Supplementary material to

Cyclic AMP induces IPC leukemia cell apoptosis via CRE- and CDK-dependent Bim transcription

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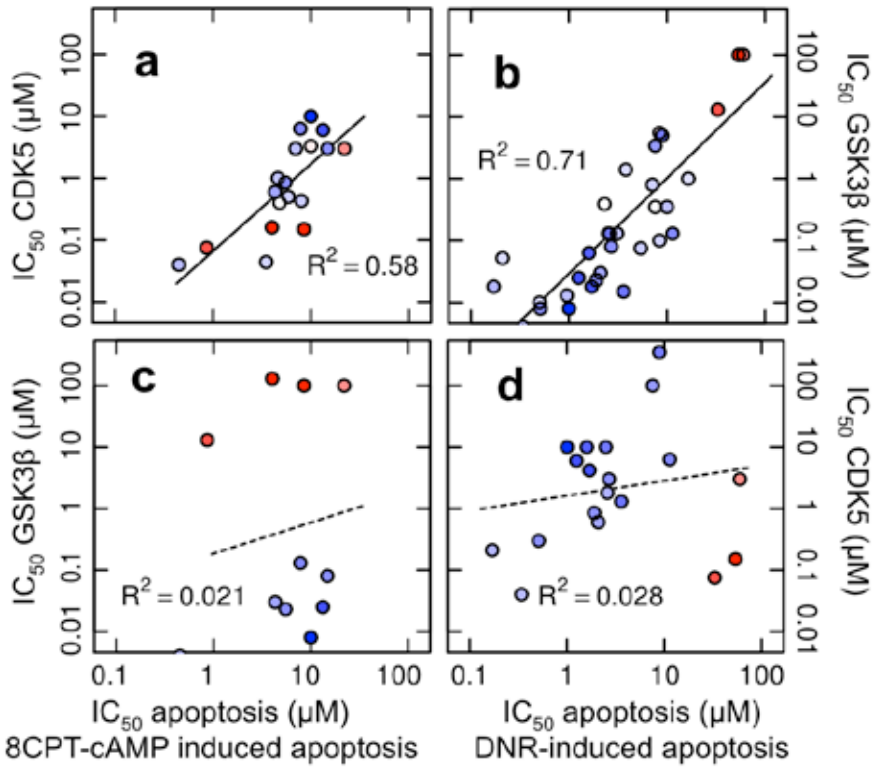
Table of Content

Tables S4, S5 are provided in a separate file (MS Excel).

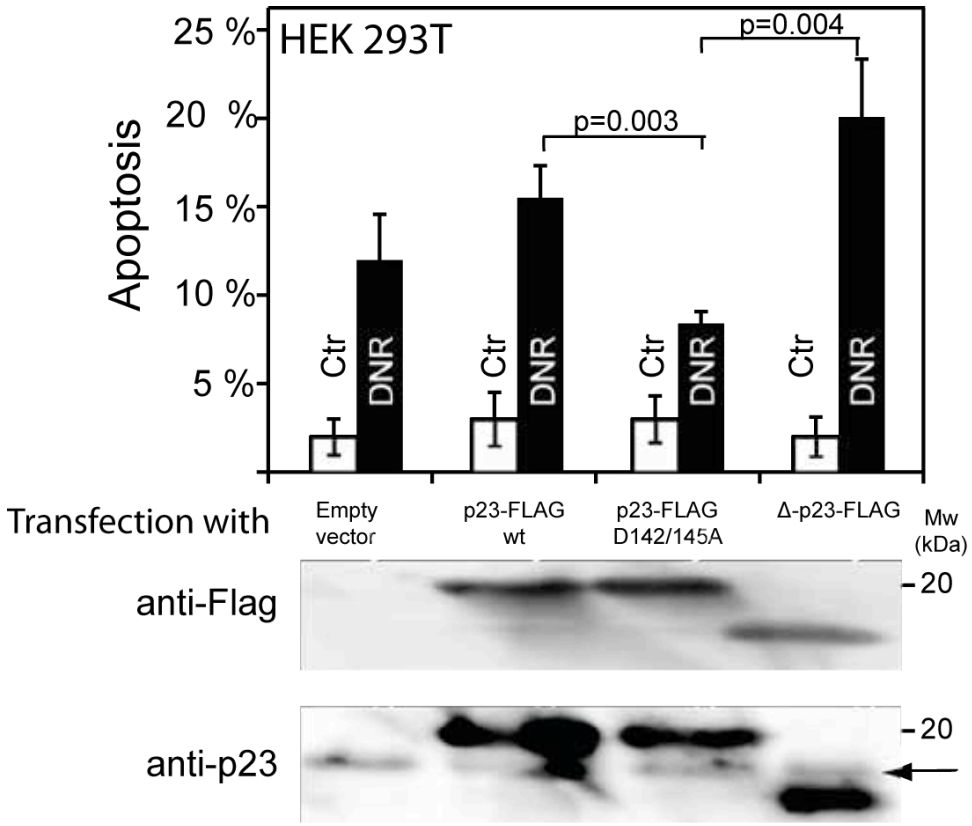
I) Supplementary Figures S1-3:

Figure S1. ICDK inhibitors protect against cAMP-induced apoptosis while GSK3 inhibitors protect against DNR-induced apoptosis.



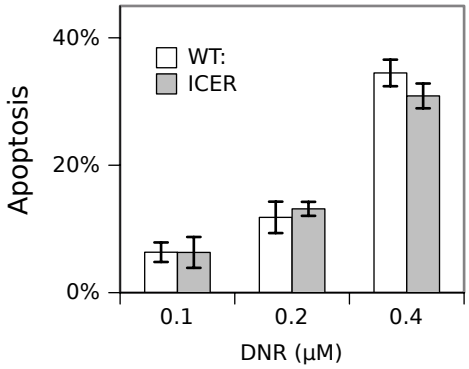
IPC_{WT} cells were exposed to 8CPT-cAMP (0.2 mM) or DNR (0.4 μM) for 6h in the absence or presence of various concentrations of active site directed CDK and GSK3 inhibitors. Panel **a** shows the correlation between the potency of each compound to inhibit cAMP-induced apoptosis and CDK5, based on the IC_{50} data of Table S6. Panel **b** shows a correlation between the potency of each compound to inhibit DNR-induced apoptosis and GSK3, based on the IC_{50} data of Table S6. Panel **c** shows lack of correlation. The figure shows plots of the inhibitor symbols in intense red signify strong CDK5/GSK3β specificity and in intense blue strong GSK3β/CDK5 specificity. A positive correlation exists between CDK5 inhibition and protection against 8CPT-cAMP (**a**) and between GSK3ß inhibition and protection against DNR (**b**). No correlation exists between between cAMP apoptosis protection and GSK3ß inhibition (**c**), or DNR apoptosis inhibition and CDK5 inhibition (**d**). The anti-apoptotic effect of GSK3 inhibitors stands in contrast to the role of this kinase as an oncogene in MLL[1]

Figure S2. Truncated p23 HSP90 cochaperone facilitates DNR-induced HEK293T cell apoptosis



HEK-293T cells were co-transfected with GFP and pRK5MCS-FLAG (empty vector), pRK5MCS-$p23_{WT}$-FLAG, pRK5MCS-$p23_{D145/145A}$-FLAG, or pRK5MCS-$p23_{1-142}$-FLAG (Δ-p23-FLAG), using the calcium phosphate method. 36 hours thereafter the cells were incubated for 7 hours with DNR (9 μM) and GFP-positive cells (routinely > 80%) scored for apoptosis (upper panel) by interference contrast microscopy[2] or lyzed and analyzed for p23 expression by immune-blotting and probing with anti-FLAG (middle panel) or anti-p23 (lower panel) antibody. Note that the deletion of the 18 C-terminal residues of p23 in Δ-p23-FLAG leads to a visible shift of gel migration. The horizontal arrow points to the endogenous p23. The error bars represent SEM (n=5). The *p*-values were calculated by the Student t-test.

Figure S3. IPC cells overexpressing the CREB antagonist ICER have intact DNR-induced IPC cell apoptosis that is stimulated further by cAMP



The figure shows similar apoptosis induction in IPC_{WT} (white columns) and IPC_{ICER} cells treated for 6h with the concentrations of DNR indicated (grey columns). The death was scored based on chromatin condensation in nuclei of cells after staining with Hoechst33342. The death in the untreated cells is subtracted. The data represent mean ± SEM (n = 3-9). The death inducing synergy between cAMP analog and DNR was diminished, but not abolished by RCV (not shown), suggesting that it was due also to RCV-resistant cAMP-induced processes.

