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Targeting CREB for Cancer Therapy: Friend or Foe

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Abstract

The cyclic-AMP response element-binding protein (CREB) is a nuclear transcription factor activated by phosphorylation at Ser133 by multiple serine/threonine (Ser/Thr) kinases. Upon phosphorylation, CREB binds the transcriptional co-activator, CBP (CREB-binding protein), to initiate CREB-dependent gene transcription. CREB is a critical regulator of cell differentiation, proliferation and survival in the nervous system. Recent studies have shown that CREB is involved tumor initiation, progression and metastasis, supporting its role as a proto-oncogene. Overexpression and over-activation of CREB were observed in cancer tissues from patients with prostate cancer, breast cancer, non-small-cell lung cancer and acute leukemia while down-regulation of CREB in several distinct cancer cell lines resulted in inhibition of cell proliferation and induction of apoptosis, suggesting that CREB may be a promising target for cancer therapy. Although CREB, as a transcription factor, is a challenging target for small molecules, various small molecules have been discovered to inhibit CREB phosphorylation, CREB-DNA, or CREB-CBP interaction. These results suggest that CREB is a suitable transcription factor for drug targeting and therefore targeting CREB could represent a novel strategy for cancer therapy.

Keywords

Cancer; CBP; CREB; inhibitors; KID; KIX; naphthol AS-E; Ro 31-8220

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INTRODUCTION

The cyclic AMP (cAMP) response element binding protein (CREB) was first identified in 1987 during an investigation for nuclear proteins that bind to a stretch of DNA containing the cAMP response element (CRE) 5′-TGACGTCA-3′ [1, 2]. This 43 KDa protein belongs to a large family of basic leucine zipper (bZIP)-containing transcription factors including c-Jun, c-Fos and c-Myc (Fig. 1) [3, 4]. A salient feature of this transcription factor is that its transcriptional activity is induced upon phosphorylation at Ser133 located in the KID (kinase-inducible domain) domain by many different serine/threonine (Ser/Thr) protein kinases to yield phosphorylated CREB (p-CREB) [4]. The kinases known to phosphorylate CREB include protein kinase A (PKA) [1], Akt/protein kinase B (PKB) [5], mitogen-activated protein kinases (MAPK) [6, 7], and p90 ribosomal S6 kinase ($p90^{RSK}$) (Fig. 2) [8]. Phosphorylation at Ser133 is required for its binding to the mammalian transcriptional co-activator, CREB-binding protein (CBP), through the KID domain in CREB and KIX (KID-interacting) domain in CBP [9]. This binding event enables recruitment of other transcriptional machinery to the gene promoter to initiate CREB-dependent gene transcription (Fig. 2) [4]. In addition to Ser133, many other sites in CREB are also known to be phosphorylated by a variety of kinases [10]. Although the biological relevance of these additional phosphorylation events is less well understood, they could potentially regulate CREB's transcription activity in a more delicate fashion [10][#].

CREB serves a variety of biological functions including cellular proliferation, differentiation and adaptive responses in the neuronal system [4, 11]. Recently, accumulating evidence has revealed that CREB participates in the regulation of immortalization and transformation of cancer cells. Therefore it is hypothesized that CREB is directly involved in oncogenesis of a variety of cancers [12–14].

CREB IN HUMAN MALIGNANCIES

The first indication of CREB's involvement in cancer stemmed from the identification of a chromosomal t(12;22)(q13;q12) translocation in clear cell sarcomas of soft tissue (CCSST) to give a fusion protein EWS-ATF1 [15]. In the resulting chimeric protein, the N-terminal region of EWS (Ewing's Sarcoma) [16], an RNA-binding protein, is fused with the C-terminal region (bZIP) of ATF-1 (activating transcription factor 1), a CREB related transcription factor which binds CRE and heterodimerizes with CREB [17]. This fusion protein loses a consensus PKA phosphorylation site mediating regulation by PKA and other kinases for transcription activation. Instead, the activation domain of the N-terminal region of EWS renders this fusion as a constitutively active transcription activator to upregulate the expression of some CREB target genes [18, 19]. This gene translocation is present in nearly all the CCSSTs [15, 18].

