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High-Throughput Screening Strategies for Targeted Identification Small-Molecule Compounds Towards Activated Pathways in Colorectal Cancer

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Summary

Recent advancement in understanding the role of both the genetics and molecular pathways in the formation and progression of colorectal cancer allowed the identification of factors that may be targeted for drug discovery. For the past decade various approaches have been developed to target specific steps or components of these pathways in order to prevent the development or progression of colorectal cancer. The innovation and optimization of high-throughput screening methods as well as the recent emphasis from the National Institutes of Health on translational sciences have enabled rapid progress in drug discovery in many fields, including colorectal cancer. Here we present a summary of the recent efforts of targeted high-throughput drug discovery towards pathways affected in colorectal cancer.

Keywords

High-throughput screen; Colorectal cancer; Molecular pathways; Chemical probes

1. High-Throughput Screen and the National Institutes of Health RoadMap Project

High-throughput screen (HTS) is an efficient strategy that lies at the heart of the drug discovery. Implementation of this method allows the identification and validation of compounds that target factors involved in the development of numerous diseases. Extensive reviews regarding the characteristics, methodology, and evaluation of HTS have been previously published [1–3]. Targeted drug discovery program is highly supported by the National Institutes of Health (NIH) Common Fund (<http://commonfund.nih.gov>). The NIH Common Fund (a Roadmap Project, <http://commonfund.nih.gov/aboutroadmap.aspx>) supports biomedical research in a variety of areas allowing for progress in understanding the mechanisms governing biological processes. The innovation and optimization of high-throughput screening methods as well as the NIH programs connected to translational sciences both play important function in targeted drug discovery. This review focuses on targeted high-throughput screens performed in the background of colorectal cancer cell lines. Examples of lead compounds successfully identified in these screens are presented in Table 1.

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2. Mechanism of Colorectal Cancer Development and Progression

Colorectal cancer is one of the leading causes of cancer mortality and morbidity in the United States (see U. S. Cancer Statistics by the Centers for Disease Control and Prevention; <http://apps.nccd.cdc.gov/uscs/>). The progression from normal epithelial tissue to metastatic form of this cancer requires several steps; each of them involving alterations in the regulation or activity of specific signaling pathways [4]. The changes in the activity of molecular pathways that lead to diseases involve mutations, gene amplification, and epigenetic alterations. There are several major pathways that abnormal activity leads to colorectal cancer: Wnt, epidermal growth factor receptor (EGFR), and p53. Alterations in these pathways that are associated with colorectal cancer are listed in Table 2.

Below we present a short description of the characteristics of these pathways in the normal intestinal epithelial cells, the consequences of their abnormal activity in the development of disease, and the results of targeted drug discovery aimed at these pathways for colorectal cancer treatment.

a. The canonical WNT signaling pathway

The schematic outline of the canonical Wnt signaling pathway is illustrated in Figure 1. Extensive description of the role of Wnt signaling in development and diseases can be found in recent reviews [4, 5]. At steady state, β -catenin (a major effector of the Wnt pathway and a transcriptional regulator) is bound to adenomatous polyposis coli (APC), glycogen synthase kinase 3- β (GSK3 β), casein kinase 1 (CK1), and Axin, forming a complex called the destruction complex. Sequential phosphorylation of β -catenin at serine 45, tyrosine 41, and serines 37 and 33 by CK1 and GSK3 β , stimulates its binding and degradation by proteosomal complex β -transducin repeat-containing protein (β -TRCP) [6]. In this fashion, tight regulation of cytoplasmic β -catenin levels controls cell proliferation (Figure 1A). Upon binding of the Wnt ligand to the Frizzled receptor (Fzd) and low-density lipoprotein receptor-related protein 5 and 6 (Lrp5/6), disheveled (Dvl) sequesters Axin and GSK3 β from the cytoplasm to the membrane [7, 8]. At this point, β -catenin cannot be trapped in the APC (destruction) complex, accumulates in the cytoplasm and nucleus where it regulates the transcription of a spectrum of genes involved in the cell proliferation (Figure 1A). The regulation of the Wnt pathway is very stringent, with the highest activity of this pathway in the crypts of the normal intestinal epithelium where proliferation occurs [9]. Constitutive activation of β -catenin leads to uncontrolled proliferation and cell growth, and can result in cancer development. The most frequent mutations that deregulate Wnt signaling and cause β -catenin stabilization occur in gene encoding APC. Mutations in the APC gene are almost all non-sense and the truncated form of APC cannot form the proper complex with GSK3 β , CK1, Axin, and β -catenin. Thus β -catenin is not phosphorylated, accumulates in the cytoplasm and nucleus, and promotes unrestrained cell proliferation. Alternatively, a minority of miss-sense mutations affect β -catenin which render it insensitive to CK1 and GSK3- β phosphorylation and elevate its levels and transcriptional activity, leading to abnormal cell proliferation (Figure 1B). As alteration of Wnt pathway in the proliferative intestinal epithelium is a hallmark of colon cancer, numerous efforts have been undertaken to identify small molecules that can inhibit the activity of this pathway in tumor cells. Moreover, the role of Wnt signaling in the intestinal stem cells renewal [10] and homeostasis is critical and it became obvious that perturbation of this pathway in stem cells leads to transformation of the progenitor cells into cancerous cells [11, 12]. More than 80% of colorectal cancers have APC mutation and around 10% are associated with a mutated form of β -catenin, both mutations cause hyperactivation of Wnt signaling. There are three major approaches for targeting Wnt pathway: i) to decrease β -catenin protein levels by affecting components of the destruction complex (APC, Axin, CKI, and GSK3 β) which is imperative for β -catenin phosphorylation and degradation, ii) to decrease transcriptional