II) Supplementary Tables S1-3:

Table S1. N^6-Bnz-8-Pip-cAMP has superior ability to discriminate cAMP binding site AI of PKA-I from those (AII, BII) of PKA-II

Compound	Aff. Site AI (K_dcA/$K_d$anal.)	Aff. Site AII (K_dcA/$K_d$anal.)	Aff. Site BII (K_dcA/$K_d$anal.)	Discr. AI/AII	Discr. AI/BII
Cyclic AMP (cAMP)	1.0	1.0	1.0	1.0	1.0
N^6-Bnz-8-Pip-cAMP	**1.7**	**0.0011**	**0.065**	**1550**	**26**
N^6-Bnz-cAMP	0.5	3.8	0.0037	1/7.6	135
8-Pip-cAMP	1.2	0.037	2.4	32	½
8-CPT-cAMP	3.9	0.054	19	72	1/4.9
N^6-MB-cAMP[a)]	3.9	0.74	0.071	5.3	55
N^6-MBC-cAMP	0.50	13	0.066	1/26	7.6

The table shows the affinity, relative to cAMP, of the new analog N^6-Bnz-8-Pip-cAMP (bold) synthesized for the present study to achieve specificity for site AI of PKA-I relative to site AII and BII of PKA-II. It shows also data for other analogs previously used to preferentially occupy site AI, as well as for 6-MBC-cAMP, used to selectively occupy site AII of PKA-II. The rel. affinity of N^6-Bnz-8-Pip-cAMP for site BI of PKA-I was 0.018, against 0.26 for N^6-Bnz-cAMP, 0.022 for 8-Pip-cAMP, 1.7 for 8-CPT-cAMP, 0.78 for N^6-MB-cAMP, and 0.086 for N^6-MBC-cAMP. N^6-Bnz-8-Pip-cAMP is the most site AI/BI selective (1550x) and AI/BI selective (94x) compound on record. The synthesis of the new analog and determination of the affinity of the various analogs for the individual sites of PKA is described in Suppl. Sect. IV.

[a)] Data from.[3]

Table S2. The 2-Cl-substituted 8-AHA-cAMP has superior site BI specificity.

Compound	Aff. Site BI (K_dcA/$K_d$anal.)	Aff. Site AII (K_dcA/$K_d$anal.)	Aff. Site BII (K_dcA/$K_d$anal.)	Discr. BI/AII	Discr. BI/BII
Cyclic AMP (cAMP)	1.0	1.0	1.0	1.0	1.0
2-Cl-8-AHA-cAMP	**3.9**	**0.0012**	**0.50**	**3250**	**7.8**
8-AHA-cAMP	3.6	0.028	0.31	128	11.6
8-NH-CH_3-cAMP	1.4	0.026	1.6	54	1/1.1
8-CPT-cAMP	1.7	0.054	19	31	1/11
Sp-5,6-DCl-cBIMPS	0.13	0.034	14	3.8	1/11

The table shows the affinity, relative to cAMP, of the new analog 2-Cl-8-AHA-cAMP for site BI of PKA-I, and site AII and BII of PKA-II. Its rel. affinity for site AI of PKA-I was 0.0052, against 0.73 for 8-AHA-cAMP, 0.09 for 8-NH-CH_3-cAMP, 3.9 for 8-CPT-cAMP, and 0.22 for Sp-5,6-DCl-cBIMPS. 2-Cl-8-AHA-cAMP has 3 orders of magnitude discriminatory power for site BI/AII (3250 x) and BI/AI (750x). The other compounds listed have been used in previous studies of preferential PKA isozyme activation. For further details see the legend to table S1.

Table S3. The expected synergy for PKA-I and PKA-II activation by selected cAMP analog pairs

cAMP analog pair (x + y)	Predicted synergy PKA-I $\frac{\sqrt{(AI^x + AI^y)(BI^x + BI^y)}}{\sqrt{(AI^x)(BI^x)} + \sqrt{(AI^y)(BI^y)}}$ -1	Predicted synergy PKA-II $\frac{\sqrt{(AII^x + AII^y)(BII^x + BII^y)}}{\sqrt{(AII^x)(BII^x)} + \sqrt{(AII^y)(BII^y)}}$ -1
N^6-Bnz-8-Pip-cAMP + 2-Cl-8-AHA-cAMP	7.2	0.09
N^6-Bnz-8-Pip-cAMP + Sp-5,6-DCl-cBIMPS	2.6	0.14
N^6-Bnz-8-Pip-cAMP + N^6-MB-cAMP	0.06	0.34
6-MBA-cAMP + Sp-5,6-DCl-cBIMPS	0.29	7.3

The Table shows how much more efficiently PKA-I or PKA-II can be activated by a mixture of two cAMP analogs (x,y) than expected from simple additive actions. The formulas are based on the analog affinity relative to cAMP, as given in Tables S1,2, for the cAMP binding sites of PKA-I (abbreviated AI^x, AI^y and BI^x,BI^y) and PKA-II (AII^x, AII^y and BII^x, BII^y). The upper term of the formulas, $[(A^x + A^y)x(B^x + B^y)]^{0.5}$, represent the expected potency of the x + y mixture, while the lower term, $(A^x \times B^x)^{0.5} + (A^y \times B^y)^{0.5}$, shows the potency expected from simple additivity. If there is no synergy the ratio between these terms is 1 (see[4] for further details), which becomes 0 after subtraction of 1, as done here. The combination of the new analogs N^6-Bnz-8-Pip-cAMP and 2-Cl-8-AHA-cAMP (first data row) produces stronger PKA-I synergism than any previously available analog combination[4], and is completely devoid of PKA-II synergy. When N^6-Bnz-8-Pip-cAMP is combined with Sp-5,6-DCl-cBIMPS (second row) a moderate PKA-I and no PKA-II synergy is expected, and when combined with N^6-MB-cAMP (row 3) almost no synergy is expected. The combination 6-MBA-cAMP + Sp-5,6-DCl-cBIMPS (row 4) produces strong PKA-II synergy.

II) Supplementary Tables S 6, 7:

Table S6. IPC cell death induced by cAMP or daunorubicin is antagonized by inhibitors of cyclin-dependent kinases (CDK5) and glycogen synthase kinase 3 (GSK3β).

Compound (code)	CDK5 IC_{50} (μM)	GSK3 IC_{50} (μM)	CDK5/GSK3 preference	cAMP-induced apoptosis IC_{50} (μM)	DNR- induced apoptosis IC_{50} (μM)	Data source	Synthesis procedure
R	0.16	130	810	4	> 100	5	
B	0.15	100	670	8.5	54	5	
P	0.075	13	170	0.86	33	5	
O	3	100	33	22	60	5	
K48	3.3	5.5	1.7	10	8.4	6	6
K447	0.4	0.39	0.98		2.3	7	7
K15	0.4	0.35	0.88	4.8	7.6	8	8
K437	3	1	0.33		17	7	7
K310	0.18	0.052	0.29		0.21	7	7
K146	5	1.4	0.28		3.8	7	7
K30	0.044	0.01	0.23	3.5	0.5	9	10
K25	0.5	0.1	0.20	5.9	8.4	9	10
K114	4	0.8	0.20		7.1	7	7
K51	0.43	0.075	0.17	8	5.4	9	10
K81	1	0.13	0.13	4.6	3.1	6	6
K82	3	0.35	0.12	7	10	11	11
K402	0.12	0.013	0.11		0.95	7	7
K49	0.04	0.004	0.10	0.45	0.34	9	10
K319	0.21	0.018	0.086		0.17	7	7
K84	1.8	0.13	0.072		2.6	11	11
K26	0.6	0.03	0.050	4.3	2.1	9	10
K129	100	3.4	0.034		7.6	12	12
K50	0.85	0.023	0.027	5.5	1.9	9	13
K172	3	0.08	0.027	15	2.7	7	7
K144	0.3	0.008	0.027		0.51	7	7
K45	6.3	0.13	0.021	7.8	11	9	10
K53	350	5	0.014		8.9	9	10
K145	10	0.13	0.013		2.5	7	7
K83	1.3	0.015	0.012		3.6	11	11
K148	10	0.063	0.0063		1.6	7	7
K85	4.2	0.018	0.0043		1.7	14	14
K242	6	0.025	0.0042	13	1.3	7	7
K238	10	0.008	0.0008	10	1	7	7

The table shows the inhibitory potency (IC_{50}) against cyclin-dependent protein kinase 5 (CDK5) and glycogen synthase kinase 3beta (GSK3) of 33 compounds, ordered according to decreasing CDK5/GSK3 preference (IC_{50}GSK3/IC_{50}CDK5). The IC_{50} to protect against IPC cell apoptosis induced by incubation for 6h with 0.4 μM daunorubicin (DNR) is shown for all compounds, and to protect against 200 μM 8-CPT-cAMP (cAMP-induced apoptosis) for 18 compounds. Details are given in the Materials and Methods section. The sources of the 29 K-x protein kinase inhibitors, R (roscovitine), O (olomoucine), B (butyrolactone), and P (purvalanol) are given in Suppl. Sect. IV. The IC_{50} values for GSK3 and CDK5 are taken from the sources indicated in the table. The compounds shown in red have more than 30-fold selectivity for CDK5 as compared to GSK3. The ones in dark blue have > 100-fold selectivity for GSK3 as compared to CDK5, those in light blue have 10 – 100 x preference.

