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Drug repurposing for targeting cyclic nucleotide transporters in acute leukemias - a missed opportunity

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Abstract

While current treatment regimens for acute leukemia can dramatically improve patient survival, there remains room for improvement. Due to its roles in cell differentiation, cell survival, and apoptotic signaling, modulation of the cyclic AMP (cAMP) pathway has provided a meaningful target in hematological malignancies. Several studies have demonstrated that gene expression profiles associated with increased pro-survival cAMP activity or downregulation of various pro-apoptotic factors associated with the cAMP pathway are apparent in acute leukemia patients. Previous work to increase leukemia cell intracellular cAMP focused on the use of cAMP analogs, stimulating cAMP production via transmembrane-associated adenylyl cyclases, or decreasing cAMP degradation by inhibiting phosphodiesterase activity. However, targeting cyclic nucleotide efflux by ATP-binding cassette (ABC) transporters represents an unexplored approach for modulation of intracellular cyclic nucleotide levels. Preliminary studies have shown that inhibition of cAMP efflux can stimulate leukemia cell differentiation, cell growth arrest, and apoptosis, indicating that targeting cAMP efflux may show promise for future therapeutic development. Furthermore, inhibition of cyclic nucleotide transporter activity may also contribute multiple anticancer benefits by reducing extracellular pro-survival signaling in malignant cells. Hence, several opportunities for drug repurposing may exist for targeting cyclic nucleotide transporters.

Keywords

Acute myelogenous leukemia (AML); Acute lymphoblastic leukemia (ALL); 3',5'-cyclic adenosine monophosphate (cAMP); ATP-binding cassette (ABC) transporters; Efflux, Inhibitors of cAMP efflux (ICE)

Acute leukemias — Novel therapeutics are urgently required

1.1 Biology and etiology of acute leukemias

While not the most prevalent malignancy in the United States, acute leukemia ranks among the top ten cancers in terms of both morbidity and mortality [1]. These hematological malignancies involve aberrant proliferation of blood cells with immature phenotypes. Acute leukemias are typically identified by the presence of > 20% blast cells in the peripheral blood or bone marrow [2]. Due to inherent characteristics related to a reduced differentiation state, and the ability to rapidly propagate, acute leukemias may have worse prognoses than chronic leukemias.

Acute myelogenous leukemia (AML) is most prominent in elderly adults over 65 years of age. Annually, 21,450 cases are diagnosed in the United States, and 10,920 cases succumb to the disease [1]. The five year overall survival for this disease is a disheartening 28.3% for adults [1] and about 60% for children [3]. T-cell lineage and B-cell precursor acute lymphoblastic leukemia (T-ALL and B-ALL) primarily affects children, adolescents and young adults, with about 6,000 new cases diagnosed and 1,500 deaths each year [4, 5]. This disease is considered > 80% curable, however there is much room for improvement, specifically for high-risk subtypes such as Ph-like ALL, leukemias harboring rearrangements of *KMT2A* gene at 11q23 or BCR-ABL1 translocations, *etc.* [6-8] Therefore, there is a strong need for the development of novel approaches to treat these malignancies and improve patient outcome.

The primary classification of acute leukemia is based on the presence of chromosomal abnormalities and translocations along with immunophenotyping. For example, 25-30% of B-ALL have hyperdiploidy [9], while a surprising 40-50% of AML are cytogenetically normal [2]. An analysis of primary AML samples determined an average of 13 mutated genes per sample [10]. It has also been reported that > 80% of B-ALL cases contain deletions within genes related to B cell development [11]. *De novo* B-ALL genomes also contain 10-20 gene coding mutations [12].

Most commonly, the etiology of acute leukemias is attributed to the combination of mutations that govern pro-survival signaling and reduce tumor suppressor genes [13]. Many factors are potentially implicated in leukemogenesis, and most include aberrancies that increase cell proliferation and induce differential arrest. Due to potential mutations, rapid growth, and deviant signaling, leukemia cells have the capacity to overcome normal mechanisms that would typically result in cell death.

1.2 Treatment paradigms in acute leukemias

Treatment for acute leukemia begins with induction therapy, wherein chemotherapeutic agents are used to induce remission of the disease. In this approach, the goals are to reduce the population of blast cells and/or induce them to differentiate. Most commonly, this stage of treatment involves drugs that are used in many other cancers and diseases, and hence their mechanisms of action are non-specific for leukemia. These drugs are aimed at killing all cells that are proliferating by inducing DNA damage or interfering with cell replication mechanisms. For some AML and ALL patients, allogeneic stem cell transplants are a treatment option as well.

The standard of care for AML is the use of cytosine arabinoside (cytarabine; AraC) with an anthracycline (e.g., daunorubicin, idarubicin, doxorubicin) for 7-10 days [14]. Over 80% of patients can achieve a complete response with this treatment, but often relapse will occur over time [15]. This is followed by consolidation therapy, which is typically high dose AraC [16]. Refractory or relapsed AML is also treated with high dose AraC, or the FLAG (fludarabine, AraC, granulocyte colony-stimulating factor (G-CSF)) regimen, but these result in modest complete remission rates of 32% and 47.5%, respectively [17].