Immunohistochemical analysis of primary and bone metastatic prostate cancer tissues from patients demonstrated that normal, benign prostate glands showed no detectable p-CREB [20]. On the other hand, positive p-CREB staining was detected in all the examined poorly-

[#]The remaining discussion of phosphorylation of CREB in this article refers to Ser133.

differentiated prostate cancers and bone metastatic tissue specimens [20]. This positive correlation between the level of p-CREB and the extent of tumor differentiation and metastasis suggests that CREB is critically involved in tumor progression and metastasis. In prostate cancer LNCaP cells, activation of CREB has been implicated in the promotion of neuroendocrine differentiation (NED) induced by ionizing radiation [21] and elevated intracellular cAMP level [22, 23]. The neuroendocrine transdifferentiation process appears to be associated with the development of cross-resistance to radiotherapy, chemotherapy and androgen-independence in prostate cancers [21, 24]. In addition to prostate cancer, increased mRNA levels of CREB were also consistently detected in breast cancer tissues compared to normal mammary tissues [25]. Notably, the level of CREB expression was correlated with disease progression and survival [25]. In non-small-cell lung cancer (NSCLC) never-smoking patients, the expression levels of CREB and p-CREB were distinctively elevated in tumor tissues compared to the adjacent normal tissues [26]. In these examined patient samples, there exists an inverse correlation between CREB overexpression and disease-free survival [26]. In an animal model of lung adenocarcinoma induced by tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the expression level of p-CREB progressively increased along the development of lung adenocarcinoma [27]. Similar observations were made in human lung adenocarcinoma NCI-H322 cells, where NKK potently stimulated phosphorylation of CREB and DNA synthesis [28].

Besides its role in the development of solid tumors, mounting evidence has shown that CREB may also play a role in the development of bone marrow neoplasms. The oncogenic virus human T-cell leukemia virus type 1 (HTLV-1) is strongly associated with aggressive cases of adult T-cell leukemia (ATL) [29, 30]. The oncogenic transformation caused by the HTLV-1 Tax oncoprotein requires intact CREB signaling [31, 32]. Furthermore, the role of CREB itself as a proto-oncogene in leukemogenesis is supported by studies of leukemia patient samples and leukemia cell cultures [33–36]. Higher CREB and p-CREB expression levels were detected in the bone marrow from patients with ALL (acute lymphoid leukemia) and AML (acute myeloid leukemia) compared to that from patients without neoplastic hematologic disorders [35, 36]. The degree of increased p-CREB level does not correlate with the cAMP levels in the leukemia patient samples [36], suggesting CREB kinases other than PKA are contributing to the enhanced phosphorylation of CREB. In addition, CREB expression clearly correlates with disease stage in the leukemia patients where high expression was observed at diagnosis and relapse, but low expression was seen upon remission from the same patients [35, 36]. Kaplan-Meier analysis showed that CREB overexpression is also associated with a decrease in time to relapse and a decrease in event-free survival [33]. On the flip side, the expression profiles of inhibitors of CREB, including ICER (inducible cyclic AMP early repressor) [36] and miR-34b (a microRNA targeting CREB) [37] are down-regulated in the bone marrow of AML patients compared to healthy individuals. These empirical expression data from human patient samples have been supported by animal models. The transgenic mice that overexpress CREB in the cells of the myeloid lineage developed increased monocytosis and myeloproliferative syndrome with splenomegaly [33]. The bone marrow cells from these transgenic mice also displayed transformed blast-cell phenotypes including increased proliferation, immortalization and growth factor-independence [33]. Overall, the data from both clinical samples and animal

models indicate that CREB not only serves as a diagnostic marker, due to its role in induction and maintenance of malignancy, but also an oncogene of its own accord when inappropriately activated.