Table S7a. The oligoDNA sequences of primers used for qRT-PCR or RT-PCR based cloning of Bim variants

Gene	Forward primer	Reverse primer
18S rRNA	cggctaccacatccaaggaa	cagctggaattaccgcggct
SDHA	catgccagggaagattacaa	gcacagtcagcctcattcaa
Bim (all isoforms)	ggccatggccaagcaaccttctga	ccagatcttcagtgccttctccagacca
BimEL	gtcctccagtgggtatttctc	attgaactcgtctccgatcc
BimL	cagacagaatcgcaagacag	attgaactcgtctccgatcc
BimS	gaatcgcaagcttccataagg	attgaactcgtctccgatcc
η-BimEL	gtcctccagtgggtatttctc	tgagttgaccataccgagac
η-BimL	cagacagaatcgcaagacag	tgagttgaccataccgagac
η-BimS	gaatcgcaagcttccataagg	tgagttgaccataccgagac

Panel a shows the sequences are in 5' – 3' sequence. The primers for Bim (all isoforms) are from the (common) translational start and stop regions. The others from variant-specific regions. See Figure 3 for further description of the Bim isoforms.

Table S7b. The oligoDNA sequences used in shRNAi directed against (pan)-Bim mRNA.

Bim1 fwd	GATCTCCgagtgtgacagagaaggtggacaattgCTGGTCcaattgtccaccttctctgtcacactc
Bim1 rev	AAAAgagtgtgacagagaaggtggacaattgGACCAGcaattgtccaccttctctgtcacactcGGA
Bim2 fwd	GATCTCCgtaaattctgagtgtgacagagaaggtCTGGTCaccttctctgtcacactcagaatttac
Bim2 rev	AAAAgtaaattctgagtgtgacagagaaggtGACCAGaccttctctgtcacactcagaatttacGGA
Luc fwd	GATCTCCgattatgtccggttatgtaaacaatccggCTGGTCccggattgtttacataaccggacataatc
Luc rev	AAAA gattatgtccggttatgtaaacaatccggGACCAGccggattgtttacataaccggacataatcGGA

Panel b shows the sequences of the two hairpin oligo DNA's used to knock down Bim mRNA in the IPC cells. The sequences directed against luciferase mRNA, used as non-target controls, are also shown.

IV) Supplementary Materials and Methods

Source and synthesis of cAMP analogs

The following cAMP analogs: 8-*para*-Chloro-Phenylthio-cAMP (8-CPT-cAMP), 8-*para*-Chloro-Phenylthio-2'-Methyl-cAMP (8-CPT-2'-Me-cAMP), 8-Pieridino-cAMP (8-Pip-cAMP), 8-Methylamino-cAMP (8-$NHCH_3$-cAMP), 8-Aminohexylamino-cAMP (8-AHA-cAMP), N^6-Monobutyryl-cAMP (N^6-MB-cAMP), N^6-Mono-*tert*-Butylcarbamoyl-cAMP (N^6-MBC-cAMP), N^6-Benzoyl-cAMP (N^6-Bnz-cAMP), and Sp-5,6-DCl-cBIMPS were supplied by BioLog Life Science Institute, Bremen, Germany (www.biolog.de). The new analog 2-Chloro-8-Aminohexylamino-cAMP (2-Cl-8-AHA-cAMP) with improved specificity for cAMP binding site BI of PKA-I was synthesized from 2-Chloroadenosine by steps described in[15–17]. The new analog N^6-Benzoyl-8-Piperidiono-cAMP (N^6-Bnz-8-Pip-cAMP) with improved specificity for site AI of PKA-I was synthesized from 8-Br-cAMP using procedures described in [17,18]. The new analogs were ≥ 99% pure as judged by analytical HPLC chromatography on two different resins.

Determination of the affinity of cAMP analogs to the cAMP binding sites of PKA-I and PKA-II

In order for PKA to be activated both of its two cAMP binding sites (A,B) must be occupied by cAMP or another activatory cAMP analog.[19] The binding sites of two PKA isozymes PKA-I and PKA-II differ only subtly, and selective activation of one isozyme demands therefore a pair of cAMP analogs, which when appropriately combined can achieve selective activation of PKA-I or PKA-II. In order to achieve selective activation of PKA-I one analog with preference for site AI of PKA-I must be combined with one preferring site BI of PKA-I. To achieve selective activation of PKA-II one analog must prefer site AII and the other one site BII (see also [4] and Table S3).

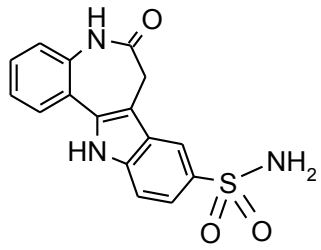
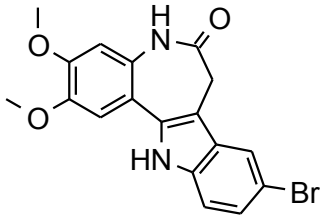
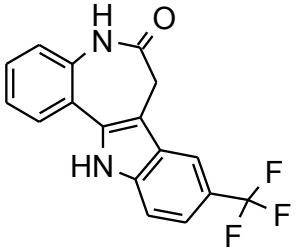
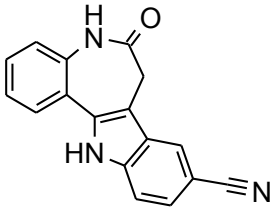
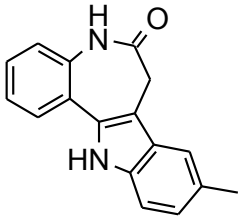
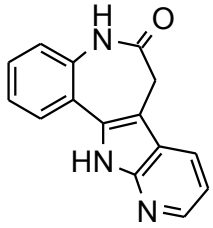
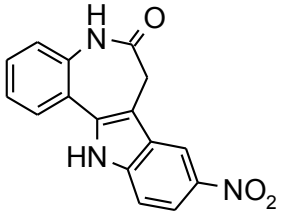
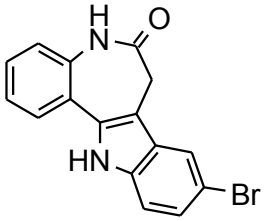
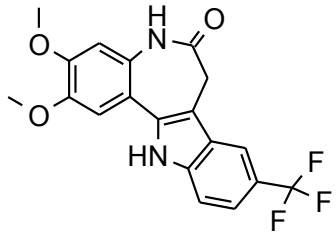
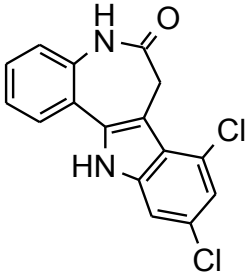
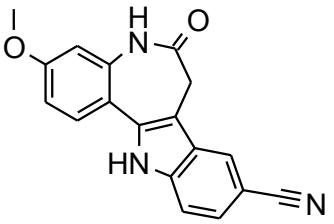
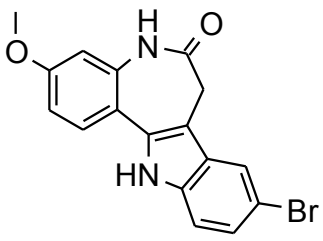
The affinity of each analog for site AI and BI of PKA-I was determined using the human recombinant regulatory, cAMP-binding subunit (hRIα) of PKA-I. For PKA-II the hRIIα subunit was used. The method is based on the ability of analogs to displace [^{3}H]cAMP from site A and B under equilibrium binding conditions, and has been thoroughly validated and described.[20] The data are given as the average of at least three determinations with a range of ± 15% of the mean. The results obtained with previously available cAMP analogs (Tables S1, S2) were similar to those previously found for rabbit RI subunit and bovine RII subunit[21] and murine RI subunit.[22] This fact and the near identical amino acid sequences of each cAMP binding site of mammalian RI subunits suggest that the present data are relevant for the rat R subunit expressed in the presently studied rat-derived IPC cells.