B-ALL induction therapy involves 4-6 weeks of a glucocorticoid (e.g., dexamethasone or prednisone), asparaginase, vincristine, and an anthracycline. This is followed by several months of consolidation and maintenance therapies, which consist of some induction agents, as well as 6-mercaptopurine or 6-thioguanine, or methotrexate [9]. Like AML, the time elapsed between remission and relapse was associated with better overall survival. In one longitudinal study of pediatric B-ALL patients, only 8.8% of participants relapsed within 5 years [18], although other reports have indicated relapse rates of about 15-20% [23]. Nonetheless, a small percentage of B-ALL patients are vulnerable to the development of extramedullary disease in the central nervous system [19].

For both AML and B-ALL, remission is often determined by the minimal residual disease in the bone marrow. Most commonly, this is classified as < 1 leukemic blast in 10,000-100,000 cells [9, 19]. The length of remission varies by patient, although some mutations and translocations have been associated with longer event free survival. For example, AML cases with *NPM1* mutations are considered to have favorable risk, while those containing an internal tandem duplication of fms-related tyrosine kinase 3 (FLT3-ITD) have adverse risk [20]. In B-ALL, the *ETV6-RUNX1* fusion protein is associated with better treatment response and overall good outcome [21]. Rearrangements of the mixed-lineage leukemia (*KMT2A*) gene are considered prognostically less favorable [7, 8, 21]. Despite the fact that these and other cytogenetic factors are characteristically associated with prognosis, many patient responses to treatment are individual, and even those with similar cytogenetic factors could have opposite responses to the same therapy.

The treatment paradigms for acute leukemias are not without side effects. Primarily due to their lack of selectivity for leukemic cells, leukemia chemotherapeutic agents (LCA) can cause systemic damage to the patient. Most notably, anthracyclines and other chemotherapeutics exhibit cardiotoxicity. This is probably because, like cancer cells, the cardiac muscle is highly metabolically active. Survivors of pediatric ALL are at increased risk for the long-term development of heart disease [22]. LCA are associated with damage to the liver and kidneys, as these are the primary organs for drug metabolism. Patients with ALL are also at risk for osteonecrosis [23, 24]. Furthermore, leukemia treatments have been related to long-term cognitive impairment, including memory, attention, and executive function deficits [25, 26]. Hence, the development of drugs that are more selective for malignant cells could seriously reduce the incidence of adverse side effects suffered by patients.

cAMP-dependent pathway - A meaningful target in hematological malignancies

2.1 The cellular roles of cAMP and its regulation

In 1958, the first second messenger in cells, 3',5'-cyclic adenosine monophosphate (cAMP) [27], was identified. This molecule is critically important for all cells and is central to many cellular processes that regulate growth, survival, differentiation, and the transcription of a myriad of genes. cAMP signal transduction can result in activation of pro-survival or death pathways, depending upon cell type and conditions [28]. The primary downstream effectors of cAMP are protein kinase A (PKA) and exchange proteins activated by cAMP (EPAC). While these two effectors can activate multiple transcription

factors, the one most commonly associated with cAMP pathway signaling is the cAMP response element binding protein (CREB). CREB activity is associated with transcription of many pro-survival genes, including cyclins A, D1, and D2 [29].

Canonical cAMP synthesis involves activation of any of nine transmembrane-associated adenylyl cyclases (tmAC), which are stimulated by $G\alpha_s$-coupled receptors. These activated receptors are capable of stimulating cAMP synthesis from within endosomes [30]. Activation of $G\alpha_i$-coupled receptors inhibits tmAC activity, and hence reduces intracellular cAMP (icAMP). tmACs are envisioned to be responsible for the regulation of cAMP level in the vicinity of the cell membrane. Additionally, the cAMP signaling is shown to be spatially segregated throughout the cytosolic compartment, where the second messenger can be generated by a different enzyme, a non-membrane associated soluble adenylyl cyclase (sAC; *ADCY10*). This enzyme is responsible for cAMP production in cytosolic microdomains, such as the cytoplasm, or within the nucleus or mitochondria [31-33]. Unlike tmACs, sAC is activated by bicarbonate (HCO_3^-) or oxidative stress [32, 34-37]. Elevated icAMP triggers intrinsic apoptosis by PKA signaling. PKA can activate the transcription factor cAMP response element binding (CREB) protein that triggers up-regulation of the expression of the pro-apoptotic protein Bim [38]. Additionally, sAC stimulation of PKA can cause translocation of pro-apoptotic Bax into mitochondria [39, 40]. All these signaling events can play a role in the activation of the mitochondria-related intrinsic apoptotic pathway.

icAMP concentrations can be modulated by two principal mechanisms. The most commonly studied cAMP regulators are phosphodiesterases (PDEs). These enzymes hydrolyze cyclic nucleotides. PDEs 1-4, 7, 8, 10, and 11 are capable of degrading cAMP to 5'-AMP [41]. The second mechanism implies active removal of cAMP from cytosol. The fact that cAMP can be released outside the cell (ecAMP) was first described in 1963 [42]. Decades later, it was determined that icAMP can be actively removed from cytosol via the ATP-binding cassette (ABC) transporters. The members of the multidrug resistance (MRP) family of transporters, ABCC4 (MRP4), ABCC5 (MRP5), and ABCC11 (MRP8) are reported to efflux cAMP [41]. In this review, we focus on the modulation of this process by small molecules.

