Cyclic AMP can promote APL progression and protect myeloid leukemia cells against anthracycline-induced apoptosis

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We show that cyclic AMP (cAMP) elevating agents protect blasts from patients with acute promyelocytic leukemia (APL) against death induced by first-line anti-leukemic anthracyclines like daunorubicin (DNR). The cAMP effect was reproduced in NB4 APL cells, and shown to depend on activation of the generally cytoplasmic cAMP-kinase type I (PKA-I) rather than the perinuclear PKA-II. The protection of both NB4 cells and APL blasts was associated with (inactivating) phosphorylation of PKA site Ser118 of the AML oncogene CREB. Either event would be expected to protect broadly against cell death, and we found cAMP elevation to protect also against 2-deoxyglucose, rotenone, proteasome inhibitor and a BH3-only mimic. The in vitro findings were mirrored by the findings in NSG mice with orthotopic NB4 cell leukemia. The mice showed more rapid disease progression when given cAMP-increasing agents (prostaglandin E2 analog and theophylline), both with and without DNR chemotherapy. The all-trans retinoic acid (ATRA)-induced terminal APL cell differentiation is a cornerstone in current APL treatment and is enhanced by cAMP. We show also that ATRA-resistant APL cells, believed to be responsible for treatment failure with current ATRA-based treatment protocols, were protected by cAMP against death. This suggests that the beneficial pro-differentiating and non-beneficial pro-survival APL cell effects of cAMP should be weighed against each other. The results suggest also general awareness toward drugs that can affect bone marrow cAMP levels in leukemia patients.

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Abbreviations: APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; cAMP, cyclic AMP; dmPGE2, 16,16-dimethyl PGE2; DNR, daunorubicin; IBMX, 3-isobutyl-1-methylxanthine; IDA, idarubicin; PDE, phosphodiesterase; PGE2, prostaglandin E2; RCV, roscovitine

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The clinical relevance of the NB4 model is supported by studies of blasts from APL and AML patients, which also were protected by cAMP against DNR-induced death in vitro. The findings indicate awareness when patients on cAMP-elevating agents like theophylline are treated with anthracyclines, or when cAMP stimulation is considered combined with ATRA to boost AML cell differentiation.

Results

CAMP agonists protect NB4 APL cells against DNR-induced death. We found that human NB4 cells, widely accepted as being representative of human APL,5 were strongly protected by cAMP agonists against death induced by the first-line anti-leukemic anthracycline drug DNR. The degree of protection (Figure 1a, main panel) correlated with the cellular cAMP level, which was raised more when the adenylate cyclase stimulator PGE2 was combined with the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Figure 1a, upper right inset). The potentiating effect of IBMX and the efficacy of the cAMP analog N6-Bz-cAMP (Figure 1a, main panel) both indicated that the protective effect of PGE2 was mediated by cAMP. As a prelude to intact animal studies, we found that the stable PGE2 analog 16,16-dimethyl PGE2 (dmPGE2) synergized with any of four different cAMP phosphodiesterase inhibitors, including the clinically relevant compounds theophylline (aminophylline), milrinone and rolipram (Figure 1b).

The effects could not be explained by coincidental blocking of one or a few features of cell death, as all the death phenotypes probed for were inhibited by cAMP, including cell membrane blebbing (Figures 1a and b), cell size reduction with diminished forward light scattering (Figure 1c) and phosphatidylserine externalization (Figure 1d). CAMP stimulation also prevented DNR-induced cleavage of pro-caspase 3 and the caspase target Hsp90 co-chaperone p23, known to have a role in leukemic apoptosis13,17 (Figure 1e). In view of reports that CAMP can affect drug transporters18,19 and concern that CAMP might lead to intracellular sequestration of DNR in intracellular vacuoles, we determined the total cell and the nuclear DNR content. We found identical total DNR concentration in cells incubated with DNR both in the absence and presence of N6-Bz-cAMP (Figure 1f), as well as similar intracellular distribution of DNR, with strong nuclear accumulation and even cytoplasmic content (data not shown).