TARGETING CREB FOR CANCER THERAPY: PROOF-OF-CONCEPT

The observation that CREB is overexpressed and/or over-activated in a variety of different clinical cancer tissues argues that CREB may represent a promising target for cancer therapy. Although the exact mechanisms by which CREB contributes to cancer development are not clear, CREB, as a transcription factor, directly regulates a number of critical genes involved in cellular proliferation, anti-apoptosis and metastasis. These targets include cyclin A1 [33], cyclin D1 [38], bcl-2 (b-cell leukemia 2) [33], VEGF (vascular endothelial growth factor) [20], type IV collagenase MMP-2 (matrix metalloproteinase 2) and cell adhesion molecule MUC18/MCAM (melanoma cell adhesion molecule) [39]. In hematopoietic cells, overexpression of CREB may also block cellular differentiation by up-regulating a member of the *hox* gene family, Meis1 (myeloid ecotropic viral integration site 1) [40, 41]. As mentioned earlier, CREB is also implicated in promoting prostate cancer cell NED.

The biological function of CREB, as a transcription factor, entails protein-DNA and protein-protein interactions. Both of these binding interfaces are challenging targets for rational small molecule design even though the high-resolution structures of CREB-CRE and KIX-KID complexes have been determined [9, 42]. Therefore, initial studies to test the hypothesis of targeting CREB as a strategy for cancer therapy are focused on biological approaches to inhibit CREB's function.

Dominant-negative CREB mutants were employed to inhibit CREB's transcription activity. A melanoma cell line (MeWo) was transfected with a dominant-negative CREB, KCREB, which is a full-length CREB with R287L mutation [43] and does not bind CRE but does heterodimerize with wild-type CREB [39, 44]. Expression of KCREB in the highly metastatic MeWo cell line resulted in 1) decreased potential for metastasis *in vitro* and *in vivo* [39]; 2) decreased tumor growth in the mouse xenograft model [39]; 3) decreased capability to grow in soft agar, indicating decreased transforming capacity [44]; and 4) reduced resistance to radiation [44]. These results are possibly due to the decreased expression of CREB target genes involved in metastasis, including type IV collagenase (*MMP-2*) and cell adhesion molecule *MUC18/MCAM* [39]. Similar tumor growth inhibition with dominant-negative CREB was also observed in hepatocellular carcinoma BNL1ME cells [45] and non-small-cell lung cancer A549 cells [46]. Inducible expression of a dominant-negative CREB mutant in the mouse basal epidermis significantly reduced the incidence of skin papillomas induced by sequential treatment with 7,12-dimethyl-benz[a]anthracene (DMBA) and phorbol-12-myristate-13-acetate, suggesting potential cancer preventive value of CREB inhibitors [47].

A second biological approach to inhibit CREB's function was the utilization of CRE "decoy" oligonucleotides. Once inside cells, these "decoy" oligonucleotides can bind CREB and sequester it away from the genomic CRE sequences and thus effectively inhibit CREB-mediated gene transcription. Introduction of a 24-mer CRE "decoy" into a variety of solid

tumor cell lines was shown to suppress the growth of cancer cells both in culture and in the xenograft models [48, 49]. On the other hand, delivery of the same CRE "decoy" oligonucleotide into normal cells did not result in toxicity [49]. These results further suggest that pharmacological inhibition of the transcription activity of CREB could be a promising strategy to develop next-generation nontoxic, anticancer drugs.

Finally, a third approach that was investigated for inhibition of CREB was RNA interference (RNAi). Our laboratory has recently shown that knockdown of CREB in human myeloid leukemia cells (K562 and TF-1) resulted in decreased cellular proliferation and viability *in vitro* [33, 50]. In a murine myeloid leukemia model, NOD-SCID (non-obese diabetic, severe combined immuodeficicent) mice injected with Ba/F3 cells transduced with Bcr-Abl and shRNA (short hairpin RNA) against CREB exhibited decreased disease burden and increased median survival [50]. More importantly, this effect was also observed in imatinib-resistant Bcr-Abl^{T315I}-expressing cells [50]. Knockdown of CREB by siRNA has also been shown to enhance oxidant- and asbestos-induced apoptosis [51, 52].