activity of the β -catenin by diminishing its interaction with nuclear transcriptional components, e.g. transcription factor-4 (TCF-4) or CREB binding protein (CBP) proteins, and iii) to directly target expression of the pro-proliferative genes regulated by Wnt/ β -catenin pathway.

To identify Wnt pathway inhibitors, Chen and colleagues [13] performed screens using luciferase-based β -catenin transcriptional readout in mouse L-cells. Two identified compounds IWP and IWR targeted the acetyltransferase Porcupine and Axin, respectively. Porcupine plays an important role in the Wnt ligand maturation, and IWP molecule directly inhibits the activity of Porcupine. IWR has been tested in DLD-1 cell line for its ability to impact elements of β -catenin destruction complex. It was shown that IWR has a positive effect on Axin stability and decreases the pool of free β -catenin that resides in the cytoplasm without any impact on β -catenin bound to E-cadherin (the fate of the membranous pool is described later on). In DLD-1 cells IWR was able to: a) inhibit aberrant Wnt pathway activity as tested with luciferase reporter assays, b) decrease cellular proliferation as measured by cell viability assays, and c) increase stability of Axin protein, but not mRNA levels. Preliminary results presented by this group suggested direct interaction between IWR compound and Axin. Tests performed in a zebrafish model showed that this compound has very strong negative effects on intestinal tissue homeostasis by disrupting progenitor and stem cells renewal. Another group [14] performed similar high-throughput assays in HEK cells and identified compound XAV939 that exerts its function by a similar mechanism as IWR – stabilization of Axin protein and nevertheless decreases protein levels of free, cytoplasmic β -catenin. Furthermore, Huang et al. [14] showed that the effect of XAV939 on Axin stability can be attributed to direct interaction of XAV939 with Tankyrase, a protein that regulates Axin degradation. Thus, XAV939 binding and inhibition of Tankyrase renders Axin stable, a step that is followed by increased β -catenin phosphorylation at tyrosine 41, and serines 37 and 33, and β -catenin degradation. The ability of XAV939 to inhibit Wnt signaling was confirmed in colorectal cancer cell lines. In DLD-1 cells bearing *APC* mutation, XAV939 was able to inhibit proliferation and colony formation; however these processes were not affected in β -catenin-independent RKO cells. Moreover they examined the role of previously identified IWR compound [13] on Tankyrase and found that this molecule is a negative regulator of Tankyrase as well. These are the first studies showing the link between Tankyrase function and Wnt pathway components, therefore extensive studies are necessary to uncover the mechanism of their *in vivo* interaction.

Thorne et al. [15] developed luciferase-based assays for high-throughput screen that permit simultaneous measuring of the activity of β -catenin–firefly luciferase and Axin–*Renilla* luciferase fusion proteins upon activation with a soluble form of LRP6 in *Xenopus laevis* eggs. In response to soluble LRP6 stimulation, β -catenin protein levels should increase and Axin decrease, as is during Wnt signaling activation. Using this assay Thorne and colleagues searched for compounds that abrogate β -catenin activation. They identified a compound, called pyrvinium pamoate, that a) decreases the levels of β -catenin in the cytoplasm and nucleus with no impact on membranous E-cadherin-bound β -catenin, b) increases the levels of Axin, c) inhibits the transcriptional activity of β -catenin as tested with the TOPFLASH reporter plasmid, and d) decreases the levels of mRNA of β -catenin target genes activated by Wnt3a signaling, e.g. *c-Myc* and *Bcl9*. Comprehensive molecular studies showed that pyrvinium pamoate directly interacts with and activates CKI, thus promoting β -catenin degradation. In addition to direct effects on β -catenin, pyrvinium has been shown to negatively regulate a downstream factor of Wnt pathway: Pygopus, which possess several potential CKI phosphorylation sites. Furthermore, the authors demonstrated that pyrvinium reduces the proliferation of colorectal cancer cell lines (DLD-1, HCT116, SW480, and SW620) and is more potent than IWR compound identified by Chen et al.[13].