Because cAMP activity occurs in discrete locations (microdomains), many cAMP-dependent regulatory proteins can be complexed to facilitate signal transduction. A-kinase anchoring proteins (AKAPs) are scaffolding proteins that allow adenylyl cyclases, PKA, and PDEs to be localized in close proximity to one another [43]. Hence, the compartmentalization of icAMP into microdomains near the plasma membrane, mitochondria, nucleus, or regulatory proteins, is a critical determinant of its concentration and activity [44-46] (Figure 1).

2.2 cAMP targets in acute leukemias

Considering that icAMP regulation is altered in hematopoietic malignancies, and that increased icAMP reduces white blood cell survival, the cAMP pathway has long been of interest as a target for leukemia therapeutics [28, 41, 47-49]. Many cAMP-associated proteins are dysregulated in acute leukemias. The primary tmAC expressed in lymphoid cells is the adenylyl cyclase type 7 (*ADCY7*) [50], and the expression of this protein inversely correlates with the overall survival of AML patients [51]. The overall PDE activity was reported to be 10-20-fold higher in certain leukemia and lymphoma cells as compared to normal blood cells [52]. It should also be noted that glucocorticoids, which are used to treat ALL, have been shown to downregulate PDE activity [53]. Of note, genomic studies on primary AML and adult ALL samples have reported overexpression of CREB and/or its active, phosphorylated form [54-59]. Another study identified downregulation of ICER (inducible cAMP early repressor), the physiological antagonist of CREB that is associated with tumor suppression activity [60]. In B-ALL samples, there is an increased incidence of mutations in transcripts for *CREBBP*, the binding protein for CREB. These *CREBBP* mutations were associated with dominant-negative or deleted activity [61, 62].

To summarize, acute leukemia cells demonstrate both increased pro-survival cAMP activity through increased CREB signaling, as well as downregulation of the pro-apoptotic factor ICER.

2.3 History of cAMP targeting in cancer with a focus on hematological malignancies

Most previous attempts to modulate cAMP for cancer therapeutics have focused on the use of cAMP analogs, or by targeting canonical proteins of the pathway through: 1) stimulation of cAMP production by $G\alpha_s$-coupled receptors, or 2) inhibition of cAMP degradation by PDEs. These efforts have had modest success, and are briefly summarized here.

Few cAMP modulating agents targeting cancer have been tested in clinical trials. Despite demonstrating anticancer effects *in vitro* and *in vivo*, phase I clinical trials with the analog 8-Cl-cAMP in patients with refractory solid tumors resulted in hypocalcemia and toxicity to normal tissues [63-65]. A phase II clinical trial evaluated the PDE-inhibitor theophylline for chronic lymphoblastic leukemia (CLL) patients. Of the 25 CLL patients treated with theophylline, only one achieved a complete response, and 18 patients maintained a stable disease state [66].

Nonetheless, cAMP pathway modulation has been well investigated as a target in hematopoietic malignancies *in vitro* and *in vivo*. Elevation of icAMP using cAMP analogs has demonstrated the ability to induce cell cycle arrest (G_1 or G_2 phase), differentiation, and/or intrinsic apoptosis in leukemia, lymphoma, myeloma, and normal B cells *in vitro*, most often through PKA-mediated mechanisms [38, 67-73]. Similar results have been reported from studies where cAMP production was stimulated via activation of tmAC [74-77]. PDE inhibitors have also been employed to increase cAMP signaling in hematopoietic cells. Meyers, *et al*. provided evidence that PDE4 inhibition induced cell death in B-CLL cell lines, but not in normal B and T cells [50]. This selectivity is plausible, since leukemia and lymphoma cells exhibit elevated PDE expression and activity [49, 52, 78]. Mitton, *et al.* used small molecule CREB inhibitors to reduce the expression of pro-survival factors in AML cells *in vitro* and *in vivo*. They showed that this approach induced cell cycle arrest and apoptosis [59]. Nevertheless, it is important to note that elevated icAMP can also rescue blood cancer cells from apoptosis, although those studies involved activation of cAMP signaling after substantial DNA damage had occurred in the treated cells [79-84]. Consequently, there is still substantial evidence to support cAMP pathway targeting in hematological malignancies.

Targeting cAMP transporters/efflux – an unexplored approach to modulation of the cyclic nucleotide pathway

3.1 Cyclic nucleotide efflux as a previously overlooked target

While the intracellular concentrations of cyclic nucleotides are canonically regulated by degradation by PDE in microdomains [46], efflux mechanisms by ABC transporters are less explored. This class of proteins consists of 7 families (ABC-A through ABC-G), in which two molecules of ATP are used to translocate substrates into the extracellular space. As previously mentioned, cAMP and cGMP are transported by members of the ABCC (MRP) family, ABCC4 (MRP4), ABCC5 (MRP5), and ABCC11 (MRP8). These transporters are distributed variably throughout the body, with ABCC4 having the highest expression in the bladder, kidney, and prostate [85]. The affinity of these transporters varies for cAMP or cGMP. ABCC5 has a greater affinity for cGMP, whereas ABCC4 is preferential for cAMP [86]. ABCC4, ABCC5, and ABCC11 regulate xenobiotic metabolism, and they can efflux antiviral drugs (PMEA, ganciclovir) as well as chemotherapeutic agents (fluorouracil, mercaptopurine, thioguanine, campothecins, methotrexate). Importantly, these proteins also serve a role in the transport of natural,

endogenous substrates. These include eicosanoids (e.g., prostaglandins E1 and E2, leukotriene C4), estradiol-17β-glucuronide, folic acid, bile acids (taurocholate, glycocholate), and some steroids (e.g., DHEAS) [87]. Furthermore, the cyclic nucleotide transporters also play a major role in relieving oxidative stress, as they remove glutathione conjugates from the cell [88].