CAMP failed to protect the AML cell lines Mv4-11 and HL60 against DNR death, whether based on morphological assessment (Figures 2a and b) or (for HL60) cleavage of p23 (Figure 2c). As cAMP agonists enhance DNR-induced cell death in the BNML-derived rat IPC-81 cell AML model,13 we conclude that AML cell lines differ greatly in their response to the combination of DNR and cAMP stimulation.

CAMP agonists can protect APL and AML patient blasts against DNR-induced death. To probe which, if any, of the studied cell line models is relevant for human acute myeloid leukemia, we tested leukemic blasts isolated from patients with diagnosed APL or AML (see Supplementary Table S1 for details of disease). None of the blast isolates showed cAMP-enhanced DNR-induced death, as observed in the IPC cells. Rather, all the three ABL blast isolates (Figures 2d–f) and six of the seven AML blast isolates (Figures 2g–m) resembled the NB4 cells by being protected against DNR by PGE2/IBMX. The APL blasts had an average of 47 ± 5.9% (mean ± S.E.M.; n = 3) protection and the AML blasts 41 ± 5.8% (n = 7). With N6-Bz-cAMP, the average protection of APL blasts was 51% (data not shown). The NB4 cells appear therefore to be the most relevant cell line to study CAMP effects on anthracycline-induced acute myeloid leukemic blast death.

CAMP agonists protect NB4 cells under in vitro conditions relevant for the leukemic bone marrow and enhance APL progression in a NB4 orthotopic NSG model. In order to better judge the intact organism relevance, additional in vitro experiments were conducted to cover DNR and IDA concentrations likely to be encountered in vivo20 and to mimic the hypoxic nature of leukemic cell bone marrow niches.21 We demonstrated that CAMP agonists protected NB4 cells also under hypoxic conditions, and against a wide range of anthracycline concentrations (0.1–5 μM DNR and 10–500 nM IDA), and for periods up to 32 h (Figure 3a, Supplementary Figure S1a).

In current standard protocols for APL treatment, DNR or IDA is administered shortly after the onset of ATRA treatment,
and later used jointly with ATRA to eliminate ATRA-resistant cells. We tested, therefore, whether cAMP agonists could counteract the DNR-induced death both of NB4 cells starting to undergo differentiation with ATRA (Figure 3b, left panel), and of the ATRA-resistant sub-clones NB4-LR2 and NB4-LR15 (Figures 1c and 3b, right panel). We found that cAMP stimulation may protect both ATRA-primed NB4 cells and ATRA-resistant NB4 cells against DNR in the presence of ATRA (Figure 3b).

To know whether the cAMP agonists protected specifically against DNR, we tested whether they also could protect against agents promoting unfolded protein stress, like the

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**Figure 3b**

**% Dead cells**

| Incubation time (h) | Ctrl | DNR | DNR+N6-Bz-cAMP |
|---------------------|------|-----|----------------|
| 3                   | 25.0 | 75.0| 5.0            |
| 4                   | 25.0 | 75.0| 5.0            |
| 5                   | 25.0 | 75.0| 5.0            |

**Figure 3c**

**SSC-H (NB4)**

| Incubation time (h) | Ctrl | DNR | DNR+N6-Bz-cAMP |
|---------------------|------|-----|----------------|
| 3                   | 92.1 | 52.9| 89.8           |
| 4                   | 92.4 | 38.8| 78.7           |
| 5                   | 76.6 | 38.2| 66.3           |

**SSC-H (NB4-LR1)**

| Incubation time (h) | Ctrl | DNR | DNR+N6-Bz-cAMP |
|---------------------|------|-----|----------------|
| 3                   | 92.1 | 52.9| 89.8           |
| 4                   | 92.4 | 38.8| 78.7           |
| 5                   | 76.6 | 38.2| 66.3           |

**SSC-H (NB4-LR2)**

| Incubation time (h) | Ctrl | DNR | DNR+N6-Bz-cAMP |
|---------------------|------|-----|----------------|
| 3                   | 92.1 | 52.9| 89.8           |
| 4                   | 92.4 | 38.8| 78.7           |
| 5                   | 76.6 | 38.2| 66.3           |