Another method that allows the selection of compounds against cancer cell lines with constitutive activation of Wnt signaling has been presented in a paper published by Ewan et al. [16]. The authors established an inducible HEK cell line stably expressing Dvl2 fusion with estrogen receptor (ER) and luciferase with green fluorescent protein (GFP) genes under the control of a minimal *c-Fos* promoter with TCF-binding sites and Xnr3 enhancer. Addition of estradiol induces the expression of Dvl2 and the transcriptional activity of β -catenin within 30 minutes. This permits the identification of compounds that block β -catenin induction upon estradiol addition. Multiple counterscreens and follow-up experiments allowed the identification of three compounds: CCT031374, CCT036477, and CCT7070535 that a) blocked the induction of β -catenin activity and its transcriptional activity, b) decreased the proliferation of colorectal cancer cell lines (HCT116, HT29 and SW480), c) had low effect on proliferation of normal cells, and d) were able to induce apoptosis in HCT116 cell line as tested by caspase 3 activation. Subsequent experiments performed in several different models: HEK, mouse L-cells, U2Os, *Xenopus*, and Zebrafish embryos, led the authors to conclude that these compounds prompt degradation of β -catenin through GSK3 β stabilization. Unfortunately, this hypothesis was not confirmed in SW480 colorectal cancer cell line; which can suggest additional mechanisms of action of these compounds independent of β -catenin.

The other approach used to search for molecules that inhibit Wnt signaling is to target the protein-protein interaction between β -catenin and relevant transcription factors that facilitate β -catenin-dependent transcription. Interaction between β -catenin and TCF-4 in the nucleus allows the expression of genes involved in cell cycle progression and proliferation: cyclin D1, c-Myc or Axin2. Thus, identification of compounds that can disrupt the interaction between β -catenin and TCF4 would be critical for preventing tumorigenesis. Lepourcelet and colleagues [17] established a method that allows the measurement of the interaction between purified β -catenin and purified N-terminal fragment of TCF4 fused with glutathione S-transferase (GST) and conjugated to alkaline phosphatase (AP) antibody. Inhibition of β -catenin-TCF4 interaction would present itself as a change in the AP activity. Out of 7,000 natural compounds they identified eight, which in a dose-dependent manner, inhibited β -catenin-TCF4 interaction with IC₅₀ value lower than 10 μ M. They showed that incubation of extracts from HCT116 cells with PKF115–584, CGP049090, and PKF222–815 decreased β -catenin-TCF4 binding in a dose-dependent manner. Proteins levels of cyclin D1 and c-Myc were downregulated in HCT116 cells treated with PKF115–584 and CGP049090, whereas ZTM000990 affected only levels of c-Myc, PKF118–310 and NPDDG1.024 only cyclin D1 levels, and PKF118–744 was inactive in both assays. Proliferation of two colorectal cancer cell lines, HCT116 and HT29, after treatment was significantly reduced by PKF115–584, CGP049090, ZTM000990, PKF118–310, PKF118–744, and NPDDG1.024. In another study, [18] an inhibitor of β -catenin dependent transcription in SW480 colorectal cancer cell line has been identified [18]. This compound, ICG-001, competes with β -catenin for binding to CBP by selectively binding to CBP. This interaction abrogates the expression of transcription targets of Wnt pathway: survivin, cyclin D1 as well as S100A4. The therapeutic potential of this agent was tested in two colorectal cancer cell lines (HCT116 and SW480) *versus* normal colonic epithelial cell line (CCD-841Co) and a selective inhibitory effect on tumor cells was demonstrated. When tested in the *Apc^{Min}* mouse model of colorectal cancer and SW620 xenograft model in nude mice, ICG-001 has the potential to decrease formation and induce regression of tumor, respectively. The RNAi screen performed by Gonsalves and colleagues monitors the transcriptional activity of β -catenin-TCF4 complex independently of upstream factor: Axin [19]. This method has been established to specifically target nuclear/active pool of β -catenin and does not disrupt the interaction between E-cadherin- β -catenin- α -catenin that is necessary for cell polarity and epithelial tissue integration [20]. The authors decreased Axin levels in *Drosophila* cells using RNAi and measured the activity of Wnt responsive luciferase reporter dTF12 [21]