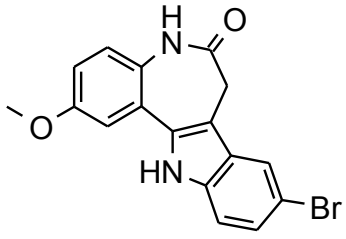
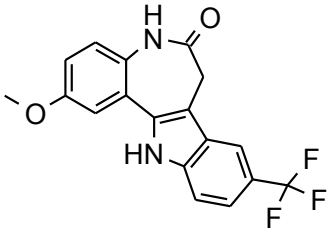
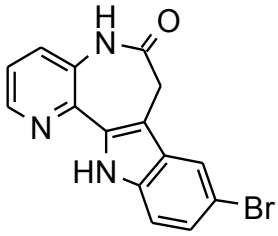
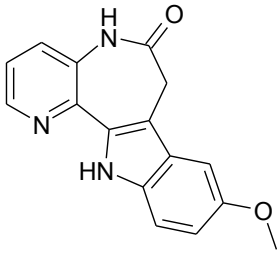
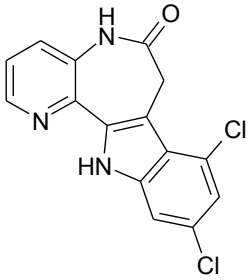
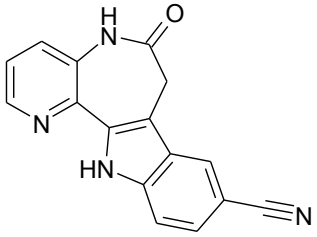
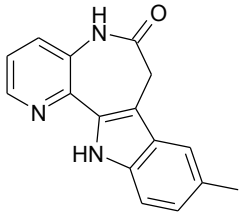
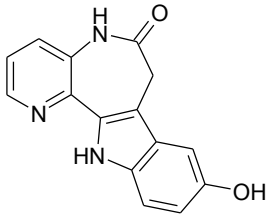
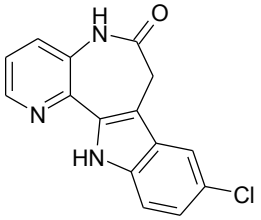
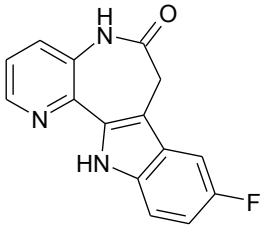
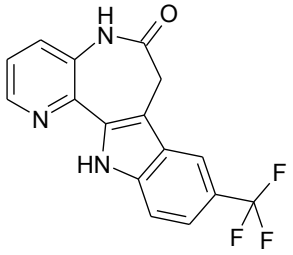
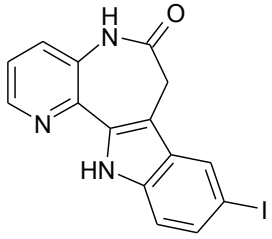
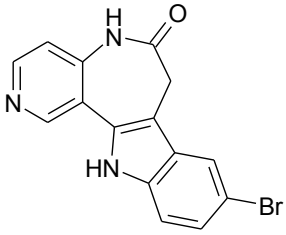
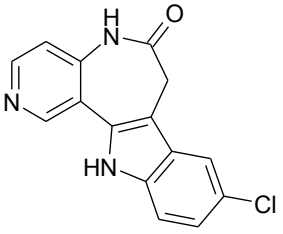
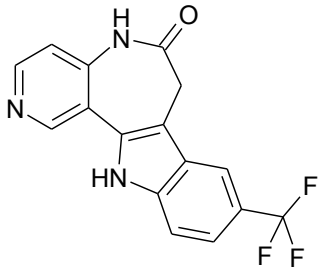
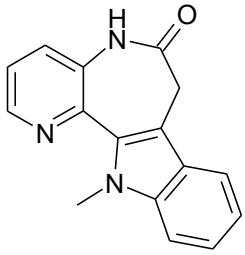
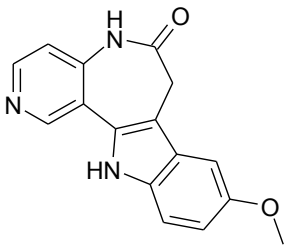
Source and synthesis of inhibitors of cyclin-dependent protein kinases and glycogen synthase kinase 3

The CDK inhibitor roscovitine (RCV) was from Sigma chem. Co. (St.Louis, MO, USA). Butyrolactone and olomoucine were from Calbiochem, and purvalanol from Alexis.

The 29 CDK/GSK3 targeting inhibitors labeled Kn (Table S6) were synthesized and provided by Dr. C. Kunick, Institut für Medizinische und Pharmazeutische Chemie, Technische Universität Braunschweig, Beethovenstraße 55, D-38106 Braunscheig, Germany. Synthesis, purification, structure identification, physicochemical characterization, and purity evaluation were carried out according to the published methods described in the references listed in Table S6.

Chart S1: Structures of dual CDK/GSK3 inhibitors labeled Kn listed in Table S6

 K15	 K25	 K26
 K30	 K45	 K48
 K49	 K50	 K51
 K53	 K81	 K82

 K83	 K84	 K85
 K114	 K129	 K144
 K145	 K146	 K148
 K172	 K238	 K242
 K310	 K319	 K402
 K437	 K447	

Source of cell lines used

The HEK293T cells were obtained from ATCC (Rockville, MD). The Phoenix cells used to produce retrovirus were a kind gift from J. Lorens. The IPC-81 cell line, also denoted IPC or IPC_{WT} in this work, was established by M. Lanotte.[23] It is derived from the transplantable rat BNML leukemia cell model, which is considered one of the most reliable intact cell animal models for the prediction of anti-leukemic drug efficiency in human AML patients.[24,25]

The IPC_{BCL2} cell line was derived from IPC_{WT} by stable retroviral transfection[26], and due to its resistance to cAMP it allowed the detection of a cAMP-induced differentiation pathway hidden by the rapid death otherwise induced by cAMP.[26] The IPC_{BCL2} line was used to study cAMP-induced gene regulation un-confounded by apoptosis and judge the ability of Bcl2 to counteract death induced by exogenously introduced Bim-L.

The IPC_{ICER} cell line was derived from the IPC_{WT}cells[27] to judge the importance of CRE-dependent transcription factors (CREB, CREM, ATF family) in cAMP-dependent death and differentiation. ICER is a naturally occurring alternative splice form of the larger CREM protein, which is closely related to CREB.[28] Since ICER contains only the CRE binding domain of CREM (see Fig. 7 for a simplified diagram) it will bind to the cAMP-responsive element (CRE) of DNA without modifying transcription, and thereby act as a competitive inhibitor towards CREB and CREM. The IPC_{ICER} cells lack both the differentiation and the apoptotic response to cAMP.[27] In the present study they were used primarily to judge the CRE-dependence of cAMP-induced IPC cell gene regulation. Since CREB has become established as a potential AML oncogene and ICER as a AML tumor supressor[29–32] we used the IPC_{ICER} cells also to judge whether ICER expression facilitated IPC death induced by the first line anthracycline daunorubicin (DNR) alone and together with cAMP.

In addition to study the abovementioned IPC cell lines we created IPC cells with stably downregulated Cα subunit of PKA, stably downregulated Bim, as well as cells overexpressing BimL. Supplementary details of their generation are given below.

Generation of PKA Cα subunit depleted IPC cells and determination of their PKA activity, and calculation of their PKA subunit concentrations

The C subunits are common for PKA-I and PKA-II. The major C subunit isoform is Cα. To generate functional RNAi hairpins against the Cα subunit of PKA, an expression library based approach described in[33] was used. The method utilizes restriction enzyme digested cDNA as a template for short hairpin coding DNA to incorporate into expression vectors. The rat PKA Cα subunit cDNA from the plasmid IMAGE:7314651, was used as a source for potential target. It was digested and the fragment was treated according to.[33] The hairpin RNAi coding DNA fragments, kindly provided by Dr. Jim Lorens, were incorporated into two different retroviral vectors designed for RNAi- and GFP expression.

Retroviruses were generated and used to infect IPC cells as described in material and methods section. The infected IPC cells were cultured for two days and sorted for GFP-positive cells using fluorescence-activated flow cytometry. The GFP positive cells were treated with 65μM 8CPT-cAMP for 48 hours to select for cAMP resistant cells. The cells surviving the selection were sub-cloned and expanded for analysis of kinase activity and testing of susceptibility to cAMP-induced apoptosis.