Past work in which cAMP efflux was inhibited by small molecules was performed in epithelial cell lines, and primarily focused on cAMP concentration changes and the activity of the pathway-associated proteins [89, 90]. The efflux capacity of leukemia cells has been reported as an important factor for predicting patient outcomes [91]. However, the analysis of cAMP-specific efflux activity has not been extensive. The expression of ABCC4 is inversely correlated with hematopoietic cell differentiation [92]. Previous studies relied on the use of indiscriminate ABC transporter inhibitors (e.g., probenecid) or silencing RNA to block expression of cAMP effluxing proteins [93-95]. These approaches, however, lacked the efficiency and breadth necessary for resolving the utility of targeting cAMP efflux for cancer therapeutics.

Recently, our group initiated studies to further explore the role of cAMP efflux targeting in acute leukemias [96, 97]. We hypothesized that malignant cells produce and remove the excess icAMP from mitochondria-containing microdomains to evade intrinsic apoptosis and to promote cell survival [96]. Consequently, inhibition of cAMP efflux should increase icAMP and selectively trigger leukemia cell death. We proposed that small molecule cAMP efflux inhibition, if achieved using existing drugs through a repurposing strategy, has the potential to expedite the translation of cAMP efflux-targeting therapeutics [96]. Because elevated cAMP efflux activity could be a specific adaptation of malignant cells that is not apparent in normal cells, drugs developed using this approach have the potential to selectively target leukemia or other tumor cells.

To monitor cAMP efflux from living cells and primary patient samples, we designed and patented a novel assay that rely on cellular retention of a fluorescent cAMP analog (F-cAMP) [98]. This assay was later adapted for use in a high throughput flow cytometry platform [97]. After screening two chemical libraries consisting of off-patent FDA-approved drugs and biologically active compounds, we identified and validated six compounds termed “inhibitors of cAMP efflux” (ICE) [96, 97].

Using the F-cAMP-based approach, our group confirmed that cAMP efflux was undetectable in normal peripheral blood mononuclear cells (PBMCs) but was active in leukemia cell lines [96]. Several interpretations for this phenomenon can be considered. As mentioned before, the cAMP efflux could be a malignancy-specific adaptation directly related to the evasion of apoptosis in cancer. It can be also related to a metabolic specificity of leukemic cells (Warburg effect). cAMP is a well known regulator of cellular respiration, metabolism and an accumulation of cAMP in cancer cells can trigger metabolic reprogramming, mitochondrial biogenesis and anti-Warburg effect [99]. The fact that normal hematopoietic progenitors exhibit an increased expression of ABC transporters capable of cAMP efflux [92], a phenotype that is shared by blasts associated with acute leukemias [100], can indirectly support this idea.

Previously, Copsel *et al.*, demonstrated that blocking cAMP efflux in an AML-relevant model system not only increased icAMP accumulation, but also triggered a series of downstream signaling events relevant to the pathway activation [93, 94]. We also validated the ability of newly identified ICE compounds to increase cAMP pathway activity by demonstrating its ability to modulate cellular downstream signaling. This included CREB (pS133) / ATF-1 (pS63) phosphorylation and CD49d/CD29 (VLA-4) integrin deactivation [96]. Active VLA-4 integrin is responsible for homing and retention of hematopoietic blasts and other cells in the bone marrow. Deactivation of this adhesion molecule by cyclic nucleotide-dependent signaling mechanism is crucial for mobilization of white blood cells into the peripheral blood [101, 102], and activation of VLA-4 integrin is known to be impaired in a subset of CLL

patients [103]. Our experiments further demonstrated that the ability of ICE to reduce cell viability was partially dependent on the activity of sAC, a source for cytosolic, mitochondrial, and nuclear cAMP production [36, 104]. Furthermore, ICE reduced the viability of leukemia cell lines and *ex vivo* primary patient samples at much lower concentrations than required for PBMCs, indicating that cAMP efflux inhibition could be a feasible target for malignant cells. Because several identified ICE are FDA approved drugs, these studies provide a potential path for drug repurposing against leukemias [96].

We also compared ABCC4 expression in several leukemic cell lines and determined that there was no apparent relationship between antibody binding and the F-cAMP efflux ability [96]. The simplest explanation here is the possibility that transporter activity is regulated by protein modification, such as phosphorylation, for example. The best-studied member of ABCC subfamily, ABCC7, was shown to be phosphorylated by PKA and this directly affects its activity [105]. Another possibility is that ICE may act by some alternative mechanism(s) unrelated to transporter inhibition *per se*. Finally, the work by Guo, *et al.* previously showed that, of the known cAMP efflux transporters, only the expression of ABCC11 was prognostically relevant in leukemia [106]. Other studies have indicated the possibility for the ABCC1 and ABCG2 transporters to efflux cyclic nucleotides [95, 107]. It is therefore plausible that acute leukemia cells may reduce intracellular cAMP using ABCC5, ABCC11, or other MRP transporters.