after treatment with a library of compounds [19]. This allowed the identification of inhibitors of β -catenin responsive transcription (iCRT 3, 5 and 14) that decreased activity of the β -catenin-TCF4 complex in the absence of Axin. These compounds were able to disrupt the interaction between β -catenin and TCF4 and decrease the ability of TCF4 DNA binding but did not affect E-cadherin- β -catenin- α -catenin complex formation. Targets of Wnt signaling pathway like cyclin D1 and c-Myc were downregulated upon iCRT treatment in HCT116 cell line. Furthermore, iCRT compounds caused cell cycle arrest in the G0/G1 phases and had anti-proliferative effects in HCT116 and HT29, but not in HEK, C57MG, MCF7 or Drosophila cells. This suggests that iCRT compounds are specific towards cell lines with constitutive, high levels of nuclear β -catenin. HCT116 and HT29 xenograft models in athymic nude mice showed promising reduction in tumor growth without any apparent cytotoxic effect on normal tissue in the tested conditions.

S1004A is a calcium-binding protein and its expression depends on extracellular stimuli, epigenetic regulation, and Wnt/ β -catenin pathway [22]. It has been shown that this protein is involved in cell migration, epithelial to metastasis transition and invasion, and its upregulation is associated with metastasis in colorectal cancer [23]. This protein has been proposed by Sack and Stein as a good target to screen for compounds that inhibit metastasis in cancer [24]. In a very elegant study they used HCT116 cell line stably expressing luciferase under the control of the promoter region of S1004A gene and identified calcimycin as a negative regulator of S1004A transcription [25]. Calcimycin was able to reduce cell migration, wound healing and anchorage-dependent and -independent growth as tested in several colorectal cancer cell lines (HCT116, SW480, SW620 and LS174T). As S1004A is a downstream effector of Wnt pathway, the authors examined the efficacy of calcimycin on Wnt pathway: transcriptional activity of β -catenin by TOP/FOP flash reporter assay, mRNA levels of cyclin D1, c-Myc, Dickkopf-1, and β -catenin. All of these assays showed that calcimycin negatively regulates the components of Wnt-signaling. Additionally, in HCT116 xenograft nude mice model calcimycin inhibited tumor metastasis, thus providing a promising lead compound for translational medicine.

b. EGFR signaling pathway

EGFR is a receptor tyrosine kinase that belongs to ERBB family of receptors. EGFR and its downstream molecular pathways are important regulators of the key cellular events and their alteration contributes to carcinogenesis [26]. There are two major pathways that depend on EGFR activation: Kirsten RAS (KRAS) and phosphatidylinositol 3-kinase/Phosphatase and Tensin Homolog (PI3K/PTEN). We describe these pathways separately, however it is important to recognize that there is a strong interaction between the KRAS and PI3K/PTEN pathways [27].

i. KRAS—The activation of this pathway starts with ligand binding to the EGFR. The dimerization of the EGFR kinase and subsequent autophosphorylation of this receptor lead to activation of KRAS. This in turn, triggers phosphorylation and activation of downstream effectors: BRAF, MEK, and ERK. The signal is transduced to the nucleus and results in increased activity of transcription of genes that encode proteins that pertain to cell proliferation (Figure 2A). In the normal intestinal epithelial cells this process is regulated strictly by cell type-specific positive and negative feedback loops. Consequently the alteration of this pathway causes changes in the cell proliferation and may contribute to cancer development. *EGFR* gene amplification, KRAS and BRAF mutations render unregulated activity of the downstream effectors and may lead to uncontrolled cell proliferation (Figure 2B). *EGFR* gene amplification and increased protein levels lead to changes in the magnitude of the primary signal of the activated pathway. Consequently, the activity of all of the factors downstream from EGFR is increased. KRAS and BRAF

mutations result in their constitutive activation. The mutations in *KRAS* and *BRAF* genes lead to increased activity of kinase pathway regardless of the status of the upstream signal (EGFR and *KRAS* in the case of *BRAF*) (Figure 2B). Hence, cell proliferation is enhanced, followed by induction of anchorage-independent growth and finally metastasis. *KRAS* alterations occur in 32–40% of colorectal cancers with the most common mutations in codons 12 or 13 (around 85–90% of all mutations) [28]. Around 15% of colorectal cancers have *BRAF* mutation and more than 95% of the *BRAF* mutations carry V600E mutation [28]. The prevalence of these mutations renders *KRAS* and *BRAF* as potential targets for drug discovery.