The PKA activity (residing in the catalytic C subunit of PKA) was determined in extract of the IPC cells as described previously.[34,35]

The content of PKA subunits in the IPC_{WT} cells is higher than in average tissues, where it is about 0.5 – 1 μM C subunit.[36] We found IPC cells to contain 8.2 pmol RI subunit / mg cell protein, 2.9 pmol RII / mg protein, and 10 pmol C subunit / mg protein. Taking the protein content to be 15% and the PKA subunits to be distributed in 70% of the cell volume these figures translate to effective cellular concentrations of 1.76 μM RI, 0.62 μM RII, and 2.1 μM C subunit.[35] If the PKA is 25% dissociated in the resting state[37] about 0.52 μM C subunit will be dissociated and active under basal conditions. Upon microinjection of protein solutions about 50-fold dilution occurs in the cell cytosol[38]The injection of C subunit from a 40 μM stock solution, as in[39] would therefore result in an extra 0.8 μM of C subunit, i.e. a total of about 1.3 μM of free C subunit, which is still considerably less than the amount expected upon complete activation of all the PKA in the IPC cells. This point is important since a costimulatory role of Epac otherwise could explain an enigmatic observation: the microinjection of the catalytic subunit of PKA in IPC_{WT} cells (IPC_{WT}) leads to much more apoptosis in the presence of a low (sub-apoptogenic) concentration of the cAMP analog 8CPT-cAMP.[40] The free catalytic subunit does not depend on cAMP for activity. Since 8CPT-cAMP binds more avidly to the Rap exchange factor Epac than to PKA[41] its co-stimulatory effect could therefore indicate a role for Epac in the cAMP-induced apoptosis.

Generation of Bim knock-down and BimL overexpressing IPC cells

Bim-deprived IPC cells were obtained by retroviral transfected short hairpin (sh) RNA 29-mer. The RNAi sequences used (table S7b) were designed based on the general guidelines from[42] and the RNAi generator provided by MWG (http://www.mwgdna.com). They were inserted into the Aar1 site of the retroviral vectors carrying puromycin resistance and red fluorescent protein (RFP) markers (GenBank Acc EU424173). Retroviral transfection of IPC cells were performed as described previously.[43] The transfected cells were selected with puromycin for two weeks, when no RFP-negative cells were detected.

To achieve enforced (over)expression of BimL in the IPC cells an expression vector was produced in HEK293T Phoenix cells. The pMIG vector[44] was used to co-express BimL and GFP. The vector does not contain the SV40 origo necessary for efficient plasmid replication in cells expressing large T antigen, like HEK293T. The Phoenix cells were therefore able to produce virus before committing Bim-induced apoptosis. Retroviral transfection of IPC cells was as described.[43]

Generation of IPC and HEK-293T cells with stable and transient enforced expression of wt, truncated or caspase-resistant HSP90 cochaperone p23

In order to judge the importance of p23 and its cleavage by caspases in IPC cell death we generated IPC cells stably overexpressing wild-type, truncated or caspase-resistant p23. Mutated and truncated versions of p23 generated as described.[45] They were subcloned into the retroviral vector CRU5-IRES-GFP (kindly provided by Dr. Jim Lorens, Univ. of Bergen, Norway) to achieve bi-cistronic expression of GFP and each variant of p23. Retrovirus production and transfection of IPC cells was as described.[43] GFP positive cells were isolated by flow cytometry on a FACS Aria (BD Biosciences).

For transient transfection of HEK293T cells the wild-type, truncated or caspase-resistant p23 was subcloned into pRK5MCS-p23-FLAG.[46] The pRK5MCS-p23-FLAG vector was a kind gift from Dr. T. Rein (Max Planck Institute of Psychiatry, Germany).

Proccessing of microarray data

Raw data were normalized with the Robust Multichip Average method using the dChip software.[47] Probe sets spanning several chromosomes according to the EMBL ensemble genes database (version 59) or having low signal strength (<100 units) were excluded. Of the remaining genes those that differed more than 2-fold in expression between control and N^6-MB-cAMP treated cells were operationally defined as being differentially expressed. The gene names were collected from the official annotations from the gene expression omnibus (www.ncbi.nlm.nih.gov/geo/, version 26). The regulated genes were in addition annotated from the ensemble database (version 59). To identify potential CREB transcriptional targets we applied the CREB Target Gene Database, (http://natural.salk.edu/CREB/), described in.[48] For this the predicted cAMP responsive elements (CREs) in the proximal promoter region (-3000 to +300 nucleotides relative to the transcriptional start site) for the rat genes in the database were extracted, and coupled to our expression data. The CRE was classified as conserved when present both in the rodent and human genomes.

V) The regulation of Bim expression

Transcriptional regulation – roles of CREB and roscovitin-inhibited cyclin-dependent protein kinases

The regulation of Bim transcription is complex and still incompletely understood.[49] The transcriptional initiation can be regulated by Foxo1/3a, B/CMyb and Jun, which may act coordinately[50] to induce Bim transcription, as well as by Egr1[51], cMyc[52] c-Fos[53] and Foxo1/3 co-activators like Runx1[54] and NF-Y.[49] Bim transcription may also be induced by relief from repressors like Bmi-1[55] and Lrf/ZBTB7A.[56] The Bim promoter has, in addition, putative sp1 binding sites.[57] The transcriptional factors involved in the postulated transcriptional regulation though the 3'-UTR of Bim have not been identified, but the 3'-UTR-dependent repression appears to depend on an intact MEK/ERK pathway.[58]

None of the abovementioned transcription factors are known to act via cAMP responsive elements (CRE), and therefore unlikely to be major direct mediators of the strong cAMP-induced increase of IPC cell Bim-mRNA observed in the present study (Figures 2,4). Rather, the blunted cAMP-induced Bim-mRNA expression in IPC cells overexpressing the CRE-binding ICER protein (Figure 2) points to transcriptional control by the CRE-binding CREB/CREM/ATF transcription factor family, which has not been implicated in Bim regulation previously.

The IPC cells may have less general CRE-dependent basal activity than many other AML cells. Apparently, their PKA activity is insufficient to affect CREB-dependent transcription. Thus, the basal state IPC transcriptome is similar in wild-type cells and cells overexpressing the CRE-blocking ICER protein. The low CRE-dependent basal transcription is not due to deficient transcriptional machinery. A number of transcripts are induced in an ICER-inhibitable manner in cells incubated with a high concentration of PKA-directed cAMP analog. The apparent lack of basal state CREB activity may explain why ICER overexpression failed to affect the DNR-induced IPC cell death (Figure S3). In contrast, ICER-transfected HL-60 cells show profound differences in gene expression without cAMP stimulation.[31] Presumably, CREB has a lower constitutive activity in IPC cells than in AML cells, where CREB is a marker for chemotherapy resistance and ICER for response.[31,32,29,30,59,60]

The possibility that the CREB requirement is indirect and through regulation of the established Bim transcription factors is improbable since neither Bmi-1, ZBTR7A, runx1, Jun, Foxo1, FOXO3a, Egr1, NF-Ya,b,c, or Myb were regulated by cAMP as detected by our gene array (not shown). The Bim transcriptional activator cMyc was downregulated by cAMP on the mRNA level (Figure 2a) as well as on the protein level (not shown), which would counteract rather than stimulate Bim expression. The cAMP-induced increase of c-Fos and cEBP-b (Figure 2a) may be of limited importance since the cAMP-stimulated increase of Bim-mRNA was unaltered in the presence of cycloheximide, which blocks the de novo synthesis of any proteins induced on the mRNA level.

CREB binds to the rat Bim promoter[61], presumably the two conserved CRE half-sites about 400 bp upstream of the transcriptional start site. The involvement of CREB in Bim transcription is nevertheless noteworthy because the Bim-promoter is TATA-less[57] and therefore, according to common dogma, unable to supply CREB/CREM/ATF with an ordinary TATA-box binding protein for complexation, as normally required for CREB-mediated gene cAMP regulation[62] (see also[63]). The CRE-dependent transcription from a TATA-less promoter can be explained if elements replace the function of TATA box.[64] An inverted CCAAT box has recently been described 29 bp upstream of the rat Bim transcriptional start site.[49] Alternatively, the distant upstream TATA-box associated CREs, found to function as alternative promoter in HEK293 cells[65] might be active in the IPC cells.

Although outside the scope of the present work, we believe that the findings of the present study should incite further studies of the control of the regulation of Bim transcription in myeloid cells, inlcuding AML.