It should be emphasized that the cross-competition between transporter substrates can theoretically perturb transport activity resulting in the inhibition of one with another. Hence, it is possible that some molecules identified as inhibitors of a given transporter may instead reduce the efflux of other substrates, without actually blocking the activity of that transporter. Extensive mechanistic studies are needed to validate the mechanism of action of efflux inhibitors on transporters. It should be noted that all substrates of a transporter have the potential to inhibit the transport other substrates of the same transporter. Therefore, molecules that affect similar pathways could initially be identified as efflux inhibitors, but may in fact potentially have mechanism(s) of action that are unrelated to antagonism of transport proteins.

3.2 ICE and distinct intracellular cAMP compartments. Design of cAMP signalosomes

cAMP signaling is highly compartmentalized and responses to cAMP are spatially and temporary restricted. Thus, it is necessary to understand the effects of ICE on major cellular functions, such as cell proliferation, differentiation and cell death. This should be done in the context of cAMP signalosomes, large multi-protein complexes that consist of key players of the cAMP signaling pathway. A representative cAMP signalosome includes several components. Scaffold AKAP proteins provide binding sites for all other signalosome components, and organize as well as target the complex to a specific cellular location. For example, AKAPs that target signalosomes to the plasma membrane are AKAP7, the small-membrane AKAP (smAKAP, C2orf88) [108]. AKAP1 (D-AKAP1, S-AKAP84, AKAP121) target signalosomes to the outer mitochondrial membrane or the endoplasmic reticulum [109, 110] and AKAP6 (mAKAP), one of the proteins that targets signalosomes to the nuclear membrane [111]. Other components of the signalosome include cAMP-dependent protein kinase PKA, and the PDE responsible for the degradation of cAMP and other proteins. This specific signalosome organization that places proteins in close proximity to one another is envisioned to provide tight regulation of cAMP/PKA signaling in specific cellular compartment. In resting cells, PDEs maintain a low concentration of localized cAMP that is insufficient to activate PKA. Upon activation of AC, increased cAMP production triggers PKA activity. The subsequent phosphorylation of PDE by PKA stimulates cAMP hydrolysis and returns cAMP levels to the resting state. Disruption of the interaction between PKA and AKAP blocks PKA induced PDE activation [112]. This suggests that spatial

organization of the signalosome, where proteins are anchored in proximity to one another is essential for proper functioning of the signaling mechanism.

To decipher how ICE may affect distinct cellular compartments, here we focus on known functions of the PKA/cAMP pathways in mitochondrial, nuclear, and plasma membrane as well as cytosolic signalosomes.

3.2.1 Mitochondrial cAMP-PKA signaling

Several excellent reviews that describe cAMP signaling in mitochondrial compartment were recently published [113, 114]. Here we will briefly focus on main functions of cAMP-dependent processes that are important for understanding the potential effects of ICE on mitochondria. The outer mitochondrial membrane is permeable for small molecules [115], and therefore, cAMP produced by tmACs and sAC in the cytosol can reach the intermembrane mitochondrial space. However, the inner mitochondrial membrane is impermeable to cAMP. As mentioned above sAC could be a source of cAMP in the mitochondrial matrix [31, 32].

In mitochondria, oxidative phosphorylation (OXPHOS) [32], protein import into mitochondria [116-118], mitophagy, mitochondrial fission and fusion [113], metabolic reprogramming and the anti-Warburg effect [99], as well as the intrinsic apoptotic pathway [38], can all be modulated by the cAMP-PKA-related signaling pathway. However, it seems that the majority of these processes can be affected by cAMP diffusing to the outer mitochondrial membrane. Only OXPHOS and metabolic reprogramming/anti-Warburg effect require cAMP level changes in the mitochondrial matrix. As ABCC transporters that represent a potential target for ICE are expressed in the plasma membrane, it is expected that blocking cAMP efflux can have direct effects on protein import, mitophagy and mitochondria-induced apoptosis. Our recent data suggest that in leukemic cells ICE can trigger four apoptotic endpoints including mitochondrial membrane depolarization and activation of effector caspases 3 and 7 [96]. Thus, it is plausible that cAMP produced by tmAC can reach the outer mitochondrial membrane and trigger AML cells apoptosis.

We also found an unexpected connection to potential dysregulation of protein import in adult AML patient samples. Our analysis of several related gene expression clusters associated with poor outcome, worse overall survival and highest rates of resistant disease revealed enrichment for genes related to transport across membranes (transporters, carriers and channels). One of the identified genes was *TOMM7*, the mitochondrial import receptor subunit translocase of outer membrane (TOM) homolog [119]. This protein regulates assembly and stability of the main translocase complex [120]. The up-regulation of TOM7 in AML cells may be related to aberrant protein import in cancer. It is envisioned that defects in protein translocation into mitochondria can be devastating for organelle function, since the TOM complex provides an entry point for ~99% of all precursor proteins in mitochondria [121, 122]. While cAMP-PKA signaling can diminish import of proteins through TOM and triggers the switch from OXPHOS to glycolysis [114], it is not known whether it can be directly related to the defects of the TOM system in leukemia. Does cytosolic sAC, activated by HCO_3^-, low pH or oxidative stress [32, 34-37], produce cAMP that triggers mitochondrial protein import dysfunction in cancer? How these changes are related to the Warburg effect? All these questions should be examined in the future.