Torrance and colleagues [29] performed HTS using a pair of isogenic colorectal cancer cell lines that differ by a single mutation in the *KRAS* gene. They searched for small compounds that are cytotoxic for DLD-1 colorectal cancer cells bearing *KRAS*G13D mutation but do not affect growth of the isogenic cell line with a wildtype *KRAS* allele. Successful screening allowed for the identification of four compounds with selective toxicity towards the cell line bearing *KRAS* mutation. Demethoxyviridin (a Wortmannin homolog) and mithramycin have been previously found to impact *KRAS* pathways activity. Triphenyl tetrazolium (TPT) and a sulfinyl cytidine derivative (SC-D) were identified as novel *KRAS* pathways inhibitors. Both were six-fold more potent in mutants than wildtype cells for the *KRAS* allele. Additionally, SC-D was tested in xenografts models with DLD-1 and HCT116 cells bearing *KRAS*G13D mutation and showed promising efficacy. The tumor volume for DLD-1 was decreased 45% and HCT116 tumor growth was reduced by 65% without any apparent cytotoxicity towards normal cells. Mallon and colleagues [30] used primarily molecular high-throughput assay that allowed them to identify inhibitors of RAS/RAF/MEK pathway using ELISA plates before moving to *in vitro* and *in vivo* studies. They measured the phosphorylation status of MEK1 on threonine 202 and tyrosine 204 and identified four competitive inhibitors of ATP binding by MEK1; class 1 and class 2 of substituted 4-anilino-3-quinolinecarbonitriles: (1a, 1b: 4-phenoxyphenylaniline and 2a, 2b: 3-chloro-4-(1-methylimidazol-2-sulfonyl) aniline). In RAF/MEK1/MAPK ELISA assays these compounds showed 1 – 7 nmol/L activity. When tested against other kinases: EGFR, RAF, cyclin dependent kinase 4 (CDK4), MK2, and AKT, showed little or no effect (IC₅₀ equal 700 nmol/L, 800 – 850 nmol/L and for latter three more than 1000 nmol/L, respectively). All four compounds were examined for their ability to reduce cell proliferation in colon cancer cell lines: LoVo, HCT116, and CaCo2. Interestingly, only LoVo cells had reduced proliferation with compounds 2a and 2b; with IC₅₀ value equal 15±7 nmol/L and 7±3 nmol/L, respectively. *In vivo* studies showed that 2b was able to inhibit the growth of LoVo cells in nude animals at 50 and 100mg/kg dose after 3 weeks of treatment (54 and 78%, respectively) and after one week of withdrawal to 81 and 56%, respectively. The mode of action of this compound still needs to be determined.

Another effector of the EGFR pathway is Krüppel-like factor 5 (KLF5), a zinc-finger containing transcription factor [31, 32]. Studies from our lab as well from the others showed that inhibition of KLF5 expression reduces proliferation rates in colorectal cancer cells and tumor formation in mouse models of intestinal tumorigenesis. HTS was performed using primary screen involving luciferase assays in the DLD-1/pGL4. 18hKLF5p cell line, which stably expresses a luciferase reporter driven by the human KLF5 promoter, and as a secondary screen we used cytotoxicity assay in rat normal intestinal epithelial cell line (IEC-6) [33]. This approach allowed the identification of compounds that decreased KLF5 levels in colorectal cancer cells and that were not cytotoxic to normal intestinal epithelial cells. The two most potent compounds, CID 439501 and CID 5951923 and two newly synthesized derivatives of CID5951923 significantly reduced endogenous KLF5 protein levels and decreased the viability of several colorectal cancer cell lines. CID439501 belongs to the family of steroid-like compounds known as cardiac glycosides and has been used for

treating congestive heart failure. This compound was tested against NCI-60 panel (NSC-25485) by NIH and showed strong cytotoxic effects in all human cancer cell lines. In contrast, compound CID 5951923, when tested in sixty human cancer cell lines in the NCI-60 panel, was found to be selectively active against colon cancer cells. The precise mechanism of action of CID5951923 and its derivatives as well as its *in vivo* applications are currently being examined.