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**Figure 3d**

**Annexin V - FL4 (log arbitrary units)**

**Cell number (% of max)**

| Incubation time (h) | Ctrl | DNR | DNR+N6-Bz-cAMP |
|---------------------|------|-----|----------------|
| 3                   | 92.1 | 52.9| 89.8           |
| 4                   | 92.4 | 38.8| 78.7           |
| 5                   | 76.6 | 38.2| 66.3           |

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**Figure 3e**

**Pro-caspase 3**

**Active caspase 3**

**p23**

**Actin**

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**Table 1**

| Incubation time (h) | Ctrl | DNR | DNR+N6-Bz-cAMP |
|---------------------|------|-----|----------------|
| 3                   | 92.1 | 52.9| 89.8           |
| 4                   | 92.4 | 38.8| 78.7           |
| 5                   | 76.6 | 38.2| 66.3           |

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**Figure 3f**

**Cell number (% of max)**

| Incubation time (h) | Ctrl | DNR | DNR+N6-Bz-cAMP |
|---------------------|------|-----|----------------|
| 2h                  | 92.1 | 52.9| 89.8           |
| 5h                  | 76.6 | 38.2| 66.3           |

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**Figure 3g**

**Cell number (% of max)**

| Incubation time (h) | Ctrl | DNR | DNR+N6-Bz-cAMP |
|---------------------|------|-----|----------------|
| 2h                  | 92.1 | 52.9| 89.8           |
| 5h                  | 76.6 | 38.2| 66.3           |
proteasome inhibitor bortezomib and the HSP90 inhibitor geldanamycin, and strong metabolic stressors like 2-deoxy-glucose and rotenone. We found significant protection by cAMP against these stressors both for NB4 cells (Figure 3c) and APL and AML blasts (Supplementary Figure S1b).

In conclusion, the elevation of cAMP is expected to promote NB4 cell (and APL blast) survival under conditions thought to prevail in the leukemic bone marrow both without and with DNR therapy.

We tested, therefore, whether cAMP agonists could promote APL cell survival also in vivo, under the assumedly adverse conditions existing in the bone marrow under APL invasion and the even more adverse conditions under DNR treatment. For this purpose, we transplanted NOD-scidIL2r^null

**Figure 2** Cyclic AMP agonists can protect APL and AML blasts against anthracycline-induced death. (a and b) Mv4-11 (a) and HL60 (b) AML cells were treated for 4 or 7 h, respectively, with DNR in the absence or presence of PGE2/IBMX (2 μM/0.15 mM) or N6-Bz-cAMP (0.4 mM). The % dead cells was scored by microscopy (chromatin condensation), and is given as mean ± S.E.M., n = 3. Significance by Student t-test: **P < 0.01. (c) Extracts isolated from HL60 cells treated with DNR (5 μM) for 3 or 5 h alone or in combination with PGE2/IBMX (2 μM/0.3 mM) were analyzed by immunoblotting for Hsp90 cochaperone p23. Actin was used as loading control. (d–m) Blasts were isolated from peripheral blood of three patients with diagnosed APL (APL1-APL3) and seven AML patients (AML1-AML7) and incubated with DNR alone or together with PGE2/IBMX (2 μM/0.3 mM) for 3 h (APL1, APL2 (8 μM DNR)) or 24 h (APL2, APL3, AML1-AML7). Samples were assessed for drug-induced cell death by FACS analysis of PI uptake (APL1, APL 2) or by Annexin V labeling and PI uptake (APL2, APL3, AML1-AML7). When present, PGE2/IBMX was added 0.5 h before DNR. Further experimental details are given in the Materials and Methods section. Similar results were obtained when PGE2/IBMX was replaced by the cAMP analog N6-Bz-cAMP (data not shown). A Wilcoxon paired two-tailed comparison test of the ratio of dead cells after incubation with DNR alone and DNR + either PGE2/IBMX or N6-Bz-cAMP (0.4 mM) for all the experiments performed on the APL1-3 blasts, yielded P < 0.004 for a protective effect of the cAMP agonists against P < 0.007 for the AML1-7 blasts.
mice (NSG) mice with NB4 cells and injected them with vehicle (control) or dmPGE2/theophylline. The NB4 cell-injected animals given only vehicle survived from 31–33 days (Figure 4a). The death was preceded by weight loss (Figure 4b). The animals were watched for development of extreme fatigue and/or dorsal limb paralysis before euthanization. The animals injected with cAMP agonists had shorter life span and more rapid weight loss than the vehicle-injected animals (Figures 4a and b). This difference was attributed to more rapid APL disease development, as the timing of fatigue and paralysis relative to death was similar, and the animals selected for autopsy showed similarly swollen