TARGETING CREB FOR CANCER THERAPY: SMALL MOLECULES

The aforementioned studies of inhibiting the transcriptional activity of CREB by various genetic methods provide proof-of-concept evidence that CREB is a promising target for anticancer drug development. However, direct translation of these methods for potential cancer therapies would require gene therapy techniques, whose clinical application is still rather limited and controversial [53]. An alternative approach to inhibit CREB-mediated gene transcription is to utilize small organic molecules, which, in general, have better pharmacokinetic properties as cancer therapeutics. Three potential intervention points for small molecules as chemical inhibitors of CREB-mediated gene transcription are depicted in Fig. (2).

The first approach of pharmacological inhibition of CREB-mediated gene transcription in cells involves the use of kinase inhibitors to inhibit phosphorylation and therefore activation of CREB. As presented in Fig. (2), multiple Ser/Thr protein kinases could phosphorylate CREB. Therefore, effective inhibition of CREB phosphorylation in cancer cells would require simultaneous inhibition of multiple CREB kinases either by combining different specific kinase inhibitors or employing non-specific kinase inhibitors. This polypharmacology approach may elicit many off-target effects [54]. However, promising results were obtained from Aggarwal *et al* who reported that Ro 31-8220 (Chart 1), an analog of staurosporine [55] with medium kinase selectivity,[$] inhibited both CREB upstream and downstream signaling components in non-small-cell lung cancer cell lines (NSCLC) [56]. Specifically, phosphorylation of CREB kinase p90^{RSK} and CREB in H1734 cells was inhibited by Ro 31-8220. The expression of CREB target genes involved in anti-apoptosis, Bcl-2 and Bcl-xL, was down-regulated in H1734 cells treated with Ro 31-8220. Furthermore, NSCLC cells treated with Ro 31-8220 exhibited caspase-dependent apoptosis while similar treatment in normal human tracheo-bronchial epithelial (NHTBE) cells did not result in apoptosis [56], suggesting that cancer cells are more sensitive to CREB inhibition.

[$]see technical article of Ro 31-8220 at www.sigma-aldrich.com

While these results are intriguing, it remains to be determined whether the observed cellular phenotype is a result of inhibition of CREB's activity because of the moderate specificity profile of Ro 31-8220 against different kinases in cells.

The second approach to inhibit CREB's activity is to inhibit CREB-CRE interaction. Unlike enzymes which have a well-defined and relatively small-sized binding pocket for small molecule binding, protein-DNA interactions are traditionally thought to be difficult to be targeted by small molecules. However, a fluorescent polarization–based high throughput screening assay was designed to identify potential inhibitors of CREB-CRE interaction from the NCI-diversity set of ~1900 compounds [57]. NSC 12155 and NSC 45576 (Chart 1) were identified as submicromolar and low micromolar inhibitors of CREB-CRE interaction. However, these compounds are not specific in inhibiting CREB-CRE interaction and their cellular activity remains to be determined [57]. Another potential approach to target CREB-CRE interaction is to employ the programmable *N*-methylpyrrole-*N*-methylimidazole polyamides [60], which can be designed to target specific DNA sequences and have been engineered to inhibit HIF-1α (hypoxia inducible factor 1α) and androgen receptor [61, 62].