ii. PI3K/PTEN—The phosphatidylinositol 3-kinases (PI3K) signaling pathway regulates a myriad of cellular processes: proliferation, survival, motility, apoptosis, and metabolism, and is one of the most affected pathways in a number of cancers [34]. For comprehensive review that covers in-depth relation between PI3K pathway and colorectal cancer we direct readers to a recent publication by Zhang et al. [35]. The schematic outline of this pathway is illustrated in Figure 3A. The activity of this pathway is stimulated by receptor tyrosine kinases, e.g. EGFR. After the binding of ligands to EGFR, PI3K is recruited to the cell membrane where it facilitates the conversion of phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol (3,4,5)-triphosphate (PIP₃). The PIP₃ functions as a scaffold for Protein Kinase B (AKT) and 3-phosphoinositide dependent protein kinase-1 (PDK1) proteins. Upon AKT and PDK1 interaction, AKT is phosphorylated and translocates to the cytoplasm and nucleus. The phosphorylated form of AKT activates a wide range of effectors that regulate many cellular processes. In normal cells, PI3K pathway is strictly regulated by phosphatase and tensin homolog (PTEN) [36, 37]. PTEN counterbalances the function of PI3K by converting PIP₃ back to PIP₂ and thus prohibiting the phosphorylation and activation of AKT. There are three main alterations of this pathway; the first is caused by EGFR copy number duplication; the second caused by gain-of-function mutation in the gene encoding PI3KCA that renders this pathway activity independent of growth factors; the third is a loss of function that leads to inactivation of PTEN and lack of negative feedback signaling. The change of the receptor copy number renders this pathway more active due to higher level of external stimulation by ligand. The somatic mutations in *PI3KCA* gene as well as the inactivation of PTEN lead to increased activity of the factors downstream from PI3K. All three scenarios lead to the deregulation of PI3K signaling pathway, uncontrolled growth and survival of the cells, and finally tumor promotion. There are some discrepancies about the frequency of PI3KCA and PTEN alterations in the colorectal cancer. PI3KCA has been reported with frequency of mutations between 15% to 40% of all CRC with the hotspot in E542K, E545K and H1047R [38–40]. PTEN is mostly inactivated due to loss of heterozygosity (23–35%), epigenetic silencing, and rarely by mutations in the gene sequence [35, 41].

Li and colleagues [42] performed HTS with isogenic colorectal cancer cells (HCT116) that differ only in the genetic status of PTEN. This method is called synthetic lethality and allows for the identification of compounds that are cytostatic and/or cytotoxic to the cells that bear mutation/mutations of the tumor suppressor gene. The main goal of this approach was to search for compounds that preferentially decrease the proliferation of HCT116 PTEN $-/-$ cells than HCT116 PTEN $+/+$, thus are more potent in the inhibition of tumor *versus* normal cells. The identified compound CID1340132 causes accumulation of the cells in subG1 phase of the cell cycle, diminished cell proliferation, and the creation of double-strand breaks in DNA. The authors determined that apoptosis induced by CID1340132 was AKT-independent though it was accompanied by decreased levels of cyclin D1 and increased levels of p53 and p27^{Kip1} (Table 1). The synthetic lethality approach used could prove very useful for identifying small molecules that target tumor cell lines with loss-of-function mutations in genes encoding tumor suppressor.

c. p53 signaling pathway

TP53 gene encodes a tumor suppressor that functions as a regulator of angiogenesis, cell growth, apoptosis, and DNA repair. Under oncogenic stress or DNA damage, p53 negatively regulates cyclin-dependent kinase 4/6 (CDK4/6) and cyclin-dependent kinase 2/cyclin E2 (CDK2/cyclin E2) complexes and decreases proliferation by inducing cell cycle arrest. It can block Bcl-2 and NOX' complex formation thus reducing survival by stimulating apoptosis [43] (Figure 4A). Mutations in this gene are one of the most frequent genetic alterations in cancer [44]. The cells lacking the proper response to external and internal stimuli and can become cancerous with uncontrolled proliferation (Figure 4B) [45].

Wang and colleagues sought to identify small molecules that can activate transcriptional response in the p53-null cancer cells and ultimately causes cell death. They used a cell-based luciferase system with p53-null SW480 cells expressing p53-responsive element and identified several compounds that at lower doses stimulate the transcription of p53-dependent genes and at higher doses lead to cell death (Table 1). The identified compounds were tested for their ability to induce cell cycle arrest (increase cells number in subG1 phase), p73 levels (transcription factor that can stimulate a pool of genes that are typically controlled by p53), as well as p21 levels (involved in growth arrest) and DR5 (an effector in p53-dependent apoptosis). Some of the tested small molecules were able to induce DR5 and p21 at the transcriptional level in the p53 wild type and p53-null cells, and irrevocably caused cell death. Additionally, authors showed that four compounds: NSC5159, NSC143491, NSC162908, and NSC254681 tested in tumor xenografts stimulated p53-dependent transcription and suppressed tumor growth.