3.2.1 Plasma membrane and cytosolic compartments

The plasma membrane and cytosolic compartments are the most studied among all cAMP-PKA-related sites. Before the concept of cAMP signaling compartmentalization was introduced, it was assumed that

the main site of cAMP-PKA signaling was the plasma membrane and cytosol. Numerous reviews of the field exist. Thus, here we discuss a few novel and previously unrecognized players.

As mentioned above, tmAC activity is controlled by two types of GPCRs, $G\alpha_s$-coupled (stimulating cAMP production) and $G\alpha_i$-coupled (inhibiting) receptors, the majority of which are localized to the plasma membrane. cAMP that is produced by tmACs diffuses in cytosol. CXCR4 ($G\alpha_i$-coupled) and CXCR7 (a non-classical GPCR that can form heterodimers with CXCR4, acting as scavengers for the CXCR4 ligand CXCL12) are both relevant to leukemia and other cancers [123]. The idea that elevated signaling thorough $G\alpha_s$-coupled H2-histamine receptor can be beneficial for treatment of AML [124], supports the notion that cAMP elevation in the cytosolic compartment using ICE is also beneficial [96]. Unfortunately, in some myeloid cells despite the elevation of the cAMP level that resulted from H2-histamine receptor stimulation, cell differentiation failed to occur. To explain this discrepancy, the authors suggested that cAMP efflux through MRP transporters could be modulating the cAMP level [124]. This notion evokes the suggestion that combining ICE with H2-histamine agonists could be an option to overcome this problem. Another report that shows an improvement in immune checkpoint therapy by activating the $G\alpha_s$-coupled G protein-coupled estrogen receptor (GPER) indirectly supports this idea [125].

Hematological malignancies are characterized by the presence of a substantial number of cells exhibiting immature phenotypes. In acute leukemias up to one fifth or more of total cells can show blast morphology in the peripheral blood [2]. This reinforces the idea that leukemic cells possess a defect in their adhesion molecules, resulting in a premature release from the bone marrow niche into the peripheral blood. VLA-4 integrin is critical for homing and retention of hematopoietic blasts in the bone marrow niche, and it can be inactivated by a cyclic nucleotide-dependent signaling mechanism [101, 102]. The finding that the cytoplasmic domain of the VLA-4 subunit (alpha4, CD49d) serves as a type I PKA-specific AKAP puts VLA-4 integrins right at the heart of the plasma membrane cAMP signalosome. Sequestering type I PKA away from VLA-4 dramatically reduced VLA-4 subunit phosphorylation and inhibited VLA-4 dependent migration toward CXCL12 [126].

The role of the VLA-4-PKA protein complex in leukemia pathogenesis is not known. Similarly to VLA-4, the cAMP efflux transporters (a likely target for ICE compounds) are localized in the plasma membrane. Thus, they can also play the roles in the regulation of plasma membrane cAMP signalosome. The fact that the effect of ICE on VLA-4 functional activity can be detected within seconds after compounds addition suggests a very close association between these signaling complexes (see Figure 4 in [96]). It is also possible that blocking cAMP efflux at the plasma membrane results in cAMP accumulation and subsequent diffusion toward the mitochondrial outer membrane, where it can affect PKA activity, modulate protein import and trigger apoptosis.

3.2.3 Nuclear compartment

CREB and activating transcription factor-1 (ATF-1) are the classical cAMP effectors that activate target genes through cAMP response elements (CRE). This pathway is directly implicated in cAMP-induced apoptosis in leukemia [38]. Because the mitochondrial proteome largely originates from nuclear DNA, CREB together with downstream transcription factors, peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1α) and nuclear respiratory factors (NRF), activates transcription of numerous mitochondrial genes and promotes biogenesis of mitochondria [127, 128].

There are multiple indications of aberrant CREB function in leukemia. CREB is shown to be overexpressed in primary samples obtained from AML and ALL patients [29, 129]. CREB expression was associated with increased risk of relapse and lower rate of the event-free survival [130]. CREB inactivation inhibited AML cell proliferation and triggered apoptosis, while had no significant effect on normal hematopoietic stem cells [54], prompting the development of small molecule modulators of

CREB [131]. A recent report shows that a small molecule inhibitor of CREB, XX-650-23 blocked interaction between CREB and its co-activator CBP, and was capable of triggering apoptosis, and cell-cycle arrest in AML cells. Moreover, it increased mice survival in human AML xenograft models, suggesting that targeting CBP–CREB interaction may represent a novel approach for AML therapy [59].

In our experiments, ICE were capable of increasing CREB (pS133) phosphorylation [96], which is known to induce the formation of a complex between CREB and CBP [132]. These findings seem to tilt the signaling balance towards CREB pathway activation, which in AML cells is interpreted as pro-survival signal. ICE were capable of inducing the loss of mitochondrial membrane potential within 2 h after treatment [96]. We proposed that ICE-induced the elevation of cAMP targeted primarily the mitochondrial cAMP-PKA compartment resulting in initiation of the intrinsic apoptotic pathway [38]. CREB (pS133) phosphorylation was simply an indicator of activation in another cAMP-dependent pathway.