The third approach to the inhibition of CREB-mediated gene transcription is to target CREB-CBP interaction, which necessarily precedes CREB-dependent gene transcriptional activation [63]. The binding interface between CREB and CBP is KID-KIX interaction and it is structurally well-characterized by NMR spectroscopy [9], but targeting protein-protein interactions by small molecules is still challenging. However, the recent successful examples of small molecule inhibitors of various protein-protein interactions suggest this is feasible provided there are binding "hotspots" on the protein surfaces [64]. Two distinct binding sites on KIX are utilized by natural transcription factors [65, 66], suggesting its potential to be targeted by small molecules. Therefore, a medium-throughput NMR screening assay was designed to identify potential small molecule binders of KIX that could disrupt the CREB-CBP interaction [58]. From a pre-selected drug-like library of 762 compounds, naphthol AS-E phosphate (Chart 1) was identified as a candidate compound to inhibit the KID-KIX interaction with IC_{50} of ~90 μM. However, this same compound displayed significantly more potent activity in inhibiting CREB-mediated gene transcription in living cells [58]. Further studies in the authors' laboratory showed that naphthol AS-E phosphate is not stable in serum or cell-permeable and the actual active species in the cellular experiments is the dephosphorylated product, naphthol AS-E (Chart 1), which is a much more potent inhibitor of the KID-KIX interaction [59]. The identification of this cell-permeable KID-KIX interaction inhibitor will enable us to further investigate the hypothesis that KID-KIX interaction inhibitors are potential anticancer agents.[&] Furthermore, the modular nature of naphthol AS-E is amenable to further structure-activity relationship studies to uncover the structural manipulations that would enhance their biological activities. Some other KIX-binding small molecules were also reported in the literature [67–69], however, their capability to inhibit CREB-mediated gene transcription in cells is unknown.

[&]Li, B.X., Bryant, D., Yamanaka, K., Dorsa, D., Xiao, X. In *238th ACS National Meeting*: Washington, DC, United States, **2009**.

TARGETING CREB: GOOD OR BAD

mRNA expression data showed that CREB was expressed in all the adult tissues examined [70] and CREB is a focal point of many different signaling pathways (see Fig. 2) [4, 11]. Thus, specific and efficacious inhibition of CREB might invoke non-specific adverse effects in the normal cells. In fact, *CREB*-null mice are lethal at birth due to post-natal lung defects [71]. However, the centrality of CREB might be advantageous for anticancer drug design because cancer cells often have multiple lesions in different signaling pathways [72, 73] and targeting CREB could potentially block a number of these aberrantly activated pathways (see Fig. 2). Although genetic knockout data are critical in inferring the biological function of a gene of interest, one has to realize that the key differences between genetic knockouts and chemical inhibitors are that chemical inhibition of CREB is reversible, transient and does not change the endogenous CREB protein level. The residual CREB activity after chemical inhibition may be sufficient to maintain the normal cell homeostasis, but below the threshold to maintain the cancer cell phenotype. As a matter of fact, the hypomorphic *CREB* mice are viable without developmental defects [74].

Studies with CRE-decoy oligonucleotides demonstrated that normal cells are not sensitive to CREB inhibition [49]. The exact mechanisms underlying this selectivity remain to be determined, but one can conceptualize the selective toxicity to cancer cells over normal cells by CREB inhibition as in Fig. (3). Cancer cells acquire an incredible amount of apoptotic stress during malignant transformation by aberrant activation of oncogenes [72]. To prevent cell death, cancer cells are able to block apoptosis by up-regulating anti-apoptotic signals [72]. Since cancer is believed to be a product of evolutionary selection [72], there must be a reason for over-activation of CREB consistently seen in different tumor specimens. This may be that activated CREB transcribes its target genes required for anti-apoptosis to maintain the cancer phenotype. Endogenous genes are often regulated by more than one transcription factor. The contribution to a given transcript from CREB might be bigger in cancer cells with over-activated CREB than that in normal cells. Therefore, inhibitors of CREB-mediated gene transcription will have a more profound effect in cancer cells than normal cells, which will be sufficient to induce apoptosis in cancer cells. But the effect in normal cells is too small to induce an adverse effect due to redundancy (Fig. 3). Alternatively, promoter usage for a given gene in cancer cells may be different from that in normal cells in that CREB is predominantly driving transcription of a particular target gene that is required to maintain the cancer phenotype. A good example of this latter point is illustrated in the transcription of *aromatase* in different tissues, where CREB is major contributor in breast cancer cells but not in other cells [75]. Therefore, chemical inhibitors of CREB-mediated gene transcription could display anticancer activity with no or acceptable minimal toxicity.