Recently, it has been shown that p53 is an important transcriptional regulator of focal adhesion kinase (FAK) [46, 47]. FAK regulates important cellular processes like adhesion, motility, invasion, and survival. It has been shown by Golubovskaya and colleagues that high expression of FAK is associated with loss of p53 function in colorectal cancer and that FAK is overexpressed in more than 89% of CRC [46]. Additionally, they were able to localize autophosphorylation site (Y397) within FAK protein that can be effectively targeted to block FAK signaling and inhibit CRC. They screened NCI database of 140,000 compounds and identified two compounds: Y11 and Y30, that potently target colon cancer cell proliferation in dose- and time-dependent manner. Pre-clinical studies will be performed after successful testing in *in vivo* animal mice models (The American Association for Cancer Research special conference on Colorectal Cancer: Biology to Therapy, October, 2010).

d. E-cadherin network

The E-cadherin- β -catenin- α -catenin complex is responsible for cell-cell junctions and cytoskeleton formation [48]. The proper regulation of E-cadherin turnover contributes to the establishment and maintenance of adherent junctions and ensures epithelial cells homeostasis. Depletion of E-cadherin alters cell-cell interaction and is a sign of epithelial-to-mesenchymal transition (EMT) necessary for tumor cells to become invasive and metastatic [48, 49]. Schematic outline of these two states is shown in Figure 5A and B, respectively. The negative regulation of E-cadherin is often caused by hypermethylation of its promoter region. Thus restoration of E-cadherin expression could lead to abrogation of cell invasion and metastasis. Stoops and colleagues [50] designed an immunofluorescent screen searching for compounds that restore E-cadherin in SW620 colorectal cancer cell line that originally has very low E-cadherin levels. The primary screen identified four compounds: R08, R09, R12, and R21 that were able to induce expression of E-cadherin as measured by antibody staining. Optimization of the structure of R21 led to the synthesis of series of analogs that were screened for their ability to increase E-cadherin expression. Compounds: 8g, 8j, 14j,

and 10k at 10 μ M concentration restored the levels of E-cadherin, its proper membrane localization and restituted E-cadherin- β -catenin complex as was shown by western and immunofluorescent analysis. Comparison of transcripts levels between cells treated with DMSO and 8j, revealed alteration of many pathways: EGFR, Notch, oxidative stress response, TGF β / β -catenin. Hence in-depth analysis is needed to fully address this problem. Re-establishment of adherent junctions as a result of induction of E-cadherin levels and restoration of E-cadherin- β -catenin complex at the cell membrane should decrease the motility of the cells. Thus, the invasion assay has been performed using metastatic SW620 cells. As suggested, compound significantly reduced invasion of the cells without showing any cytotoxic effects.

3. Conclusion

Colorectal cancer is a heterogeneous disease accompanied by the alteration of multiple signaling pathways. The intricacy of the pathways, negative and positive feedback loops as well as the interaction between the pathways, add additional layers of complexity to targeted drug discovery. It becomes more evident with discovery of a new anti-cancer compounds how important it is to understand function on the lead compound on the molecular level at variety of pathways, and the carefully examine the feedback loops regulation. HTS is a very useful method; however its success depends on careful design and appropriate follow up experiments.

4. Perspective

Integration of different branches: chemistry, molecular, and translational biology are necessary to the development of lead compounds, the understanding their mechanisms of action, and finally their effectiveness as anti-tumor compounds in *in vivo* models. Thus the program launched by NIH could be very useful for researchers to design efficient drug discovery strategy and help with subsequent lead compound validation and optimization through the Molecular Libraries and Imaging program. This program consists of three branches: Molecular Libraries Probe Production Center Network (MLPCN), PubChem, and Technology Development. MLPCN constitutes a network of laboratories with the capability to perform high-throughput screens and medicinal chemistry within (the realm of) Comprehensive, Specialized Screening, and Specialized Chemistry Centers. The MLPCN's main role is to help scientists develop and/or improve already existing screening technologies, perform high-and ultra-throughput screens and to assist with the analysis and chemistry of the identified compounds. PubChem, developed by National Center for Biotechnology Information (NCBI), contains databases of the chemical structures and activities of the small-molecule compounds as well as information regarding high-throughput screens performed in collaboration with the NIH. Finally, technology development department supports the development of chemical libraries and new screening tools for testing biological assays. It is clear that in-depth analyses of the capability of the identified small molecules in *in vitro* and *in vivo* settings are both important and necessary. Comprehensive studies of lead compounds will help to better understand the mechanism of cancer development and progression, to identify new pathways and factors involved in tumorigenesis, and to develop series of compounds that separately or in combination can be used against the disease.

5. Executive summary

- HTS is a method that can be successfully used in targeted drug discovery for colorectal cancer.

- Alteration of multiple pathways (e.g. Wnt/ β -catenin, EGFR, p53) can lead to transformation of the normal intestinal epithelium to cancerous cells. Hence, targeting the components of these pathways will allow finding the leads that can restore proper pathway function and inhibit CRC development, progression and metastasis.
- Careful selection of the targeted factor, diligent design of the screening assays and comprehensive biochemical and molecular analysis of the lead compounds are central for successful drug discovery.