In T-cell leukemia both glucocorticoid and cAMP can induce Bim and death, albeit much more slowly than in the IPC cells.[66] In the IPC cells the synthetic glucocorticoid dexamethasone (at 0.1, 1 or 10 μM and present for 6 or 24 hours) failed to induce cell death alone and was also unable to enhance the cAMP-induced death (not shown). This demonstrates another distinction between the IPC and S49 cell systems.

The ability of the CDK-directed inhibitor roscovitin (RCV) to block near completely the cAMP-induced Bim-mRNA expression (Figure 4a) is presumably due to inhibition of CDK7 and CDK9.[67,68] CDK7,9 stimulate translational elongation by catalyzing multiple phosphorylations of RNA polymerase II[68–70] (see also Figure 7). The alternative explanation of inhibition of Bim transcriptional initiation factor activity by RCV is less likely. While CDK4 can activate the Bim activator Myb through the E2F pathway,[71] neither it nor CDK1 or CDK2 appear linked to the cAMP-induced IPC cell apoptosis.[72] Furthermore, Myb is not expected to require CRE for its activity.

It is known that RCV through inhibition of CDK7,9[68–70] can depress the expression of anti-apoptotic genes like Mcl1 and XIAP.[73–75] These effects together with the cell cycle arrest resulting from inhibition of CDK1,2,4 may explain the beneficial anti-cancer effect of RCV. The inhibition of Bim expression by RCV has not been observed before[76], and suggests caution in using RCV in tumors depending on Bim expression for eradication.[77–79] A possible reason why Bim is highly sensitive to CDK7 -9 inhibition for efficient transcriptional elongation may be its long 3'-UTR, which is essential for Bim-mRNA repression by the MEK-ERK pathway.[58]

We have shown previously that overexpressed CDK5 promotes cAMP-induced IPC cell apoptosis while kinase activity deficient (dominant negative) CDK5 counteracts it.[72] The overexpressed dominant negative CDK5 was a more moderate apoptosis inhibitor than RCV, indicating that the main effect of RCV was not by inhibition of CDK5 alone, but mainly by the inhibition of other CDK's like CDK7,9. The effect of CDK5 to enhance apoptosis may be by phosphorylating nuclear transcription factors[80]. CDK5 may also regulate the cellular response to DNA damage[81] and the translational efficiency of selected mRNA transcripts, as recently demonstrated in myeloid cells.[82]

Post-transcriptional regulation of Bim expression.

The majority of reported full length Bim transcripts in mouse and human (GI:323362953; GI:90093356, accessed 2011.07.30) have a large (about 4.2 kb) 3′UTR. The AU-rich elements in 3'UTR contribute to Bim mRNA stability by binding the heat-shock cognate protein p70.[83] The 3´UTR of Bim-mRNA is also targeted by the miR-106b-25 polycistron, which suppresses Bim translation.[84] Also miR17-92[85–87] and miRNA 221[88] binding supress Bim expression.

In view of the importance of Bim it is not unsurprising that its expression is regulated also at the level of protein stability and association to the cytoskeleton. Such regulation is documented mainly for the longer isoforms. In BimEL, 8 or more sites are phosphorylated by ERK and other kinases. The phosphorylations appear to regulate the interaction with other cellular components including ubiquitin ligase. ERK is reported to promote ubiquitin-dependent proteasomal degradation of Bim.[89,90] The isolated phosphorylation of mouse BimEL Ser83 by PKA appears to stabilize Bim[91] although Bim may be destabilized under conditions when the same site is phosphorylated by Akt.[92]

VI) Supplementary References

1. Wang Z, Smith KS, Murphy M, Piloto O, Somervaille TCP, Cleary ML. Glycogen synthase kinase 3 in MLL leukaemia maintenance and targeted therapy *Nature.* 2008; **455**: 1205-1209

2. Krakstad C, Herfindal L, Gjertsen BT, Bøe R, Vintermyr OK, Fladmark KE, et al. CaM-kinaseII-dependent commitment to microcystin-induced apoptosis is coupled to cell budding, but not to shrinkage or chromatin hypercondensation *Cell Death Differ.* 2006; **13**: 1191-1202

3. Byeon I-JL, Dao KK, Jung J, Keen J, Leiros I, Døskeland SO, et al. Allosteric communication between cAMP binding sites in the RI subunit of protein kinase A revealed by NMR *J. Biol. Chem.* 2010; **285**: 14062-14070

4. Christensen AE, Viste K, Døskeland SO. Cyclic nucleotide analogs as tools to investigate cyclic nucleotide signaling In: Dennis EA, Bradshaw RA, editors. Handbook of Cell Signaling. Elsevier; 2009. p. 1556-1562.

5. Leclerc S, Garnier M, Hoessel R, Marko D, Bibb JA, Snyder GL, et al. Indirubins inhibit glycogen synthase kinase-3 beta and CDK5/p25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? *J. Biol. Chem.* 2001; **276**: 251-260

6. Kunick C, Lauenroth K, Wieking K, Xie X, Schultz C, Gussio R, et al. Evaluation and comparison of 3D-QSAR CoMSIA models for CDK1, CDK5, and GSK-3 inhibition by paullones *J. Med. Chem.* 2004; **47**: 22-36

7. Stukenbrock H, Mussmann R, Geese M, Ferandin Y, Lozach O, Lemcke T, et al. 9-cyano-1-azapaullone (cazpaullone), a glycogen synthase kinase-3 (GSK-3) inhibitor activating pancreatic beta cell protection and replication *J. Med. Chem.* 2008; **51**: 2196-2207

8. Pies T. 9-Substituted paullones: Synthesis and analysis of structure-activity relationships 2003;

9. Leost M, Schultz C, Link A, Wu YZ, Biernat J, Mandelkow EM, et al. Paullones are potent inhibitors of glycogen synthase kinase-3beta and cyclin-dependent kinase 5/p25 *Eur. J. Biochem.* 2000; **267**: 5983-5994

10. Schultz C, Link A, Leost M, Zaharevitz DW, Gussio R, Sausville EA, et al. Paullones, a series of cyclin-dependent kinase inhibitors: synthesis, evaluation of CDK1/cyclin B inhibition, and in vitro antitumor activity *J. Med. Chem.* 1999; **42**: 2909-2919

11. Wieking K. Strukturmodifikationen und Affinitätsuntersuchungen zur Aufklärung des Wirkmechanismus von Paullonen 2001;

12. Stukenbrock H. Design und Synthese neuartiger GSK-3-Inhibitoren: Wirkstoffe zur Protektion und Proliferationsstimulation pankreatischer beta-Zellen 2008;

13. Kunick C. Synthese von 7,12-Dihydro-indolo[3,2-d][1]benzazepin-6(5H)-onen und 6,11-Dihydro-thieno[3',2':2,3]azepino[4,5-b]indol-5(4H)-on *Arch. Pharm. (Weinheim).* 1992; **325**: 297-299

14. Kunick C, Lauenroth K, Leost M, Meijer L, Lemcke T. 1-Azakenpaullone is a selective inhibitor of glycogen synthase kinase-3 beta *Bioorg. Med. Chem. Lett.* 2004; **14**: 413-416

15. Holmes RE, Robins RK. Purine Nucleosides. VII. Direct Bromination of Adenosine, Deoxyadenosine, Guanosine, and Related Purine Nucleosides *Journal of the American Chemical Society.* 1964; **86**: 1242-1245

16. Genieser H-G, Dostmann W, Bottin U, Butt E, Jastorff B. Synthesis of nucleoside-3', 5'-cyclic phosphorothioates by cyclothiophosphorylation of unprotected nucleosides *Tetrahedron Letters.* 1988; **29**: 2803-2804

17. Long RA, Robins RK, Townsend LB. Purine nucleosides. XV. Synthesis of 8-amino- and 8-substituted aminopurine nucleosides *The Journal of Organic Chemistry.* 1967; **32**: 2751-2756

18. Falbriard J-G, Posternak T, Sutherland EW. Preparation of derivatives of adenosine 3',5'-phosphate *Biochimica et Biophysica Acta (BBA) - General Subjects.* 1967; **148**: 99-105

19. Kleppe R, Krakstad C, Selheim F, Kopperud R, Døskeland SO. The cAMP-dependent protein kinase pathway as therapeutic target: possibilities and pitfalls *Curr Top Med Chem.* 2011; **11**: 1393-1405

20. Dao KK, Teigen K, Kopperud R, Hodneland E, Schwede F, Christensen AE, et al. Epac1 and cAMP-dependent protein kinase holoenzyme have similar cAMP affinity, but their cAMP domains have distinct structural features and cyclic nucleotide recognition *J. Biol. Chem.* 2006; **281**: 21500-21511