Figure 3 Cyclic AMP-elevating agents protect NB4 cells against death induced by a number of cell stressors under conditions relevant for the leukemic bone marrow. (a) NB4 cells were treated for various periods of time and various concentrations of DNR in the absence or presence of PGE2/IBMX (2 μM/0.15 mM) in standard (19% O2, 5% CO2) or hypoxic (2% O2, 5% CO2) atmosphere. The % dead cells was scored by microscopy (chromatin condensation and surface morphology), and is given as mean ± S.E.M., n = 3. Significance by Student t-test: **P<0.01, *P<0.05. (b) NB4 and NB4-LR2 cells were primed with ATRA (1 μM, 24 h). They were next incubated for 5 h with DNR (D; 5 μM) or 6 h with IDA (I; 0.5 μM) or vehicle in the absence or presence of PGE2/IBMX (2 μM/0.1 mM). The % dead cells are shown as upwards solid bars. The % of matured cells was determined by the nitroblue tetrazolium (NBT) reduction assay, and is given as open, downward bars. The ATRA-resistant NB4-LR2 cells were, as expected, NBT negative. The data are given as mean ± S.E.M., n = 3. Significance by Student t-test: **P<0.01, *P<0.05. (c) NB4 cells were treated with the HSP90 antagonist geldanamycin (5 μM), the mitochondrial toxin rotenone (5 μM), the proteasome inhibitor bortezomib (0.2 μM) or the glucose antagonist 2-deoxyglucose (10 mM) for the periods of time indicated in the absence or presence of dmPGE2 (2 μM)/IBMX (0.15 mM) or dmPGE2 (2 μM)/aminophylline (0.15 mM). The scoring of dead cells was as a and b.
The NB4 cell expresses both RIα and RIIγ (Figure 5a, left panel). The defined localization of the RII subunits, and thereby PKA-II, can channel PKA-II to a subset of substrates,24,25 which might be essential for cAMP protection against death. In order to probe whether PKA-II had a specific role, we produced NB4RIIγ/KD cells by RNA-mediated double knockdown of RIIγ and RIIβ.

The NB4RIIγ/KD cells lacked nearly all the RIIγ and most of the RIIβ (Figures 5a and c, right panels), but had a compensatory increase of RIIα (Figure 5a, right panel) and unchanged total PKA concentration and basal PKA activity (data not shown). The knockdown of RIIγ was not undertaken, in part because a selective inhibitor Rp-8-Br-cAMPS26 is available, and in part because downregulation of RIIγ in our hands is not compensated by RIIγ upregulation. Hence, the resulting excess of free cAMP-unregulated C subunit leads to high PKA activity already in the absence of cAMP stimulation.

The NB4RIIγ/KD cells were as well protected against DNR by N6-Bz-cAMP as the NB4wt (Figure 1) and control-transfected NB4v0 cells (Figure 5d), indicating that activation of PKA-I alone suffices to protect. This does not exclude that both isoforms can contribute in the NB4wt or NB4v0 cells. We probed therefore such cells for cAMP analog pair synergy regarding survival, using both PKA-I- and PKA-II-directed pairs. The isozyme-specific synergy of activation is based on their differential complementarity for binding to sites AI and BI of PKA-I or AII and BII of PKA-II of each member of a pair (see Supplementary Tables S2 and S3 for details of the analogs, including the new 2-CI-8-HA-cAMP). The PKA-I directed analog pair synergized both in the NB4wt and NB4RIIγ/KD cells, the length of the horizontal arrows indicating the magnitude of the analog complementarity (Figures 6a and b; see Supplementary Figure S2a,b for alternative plots and additional data). In contrast, the PKA-II-directed analog pair failed to show synergism (Figure 6c, Supplementary Figure S2c). We conclude therefore that RI subunit of PKA-I is the cAMP receptor mediating the protective action of cAMP in NB4 cells. The conclusion is supported by the ability of the PKA-I-specific competitive inhibitor Rp-8-Br-cAMPS26 to counteract the protective effect of N6-Bz-cAMP against DNR (Figure 6d). The pivotal role of PKA suggested that the protective action of cAMP was mediated primarily by phosphorylation of PKA substrate(s).