To summarize, ABCC4, ABCC5 and ABCC11 are localized in the plasma membrane. Therefore, targeting cAMP efflux by ICE is expected to affect intracellular compartments that are spatially adjacent to this compartment. Also, it is anticipated that the cAMP compartment that provides easy access to cytosolically produced cAMP would be primarily affected by diminished cAMP efflux. Thus, ICE are expected to target processes that can be modulated by plasma membrane and cytosol, outer mitochondrial membrane and nuclear signalosomes.

3.3 Cyclic nucleotide transporter inhibition potentially provides multiple anticancer benefits

Because the inhibition of cyclic nucleotide efflux obviously results in increased intracellular concentrations of transporter substrates, it can be expected that signaling processes will be affected accordingly. As previously mentioned, elevated icAMP signaling can result in the activation of the intrinsic apoptotic pathway. Conversely, extracellular cAMP (ecAMP) can result in autocrine and paracrine signaling. ecAMP has been shown to reduce immune cell activation [133], and thus cAMP efflux may provide another survival advantage for malignant cells by allowing them to avoid clearance by a normal immune response. Furthermore, Bhang, *et al.* have demonstrated that extracellular PKA, a downstream effector of ecAMP, was significantly elevated in the sera of dogs with cancer in comparison to healthy dogs or those with other diseases [134]. The authors do not speculate as to the purpose of extracellular PKA, although others have indicated that it may aid in the activation of prostaglandin H synthase [135], a factor related to cancer angiogenesis [136].

Another interesting aspect of ecAMP signaling relates to the existence of an ecAMP/adenosine pathway. Here, extracellular cAMP, which presumably has exited the cell through an efflux transporter (or leaked passively), is converted into adenosine by the activity of the ectonucleoside triphosphate diphosphohydrolase-1 (ENTPDase1) and ecto-5'-nucleotidase (NT5E), CD39 and CD73, respectively [137]. Some researchers have suggested that this pathway exists because free adenosine has a very short half-life, whereas ecAMP is stable in plasma, allowing it to participate in regulated signaling processes [138].

The adenosine produced by the hydrolysis of ecAMP can play many roles. The adenosine generated by CD73 activity is associated with increased tumor growth and suppression of normal immune responses [139-142]. Leukocytes express the adenosine receptor A2A (A2AR), a $G\alpha_s$-coupled receptor [143]. The activation of A2AR can induce several downstream signaling cascades. Importantly, A2AR can stimulate a positive feedback mechanism where cAMP production, cAMP efflux through ABCC4, and ecAMP hydrolysis can promote more A2A stimulation [144, 145]. As such, inhibition of A2A receptors is capable of inhibiting the growth and metastasis of tumor cells [146-150].

It is conceivable that leukemia cells exploit this ecAMP/adenosine pathway activity, though the linkage is not straightforward. Expression of ectonucleotidases may be triggered by hypoxia [151], which is a key characteristic of the bone marrow microenvironment. An analysis of whole blood from patients indicated that CD39 was expressed on > 90% of both normal and malignant B-cells, and 8% of normal T cells. Elevated CD39 activity was associated with earlier stages of CLL, whereas decreased CD39 was associated with worse disease [152]. Inhibition of CD39 activity has been proposed as a potential anticancer target [153-155]. Another study has shown that 32% of CLL patients expressed CD73, and that this enzyme was associated with reduced response to therapy [156]. Similarly, CD73 expression on ALL cells was significantly related to expression of CD10, a protein associated with progenitor phenotypes [157]. Moreover, gene expression analysis of a drug resistant T-ALL cell line identified increased CD73 expression and resistance to receptor-mediated apoptosis, although the authors hypothesized that a direct interaction between CD73 and the cell death receptor, rather than CD73 enzymatic activity, may have been the mechanism of action [158]. Furthermore, CD73 appeared to have no prognostic value in predicting pediatric ALL response to therapy [159].

Another substrate of cyclic nucleotide transporters merits mention here, prostaglandin E2 (PGE2). PGE2 itself is associated with pro-survival signaling, through its binding to the $G\alpha_s$-coupled receptors EP2 (*PTGER2*) and EP4 (*PTGER4*). Here, the downstream signaling is analogous to A2AR. Acute leukemia blast cells express EP2 [160], hypothetically in response to PGE2 secreted by bone marrow mesenchymal cells [161]. In principle, PGE2 could inhibit bone formation via increased icAMP signaling through EPAC, allowing for remodeling of the bone marrow microenvironment [162]. We propose that PGE2-mediated signaling may occur in an autocrine manner, by the PGE2 effluxed by cyclic nucleotide transporters.

Conclusions

This review has highlighted the need for new treatment regimens for acute leukemias. We have described the vital roles that cAMP signaling plays in the regulation of cell proliferation, survival, and apoptosis. We provided justification for targeting the cAMP pathway, since pathway-associated proteins are dysregulated in these malignancies. We also introduced the concept that the reduction of cyclic nucleotide efflux activity could potentially provide multiple anticancer benefits.