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Figure 1.

The activity of Wnt canonical signaling pathway in the normal and tumor cells. Left panel presents the activity of the Wnt pathway in normal cells (steady and active states, respectively). Right panel shows hyperactivation of the Wnt signaling pathway in the intestinal tumor cells upon APC and β -catenin mutations, respectively. Red star – activation; red crossed lines – inactivation.

Figure 2.

The activity of the EGFR/KRAS signaling pathway in normal and tumor cells. Left panel represents the activity of the pathway in the normal intestinal epithelial cells. Right panel shows three models of hyperactivation of EGRF/KRAS pathway in the colorectal cancer cells caused by EGFR copy number increased, KRAS and BRAF mutations, respectively. Red star activation; dashed lines – irrelevant signal.

Figure 3.

The activity of PI3K signaling pathway in normal and tumor cells. Left panel represents the activity of the pathway in the normal intestinal epithelial cells. Right panel shows three models of hyperactivation of the PI3K pathway in the colorectal cancer cells caused by EGFR copy number increased, PI3K and PTEN mutations, respectively. Red star – activation; red crossed lines – inactivation; dashed lines – irrelevant signal.

Figure 4.

The p53 function in normal and tumor cells. Left panel demonstrates the role of p53 in normal cells. Right panel shows the changes upon p53 tumor suppressor inactivation in the tumor cells. Red crossed lines – inactivation.

Figure 5. The role of E-cadherin in regulation of cells junctions in normal and tumor cells. Left panel shows junctions formed in normal cells. Right chows changes that occur upon E-cadherin inactivation. Red crossed lines – inactivation.

Table 1

List of inhibitors identified with high-throughput screen against pathways in CRC.

Screening target	Compound	References
β -catenin activity	IWP, IWR; XAV939; CCT031374, CCT036477, CCT7070535	[13]; [14]; [16]
β -catenin/Axin	pyrvinium pamoate	[15]
β -catenin-TCF4 interaction	PKF115-584, PKF118-310, PKF118-744, CGP049090, NPDDG1.024, ZTM0009900; iCRT3, 5 and 14	[17]; [19]
β -catenin-CBP interaction	ICG-001	[18]
S1004A	calcimycin	[25]
KRAS	demethoxyviridin, mithramycin, triphenyl tetrazolium (TPT), sulfinyl cytidine derivative (SC-D)	[29]
MEK1	1a, 1b, 2a, 2b,	[30]
KLF5	CID439501, CID595193	[33]
PTEN	CID1340132	[42]
P53	NSC5159, NSC28992, NSC49692, NSC105900, NSC127133, NSC143491, NSC146109, NSC150412, NSC162908, NSC211340, NSC254681, NSC407807, NSC639174	[51]
FAK E-cadherin	Y11, Y30 R08, R09, R12, 8g, 8j, 14J and 10k	Golubovskaya, The AACR, October, 2010 [50]

Table 2

Alterations in the pathways involved in colorectal cancer development and progression.

Gene name	Alterations	Frequency	Reference
<i>APC</i>	frameshift, nonsense mutations	>90% in FAP	[4]
<i>CTNNB1</i>	mutations (hotspots: codons 31, 33, 37, and 45)	<10%	[4]
<i>EGFR</i>	protein expression mutation copy number amplification	25%–90% rare 0–50%	[26]
<i>KRAS</i>	mutations (hotspots: codons 12, 13, 61 and 146)	30%–40%	[26]
<i>BRAF</i>	mutations (V600E)	5%–12%	[26]
<i>PIK3CA</i>	mutations in exon 9 and 20 (hotspots: E542K, E545K, H1047R)	15%	[35]
<i>PIK3CB</i>	increased enzyme activity and protein expression	uncertain	[35]
<i>PIK3RI</i>	9-bp deletion in exon 12	<1%	[35]
<i>PTEN</i>	mutation loss of heterozygosity epigenetic silencing	<5% 23%–35% 19% of MSI-high tumors	[35]
<i>AKT1</i>	PH domain mutation, E17K	6%	[35]
<i>AKT2</i>	kinase domain mutation amplification	<1% ~1%	[35]
<i>PDK1</i>	kinase domain mutation	<1%	[35]
<i>TP53</i>	mutations (hotspots: R175, G245, R248, R249, R273, and R282)	50%–70%	[45]
<i>FAK</i>	protein overexpression	>89%	Golubovskaya, The AACR, October, 2010
<i>E-cadherin</i>	epigenetic silencing	46%	[52]