The phosphorylation state of Bad and CREB in leukemic cells exposed to DNR and cAMP agonists. PKA has a vast number of direct substrates in the intact cell.2,27 Some of these are convergence points between PKA and kinases involved in general survival signaling, such as ERK and members of the PI3-kinase cascade. They may therefore be particularly important for cell survival. Examples are phosphorylation of Serine 75 and 118 in the BH3-only protein Bad,28,29 (S112 and S155 in murine Bad) and Serine 133 of the transcription factor and AML oncogene CREB.30,31

We found that cAMP stimulation increased S118Bad phosphorylation in both NB4 cells and APL3 patient blasts (Figures 7a and b; Supplementary Figure S3a). The S118 phosphorylation liberates Bcl-2/Bcl-Xl sequestered by Bad.29 The phosphorylation of S75 of Bad was high already in non-stimulated NB4 cells (Figure 7a). Bad was presumably constitutively phosphorylated also on S99 in NB4 cells.
as their Bad migrated more slowly (apparent $M_W \approx 20$ kDa) upon SDS gel electrophoresis than expected from its $M_W$ of 18.5 kDa (Figure 7a, upper lane), a feature typical for P-S75/S99Bad.\textsuperscript{28,32}

HL60, Mv4-11 AML cells and AML3 patient blasts were not protected by cAMP (Figures 2a, b and i) and did not show any cAMP stimulation of S118Bad phosphorylation. In the HL60 and Mv4-11 cells, S118Bad was highly phosphorylated already in the resting state (Figures 7c and d), and if anything, P-S118Bad decreased slightly upon prolonged incubation with DNR and PGE\textsubscript{2}/IBMX (Figures 7c and d). The AML3 blasts had very low Bad expression and P-S118Bad was below the detection limit even with strong cAMP stimulation (data not shown).

The net effect of the cAMP-stimulated S118Bad phosphorylation can be modulated if cAMP also affects the expression of other Bcl-2 family members. cAMP-stimulated NB4, HL60 or Mv4-11 cells had unaltered level of Bax, Bcl-2 and Mcl-1 (Supplementary Figures S3 and S4), whereas DNR caused a rapid decrease of Mcl-1 (Supplementary Figures S3b,c and S4a–c). This suggests that DNR may promote death in all cell lines studied by downregulating Mcl-1.

The consequence of the Mcl-1 downregulation can be compensated by release of Bcl-2 from its complex with Bad in cells showing cAMP-induced S118Bad phosphorylation, like NB4 cells and APL blasts. The BH3-mimetic HA14-1 blocks the ability of Bcl-2 to bind and neutralize Bax\textsuperscript{33} and should therefore abolish the beneficial effect of S118Bad phosphorylation. At maximally effective concentration (10 $\mu$M), HA14-1 induced strong NB4 cell death, which was only moderately antagonized by cAMP (Figure 7e, left subpanel). This is compatible with much of the protective action of cAMP being mediated by S118Bad phosphorylation. At lower HA14-1 concentration (4 or 6 $\mu$M) only moderate cell death was observed (Figure 7e, right panel), presumably because only a proportion of the Bcl-2 is occupied by HA14-1. Under such conditions also the Bcl-2 liberated from Bad through S118Bad phosphorylation will be incompletely occupied by HA14-1, which may explain why cAMP provided robust protection against HA14-1 at 4 and 6 $\mu$M (Figure 7e, right panel).