Because chemotherapy resistance can occur by cAMP efflux transporters [106, 163], there is a possibility of using cAMP in combination with current therapeutics to produce synergistic effects against leukemias. Synergism produced by these drug combinations would be beneficial for decreasing leukemia cell burden, and potentially to reduce the likelihood of resistance and/or relapse. Because several FDA approved drugs showed ICE activity, an opportunity for an accelerated introduction of novel therapeutics thorough a drug repurposing mechanism represents an exciting possibility [96].

In sum, this review could provide support for a new class of antileukemia drugs, cAMP efflux inhibiting compounds, to be tested in clinical trials. This new approach would be significant because it could substantiate inhibition of efflux as a paradigm shift in cAMP pathway targeting for cancer therapeutics.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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Figure legend

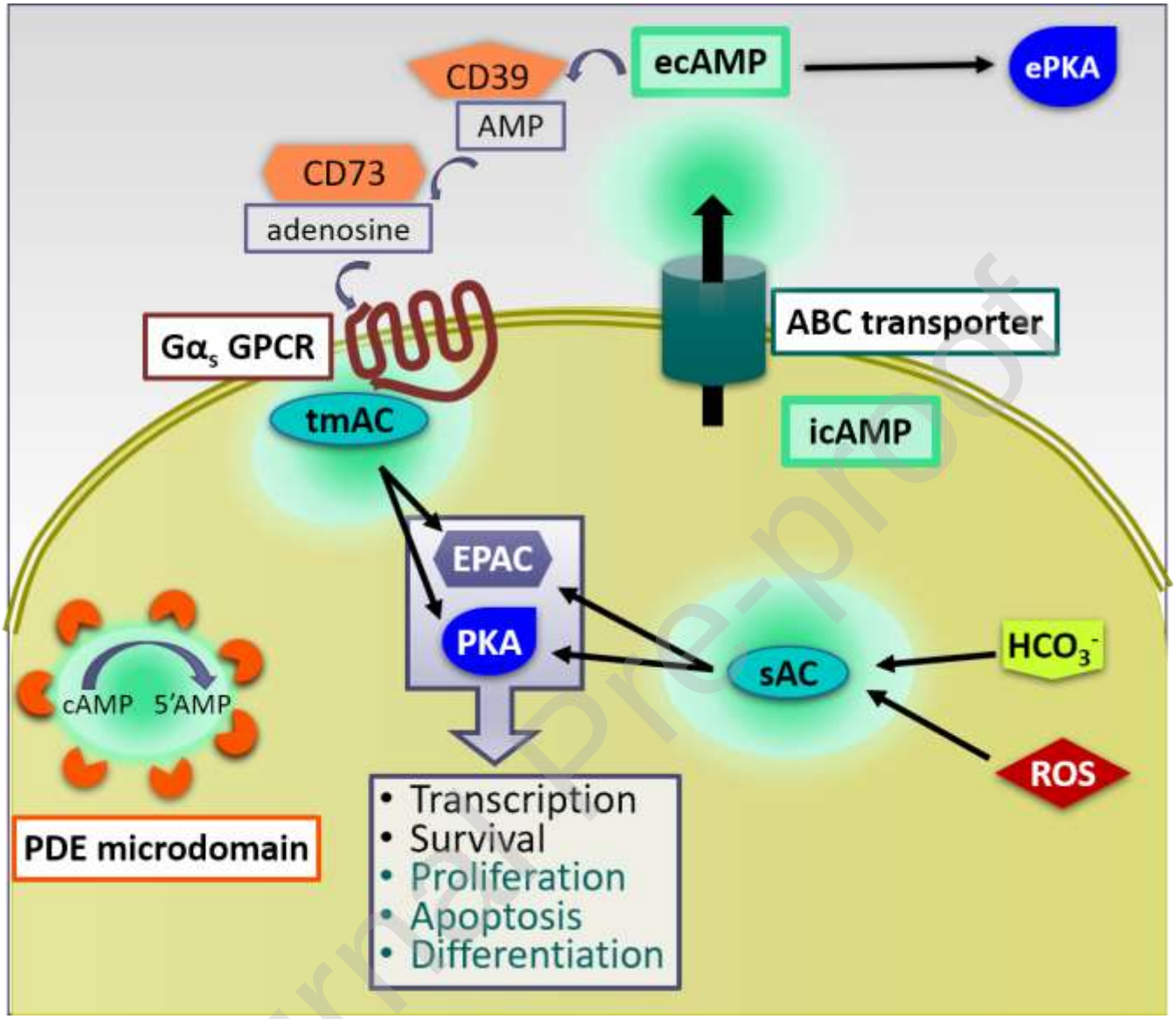

Figure 1. Basic schematic of cAMP compartmentalization and effectors.
cAMP (green) is produced by transmembrane (tmAC) or soluble (sAC) adenylyl cyclases. Intracellular cAMP (icAMP) is downregulated by PDEs or efflux by ABC transporters to the extracellular space (ecAMP). icAMP can activate the downstream effectors EPAC and PKA. ecAMP can activate ePKA, or it can be hydrolyzed by the enzymes CD39 and CD73 into adenosine, which can then stimulate its receptors. *EPAC*, exchange proteins activated by cAMP. *$G\alpha_s$-GPCR,* stimulatory G-protein coupled receptor. *PKA*, protein kinase A. *ROS*, reactive oxygen species.