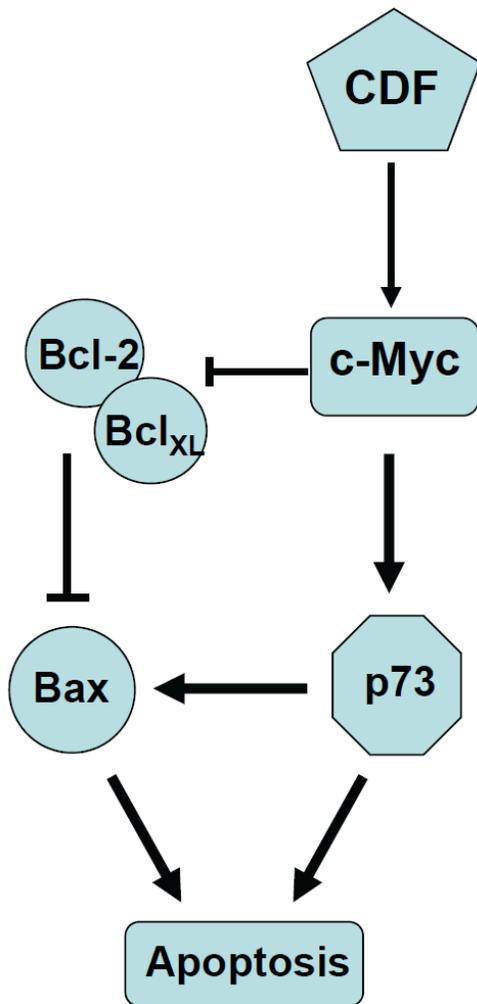


Figure 5. Novel c-Myc-p73 apoptotic network of CDF. Simplistic diagram showing novel c-Myc driven network that drives p73 mediated apoptosis. In the absence of functional p53 c-Myc can activate p73 and its downstream apoptotic effector Bax through down-regulation of Bcl-2 leading to effective apoptosis.

A number of novel strategies have been developed to overcome the target-shortage in PC; a disease with no clear single drugable marker as target. Pharmaceutical industry has rapidly and exponentially added many novel classes of drugs to drug-pipeline. Most of these new drugs are aimed at influencing multiple targets in a parallel fashion. Several clinically proven and efficient drugs, such as salicylate, non-steroidal anti-inflammatory drugs (NSAIDs), metformin (anti-diabetic), antidepressants, anti-neurodegenerative agents, multi-target kinase inhibitors (such as Gleevec™, or the inhibitors of

the kinase-maturing molecular chaperone, Hsp90) affect many targets simultaneously [34,35]. These multi-targeted drugs are increasingly being used in cancer therapy to delay the development of resistance. CDF is one such multi-targeted compound that has shown promise as an anticancer agent in pre-clinical studies [7,9]. Like other multi-targeted agents, CDF was found to have lower target binding affinity than a single-target drug. This is because it is unlikely that a small, drug-like molecule can bind to a number of different targets with equally high affinity. However, it has to be stressed that low-affinity binding does not mean low-efficiency. Systems biology and network modeling have proven that a vast majority (>80%) of the cellular protein, signaling and transcriptional networks are in a low-affinity, or transient 'weak linkage' with each other i.e. forming a complex network. It is hypothesized that a weak targeting by CDF on critical hubs may be sufficient to orchestrate a cascade of events leading to substantial effects at the cellular level. This may explain for the newly discovered apoptotic activity of CDF through the c-Myc-p73 network in PC (Fig-5). It is highly possible that there may be other potential targets of CDF that are context driven and tumor dependent and yet to be discovered. The important and burning issue is how should we find the relevant target-sets of this multi-targeting drug? The results presented in this article, although a small step certainly provided a "proof-of-concept" documenting that systems biology and network modeling could be useful in the quest of finding novel targets of therapeutic agents, which would likely aid in designing targeted and tailored therapy for patient diagnosed with PC.

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