21. Øgreid D, Ekanger R, Suva RH, Miller JP, Døskeland SO. Comparison of the two classes of binding sites (A and B) of type I and type II cyclic-AMP-dependent protein kinases by using cyclic nucleotide analogs *European Journal of Biochemistry.* 1989; **181**: 19-31

22. Steinberg RA, Gorman KB, Ogreid D, Døskeland SO, Weber IT. Mutations that alter the charge of type I regulatory subunit and modify activation properties of cyclic AMP-dependent protein kinase from S49 mouse lymphoma cells *J. Biol. Chem.* 1991; **266**: 3547-3553

23. Lacaze N, Gombaud-Saintonge G, Lanotte M. Conditions controlling long-term proliferation of Brown Norway rat promyelocytic leukemia in vitro: Primary growth stimulation by microenvironment and establishment of an autonomous Brown Norway [`]leukemic stem cell line' *Leukemia Research.* 1983; **7**: 145-154

24. van Bekkum DW, van Oosterom P, Dicke KA. In vitro colony formation of transplantable rat leukemias in comparison with human acute myeloid leukemia *Cancer Res.* 1976; **36**: 941-946

25. McCormack E, Bruserud O, Gjertsen BT. Review: genetic models of acute myeloid leukaemia *Oncogene.* 2008; **27**: 3765-3779

26. Seite P, Ruchaud S, Hillion J, Gendron MC, Bruland O, Segal-Bendirdjian E, et al. Ectopic expression of Bcl-2 switches over nuclear signalling for cAMP-induced apoptosis to granulocytic differentiation. *Cell Death Differ.* 2000; **7**: 1081–1089

27. Ruchaud S, Seite P, Foulkes NS, Sassone-Corsi P, Lanotte M. The transcriptional repressor ICER and cAMP-induced programmed cell death. *Oncogene.* 1997; **15**: 827–36

28. Sassone-Corsi P. Coupling gene expression to cAMP signalling: role of CREB and CREM *Int. J. Biochem. Cell Biol.* 1998; **30**: 27-38

29. Shankar DB, Cheng JC, Kinjo K, Federman N, Moore TB, Gill A, et al. The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia *Cancer Cell.* 2005; **7**: 351-362

30. Pigazzi M, Ricotti E, Germano G, Faggian D, Arico M, Basso G. cAMP response element binding protein (CREB) overexpression CREB has been described as critical for leukemia progression *Haematologica.* 2007; **92**: 1435-1437

31. Pigazzi M, Manara E, Baron E, Basso G. ICER expression inhibits leukemia phenotype and controls tumor progression. *Leukemia.* 2008; **22**: 2217–25

32. Pigazzi M, Manara E, Beghin A, Baron E, Tregnago C, Basso G. ICER evokes Dusp1-p38 pathway enhancing chemotherapy sensitivity in myeloid leukemia *Clin. Cancer Res.* 2011; **17**: 742-752

33. Sen G, Wehrman TS, Myers JW, Blau HM. Restriction enzyme-generated siRNA (REGS) vectors and libraries *Nat. Genet.* 2004; **36**: 183-189

34. Ekanger R, Vintermyr OK, Houge G, Sand TE, Scott JD, Krebs EG, et al. The expression of cAMP-dependent protein kinase subunits is differentially regulated during liver regeneration *J. Biol. Chem.* 1989; **264**: 4374-4382

35. Gjertsen BT, Mellgren G, Otten A, Maronde E, Genieser HG, Jastorff B, et al. Novel (Rp)-cAMPS analogs as tools for inhibition of cAMP-kinase in cell

culture. Basal cAMP-kinase activity modulates interleukin-1 beta action *J. Biol. Chem.* 1995; **270**: 20599-20607

36. Hofmann F, Bechtel PJ, Krebs EG. Concentrations of cyclic AMP-dependent protein kinase subunits in various tissues *J. Biol. Chem.* 1977; **252**: 1441-1447

37. Duprez E, Gjertsen BT, Bernard O, Lanotte M, Døskeland SO. Antiapoptotic effect of heterozygously expressed mutant RI (Ala336-->Asp) subunit of cAMP kinase I in a rat leukemia cell line *J. Biol. Chem.* 1993; **268**: 8332-8340

38. Mellgren G, Vintermyr OK, Bøe R, Døskeland SO. Hepatocyte DNA replication is abolished by inhibitors selecting protein phosphatase 2A rather than phosphatase 1 *Exp. Cell Res.* 1993; **205**: 293-301

39. Vintermyr OK, Bøe R, Bruland T, Houge G, Døskeland SO. Elevated cAMP gives short-term inhibition and long-term stimulation of hepatocyte DNA replication: roles of the cAMP-dependent protein kinase subunits *Journal of Cellular Physiology.* 1993; **156**: 160–170

40. Vintermyr OK, Gjertsen BT, Lanotte M, Doskeland SO. Microinjected catalytic subunit of cAMP-dependent protein kinase induces apoptosis in myeloid leukemia (IPC-81) cells. *Exp Cell Res.* 1993; **206**: 157–61

41. Christensen AE, Selheim F, de Rooij J, Dremier S, Schwede F, Dao KK, et al. cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension *J. Biol. Chem.* 2003; **278**: 35394-35402

42. Siolas D, Lerner C, Burchard J, Ge W, Linsley PS, Paddison PJ, et al. Synthetic shRNAs as potent RNAi triggers *Nat. Biotechnol.* 2005; **23**: 227-231

43. Huang T-sheng, Myklebust LM, Kjarland E, Gjertsen BT, Pendino F, Bruserud Ø, et al. LEDGF/p75 has increased expression in blasts from chemotherapy-resistant human acute myelogenic leukemia patients and protects leukemia cells from apoptosis in vitro *Mol. Cancer.* 2007; **6**: 31

44. Carlesso N, Aster JC, Sklar J, Scadden DT. Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics *Blood.* 1999; **93**: 838-848

45. Gausdal G, Gjertsen BT, Fladmark KE, Demol H, Vandekerckhove J, Døskeland S-O. Caspase-dependent, geldanamycin-enhanced cleavage of co-chaperone p23 in leukemic apoptosis *Leukemia.* 2004; **18**: 1989-1996

46. Wochnik GM, Young JC, Schmidt U, Holsboer F, Hartl FU, Rein T. Inhibition of GR-mediated transcription by p23 requires interaction with Hsp90 *FEBS Lett.* 2004; **560**: 35-38

47. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias *Bioinformatics.* 2003; **19**: 185-193

48. Zhang X, Odom DT, Koo S-H, Conkright MD, Canettieri G, Best J, et al. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues *Proceedings of the National Academy of Sciences of the United States of America.* 2005; **102**: 4459-4464

49. Hughes R, Kristiansen M, Lassot I, Desagher S, Mantovani R, Ham J. NF-Y is essential for expression of the proapoptotic bim gene in sympathetic neurons *Cell Death Differ.* 2011; **18**: 937-947

50. Biswas SC, Shi Y, Sproul A, Greene LA. Pro-apoptotic Bim Induction in Response to Nerve Growth Factor Deprivation Requires Simultaneous Activation of Three Different Death Signaling Pathways *Journal of Biological Chemistry.* 2007; **282**: 29368 -29374

51. Xie B, Wang C, Zheng Z, Song B, Ma C, Thiel G, et al. Egr-1 transactivates Bim gene expression to promote neuronal apoptosis *J. Neurosci.* 2011; **31**: 5032-5044

52. Hemann MT, Bric A, Teruya-Feldstein J, Herbst A, Nilsson JA, Cordon-Cardo C, et al. Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants *Nature.* 2005; **436**: 807-811

53. Ishihara Y, Ito F, Shimamoto N. Increased expression of c-Fos by extracellular signal-regulated kinase activation under sustained oxidative stress elicits BimEL upregulation and hepatocyte apoptosis *FEBS J.* 2011; **278**: 1873-1881

54. Wildey GM, Howe PH. Runx1 is a co-activator with FOXO3 to mediate transforming growth factor beta (TGFbeta)-induced Bim transcription in hepatic cells *J. Biol. Chem.* 2009; **284**: 20227-20239

55. Jagani Z, Wiederschain D, Loo A, He D, Mosher R, Fordjour P, et al. The Polycomb group protein Bmi-1 is essential for the growth of multiple myeloma cells *Cancer Res.* 2010; **70**: 5528-5538