CONCLUSIONS AND OUTLOOK

In conclusion, there is a great deal of evidence to implicate CREB as an important regulator of tumor initiation, progression and metastasis. Proof-of-principle studies using various strategies have demonstrated therapeutic potential of inhibitors of CREB-mediated gene transcription. In the coming years, we anticipate that more chemical inhibitors will be

developed to allow investigators to further test the hypothesis of CREB inhibitors for cancer therapy in preclinical and clinical settings. However, the following challenges need to be addressed in order to fully appreciate the clinical utility of any chemical inhibitor of CREB-mediated gene transcription: 1) What are the mechanisms responsible for the anti-cancer activity/selectivity of CREB inhibitors? 2) How specific are the small molecule inhibitors in inhibiting CREB-mediated gene transcription? 3) What patients are appropriate for the trials of CREB inhibitors? 4) How and whether these inhibitors should be used in combination with other existing cancer therapies? 5) What are the endpoint markers to evaluate the efficacy and on-target effects? The discovery of various inhibitors presented in Chart 1 represents just the opening chapter of an entire book of a novel strategy for cancer therapy requiring more extensive investigations.

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ABBREVIATIONS

Abl	Abelson murine leukemia viral oncogene homolog
ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
ATF-1	Activating transcription factor 1
ATL	Adult T-cell leukemia
Bcr	Breakpoint cluster region
bcl-2	b-cell leukemia 2
bZIP	Basic leucine zipper
CBP	CREB binding protein
CCSST	Clear cell sarcomas of soft tissue
CRE	Cyclic-AMP response element
CREB	CRE-binding protein
DMBA	7,12-dimethylbenz[a]anthracene
EWS	Ewing's Sarcoma
HIF-1α	Hypoxia inducible factor 1α
HTLV-1	Human T-cell leukemia virus type 1
ICER	Inducible cyclic AMP early repressor
KID	Kinase-inducible domain

KIX	KID-interacting
MAPK	Mitogen-activated protein kinase
MCAM/MUC18	Melanoma cell adhesion molecule
Meis1	Myeloid ecotropic viral integration site 1
MMP-2	Matrix metalloproteinase 2
NCI	National Cancer Institute
NED	Neuroendocrine differentiation
NMR	Nuclear magnetic resonance
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NSCLC	Non-small-cell lung cancer
p-CREB	Phosphorylated CREB
PKA	Protein kinase A
PKB/Akt	Protein kinase B
$p90^{RSK}$	p90 ribosomal S6 kinase
VEGF	Vascular endothelial growth factor

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Fig. 1.

Domain structure of rat CREB. The basic leucine zipper (bZIP) is located at the C-terminus for DNA binding and homodimerization or heterodimerization with other bZIP family members. The kinase-inducible domain (KID) is the inducible activation domain activated by phosphorylation at Ser133. The glutamine rich regions [Q1 and Q2 or constitutive activation domain (CAD)] are the basal transcriptional activation domains. Human CREB lacks the α domain, which forms a presumed amphipathic α-helix to regulate the transcription activity of CREB.

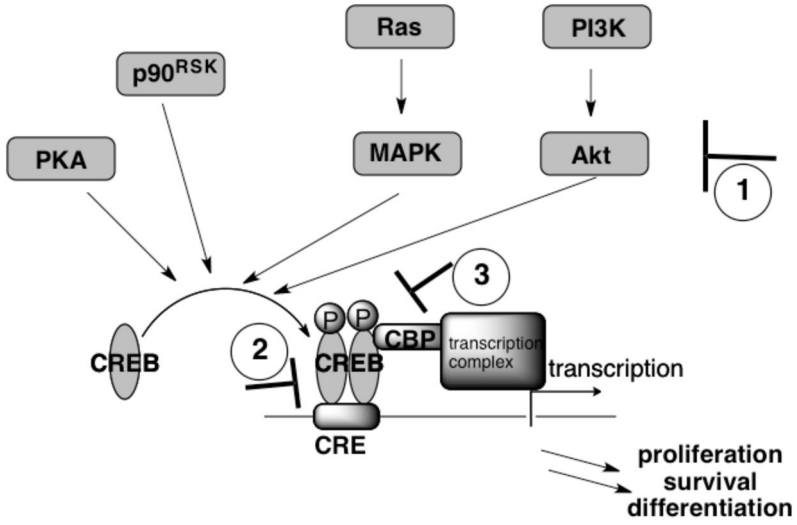

Fig. 2.

Multiple signaling pathways activate CREB by phosphorylation. Illustrated are potential points of intervention by chemical inhibitors: 1) inhibition of kinases; 2) inhibition of CREB-CRE interaction; and 3) inhibition of CREB-CBP interaction.

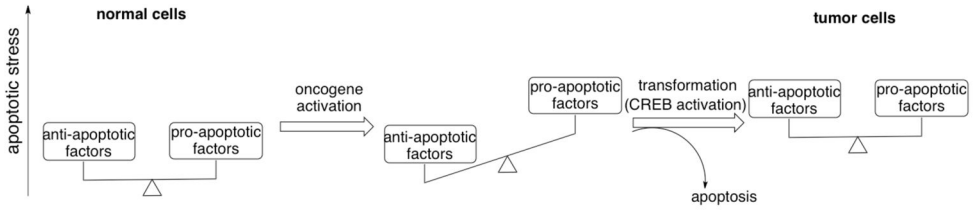

Fig. 3.

Cancer cells bear higher apoptotic stress than normal cells. Normal cells (left) maintain a homeostasis with low levels of pro-apoptotic factors and anti-apoptotic factors. Upon aberrant activation of oncogenes, the cells start to accumulate apoptotic stress (middle) and most of the cells will die. However, a small percentage of the cells will survive through activation or inactivation of other cell signaling components (e.g. activation of CREB) resulting in up-regulation of anti-apoptotic factors. These transformed cancer cells (right) will maintain this new cellular homeostasis with overall higher levels of both pro-apoptotic factors and anti-apoptotic factors than their normal counterparts.

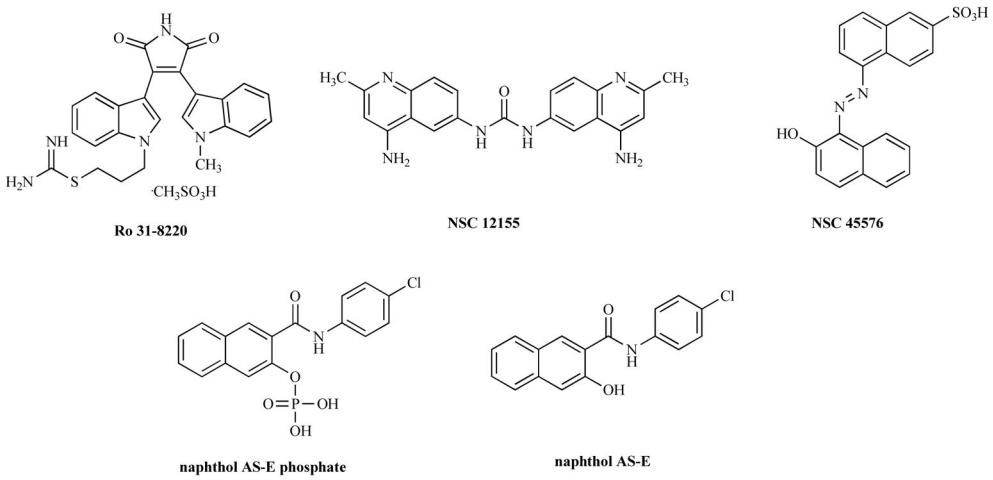

Chart 1.
Chemical inhibitors of CREB-mediated gene transcription [56–59].