56. Maeda T, Ito K, Merghoub T, Poliseno L, Hobbs RM, Wang G, et al. LRF is an essential downstream target of GATA1 in erythroid development and regulates BIM-dependent apoptosis *Dev. Cell.* 2009; **17**: 527-540

57. Bouillet P, Zhang LC, Huang DC, Webb GC, Bottema CD, Shore P, et al. Gene structure alternative splicing, and chromosomal localization of pro-apoptotic Bcl-2 relative Bim *Mamm. Genome.* 2001; **12**: 163-168

58. Hughes R, Gilley J, Kristiansen M, Ham J. The MEK-ERK pathway negatively regulates bim expression through the 3' UTR in sympathetic neurons *BMC Neuroscience.* 2011; **12**: 69

59. Cheng JC, Kinjo K, Judelson DR, Chang J, Wu WS, Schmid I, et al. CREB is a critical regulator of normal hematopoiesis and leukemogenesis *Blood.* 2008; **111**: 1182-1192

60. Sakamoto KM, Frank DA. CREB in the Pathophysiology of Cancer: Implications for Targeting Transcription Factors for Cancer Therapy *Clin Cancer Res.* 2009; **15**: 2583-2587

61. Impey S, McCorkle SR, Cha-Molstad H, Dwyer JM, Yochum GS, Boss JM, et al. Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions *Cell.* 2004; **119**: 1041-1054

62. Conkright MD, Guzmán E, Flechner L, Su AI, Hogenesch JB, Montminy M. Genome-wide analysis of CREB target genes reveals a core promoter requirement for cAMP responsiveness *Mol. Cell.* 2003; **11**: 1101-1108

63. Altarejos JY, Montminy M. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals *Nat. Rev. Mol. Cell Biol.* 2011; **12**: 141-151

64. Smale ST. Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes *Biochim. Biophys. Acta.* 1997; **1351**: 73-88

65. Gaviraghi M, Caricasole A, Costanzo C, Diamanti D, Dandrea M, Donadelli M, et al. Identification of a candidate alternative promoter region of the human Bcl2L11 (Bim) gene *BMC Mol. Biol.* 2008; **9**: 56

66. Zhang L, Insel PA. The Pro-apoptotic Protein Bim Is a Convergence Point for cAMP/Protein Kinase A- and Glucocorticoid-promoted Apoptosis of Lymphoid Cells *J. Biol. Chem.* 2004; **279**: 20858-20865

67. Meijer L, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, et al. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5 *Eur. J. Biochem.* 1997; **243**: 527-536

68. Meijer L, Bettayeb K, Galons H. (R)-Roscovitine (CYC202, Seliciclib) In: Smith PJ, Yue EW, editors. Inhibitors of cyclin-dependent kinases as anti-tumor agents. CRC/Taylor & Francis; 2006. p. 187-227.

69. Wesierska-Gadek J, Krystof V. Selective cyclin-dependent kinase inhibitors discriminating between cell cycle and transcriptional kinases: future reality or utopia? *Ann. N. Y. Acad. Sci.* 2009; **1171**: 228-241

70. Nechaev S, Adelman K. Pol II waiting in the starting gates: Regulating the transition from transcription initiation into productive elongation *Biochim. Biophys. Acta.* 2011; **1809**: 34-45

71. Biswas SC, Liu DX, Greene LA. Bim is a direct target of a neuronal E2F-dependent apoptotic pathway *J. Neurosci.* 2005; **25**: 8349-8358

72. Sandal T, Stapnes C, Kleivdal H, Hedin L, Doskeland SO. A novel, extraneuronal role for cyclin-dependent protein kinase 5 (CDK5): modulation of cAMP-induced apoptosis in rat leukemia cells. *J Biol Chem.* 2002; **277**: 20783–93

73. Hahntow IN, Schneller F, Oelsner M, Weick K, Ringshausen I, Fend F, et al. Cyclin-dependent kinase inhibitor Roscovitine induces apoptosis in chronic lymphocytic leukemia cells *Leukemia.* 2004; **18**: 747-755

74. Leitch AE, Riley NA, Sheldrake TA, Festa M, Fox S, Duffin R, et al. The cyclin-dependent kinase inhibitor R-roscovitine down-regulates Mcl-1 to override pro-inflammatory signalling and drive neutrophil apoptosis *Eur. J. Immunol.* 2010; **40**: 1127-1138

75. Garrofé-Ochoa X, Cosialls AM, Ribas J, Gil J, Boix J. Transcriptional modulation of apoptosis regulators by roscovitine and related compounds *Apoptosis.* 2011; **16**: 660-670

76. Mohapatra S, Chu B, Zhao X, Djeu J, Cheng JQ, Pledger WJ. Apoptosis of metastatic prostate cancer cells by a combination of cyclin-dependent kinase and AKT inhibitors *Int. J. Biochem. Cell Biol.* 2009; **41**: 595-602

77. Jiang N, Koh GS, Lim JY, Kham SK, Ariffin H, Chew FT, et al. BIM is a prognostic biomarker for early prednisolone response in pediatric acute lymphoblastic leukemia *Exp. Hematol.* 2011; **39**: 321-329, 329.e1-3

78. Mehnert JM, Tan AR, Moss R, Poplin E, Stein MN, Sovak M, et al. Rationally Designed Treatment for Solid Tumors with MAPK Pathway Activation: A Phase I Study of Paclitaxel and Bortezomib Using an Adaptive Dose-Finding Approach *Mol Cancer Ther [Internet].* 2011; [cited 2011 Jul 28] Available from: http://www.ncbi.nlm.nih.gov/pubmed/21680752

79. Akiyama T, Dass CR, Choong PFM. Bim-targeted cancer therapy: a link between drug action and underlying molecular changes *Mol. Cancer Ther.* 2009; **8**: 3173-3180

80. Rosales JL, Lee K-Y. Extraneuronal roles of cyclin-dependent kinase 5 *Bioessays.* 2006; **28**: 1023-1034

81. Turner NC, Lord CJ, Iorns E, Brough R, Swift S, Elliott R, et al. A synthetic lethal siRNA screen identifying genes mediating sensitivity to a PARP inhibitor *EMBO J.* 2008; **27**: 1368-1377

82. Arif A, Jia J, Moodt RA, DiCorleto PE, Fox PL. Phosphorylation of glutamyl-prolyl tRNA synthetase by cyclin-dependent kinase 5 dictates transcript-selective translational control *Proceedings of the National Academy of Sciences.* 2011; **108**: 1415 -1420

83. Matsui H, Asou H, Inaba T. Cytokines direct the regulation of Bim mRNA stability by heat-shock cognate protein 70 *Mol. Cell.* 2007; **25**: 99-112

84. Kan T, Sato F, Ito T, Matsumura N, David S, Cheng Y, et al. The miR-106b-25 Polycistron, Activated by Genomic Amplification, Functions as an Oncogene by Suppressing p21 and Bim *Gastroenterology.* 2009; **136**: 1689-1700

85. Xiao C, Srinivasan L, Calado DP, Patterson HC, Zhang B, Wang J, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes *Nat Immunol.* 2008; **9**: 405-414

86. Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ, et al. Targeted Deletion Reveals Essential and Overlapping Functions of the miR-17∼92 Family of miRNA Clusters *Cell.* 2008; **132**: 875-886

87. Molitoris JK, McColl KS, Distelhorst CW. Glucocorticoid-mediated repression of the oncogenic microRNA cluster miR-17∼92 contributes to the induction of Bim and initiation of apoptosis *Mol. Endocrinol.* 2011; **25**: 409-420

88. Terasawa K, Ichimura A, Sato F, Shimizu K, Tsujimoto G. Sustained activation of ERK1/2 by NGF induces microRNA-221 and 222 in PC12 cells *FEBS J.* 2009; **276**: 3269-3276

89. Ley R, Ewings KE, Hadfield K, Cook SJ. Regulatory phosphorylation of Bim: sorting out the ERK from the JNK *Cell Death Differ.* 2005; **12**: 1008-1014

90. Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, et al. ER stress triggers apoptosis by activating BH3-only protein Bim *Cell.* 2007; **129**: 1337-1349

91. Moujalled D, Weston R, Anderton H, Ninnis R, Goel P, Coley A, et al. Cyclic-AMP-dependent protein kinase A regulates apoptosis by stabilizing the BH3-only protein Bim *EMBO Rep.* 2011; **12**: 77-83

92. Qi X-J, Wildey GM, Howe PH. Evidence that Ser87 of BimEL is phosphorylated by Akt and regulates BimEL apoptotic function *J. Biol. Chem.* 2006; **281**: 813-823