cAMP provided some protection against DNR also in the presence of 10 $\mu$M HA14-1 (Figure 7e, left panel), suggesting that cAMP may protect also via other mechanisms than Bad phosphorylation. A search for additional survival-associated potential PKA targets by quantitative cell phospho-protein flow cytometry\textsuperscript{34} revealed increased phosphorylation of the PKA substrate site Ser133 of CREB in NB4 cells incubated with the PKA-directed activator PGE\textsubscript{2}/IBMX and N\textsuperscript{6}-Bz-cAMP, but not with the Epac-directed activator 8-CPT-2’-Me-cAMP (Figure 7f). We tested next the cAMP-protected APL3 and AML4 and the cAMP-nonresponsive AML3 patient blasts (Figures 2f, i and j) for cAMP-induced P-S133CREB. Only in the two cAMP-protected blasts did cAMP increase P-CREB (Figure 7f). The broadly acting cyclin-dependent protein kinase (CDK) 7/9 inhibitor roscovitine (RCV) blocks transcription stimulated by CREB in a rat AML cell line\textsuperscript{13} and induces death through transcriptional inhibition in human neutrophils.\textsuperscript{35} In the NB4 cells, RCV attenuated the protective effect of cAMP stimulators against DNR (Supplementary Figure S5) without affecting the phosphorylation of Bad (data not shown).

This supported the existence of protective actions of cAMP involving CDK-dependent events, possibly mediated via PKA activation of CREB.

Several other survival-related protein phosphorylation events were studied, but were not modulated by cAMP. ERK1/2 and PKB/Akt showed no activatory phosphorylation (Figure 7f). The STAT transcription factors showed unaltered phosphorylation both at sites associated with poor (P-STAT5\textsuperscript{34}, P-STAT3\textsuperscript{36}) and favorable (P-STAT1\textsuperscript{34}) treatment response in AML (Figure 7f). Furthermore, DNR or cAMP stimulation both failed to affect the phosphorylation
of the mTOR substrates p70 S6-kinase and 4E-BP1 (Supplementary Figure S6).

We considered also that clinical, cytogenetic or other parameters available for the APL/AML patients and their blasts might give clues to why only the AML3 blasts failed to be protected by cAMP. The AML3 patient has low-risk AML based on cytogenetics (inv8) and the clinical response (full response to induction therapy including DNR). The low-risk status alone does not explain the lack of cAMP response, as other blasts with low-risk cytogenetics Inv8 (AML1 and AML4) and t 8,37 (AML6) are sensitive to cAMP (Figure 2, Supplementary Table S1). Both NPM1, p53 and Flt3, are wild type in AML3, like for the cAMP-responsive APL3 and AML2 (Supplementary Table S1). Wild-type p53, found important for cAMP to protect lymphoblasts against death, 38 was expressed both in the cAMP-resistant AML3 blasts and Mv4-11 cells (see legend to Supplementary Table S1 for details). In conclusion, cAMP protection of DNR-exposed NB4 cells and APL and AML blasts was associated with inactivating phosphorylation of Bad and activating phosphorylation of CREB.

Discussion

As cAMP stimulates ATRA-induced APL blast maturation, 3–5 cAMP agonists have been considered as adjuncts to APL therapy. 6–8 In current APL therapy, an anthracycline is combined with ATRA to eradicate APL blasts resisting ATRA-induced maturation. 15,39 We show here that cAMP agonists accelerate APL progression in mice carrying the NB4 APL cell line and protect APL patient blasts and NB4 cells against anthracycline-induced death in vitro. Also the ATRA-subsensitive (NB4-LR1) and ATRA-insensitive (NB4-LR2) APL cells were protected by cAMP against DNR. The expected beneficial effect of cAMP on APL cell maturation may therefore be outweighed by boosted survival of ATRA-resistant APL blasts.
The cAMP protects not only against the multi-targeting anthracycline drugs, but also the glucose metabolism antagonist 2-deoxyglucose, hypoxia/rottenone and ER stress/misfolded protein inducers (the proteasome inhibitor bortezomib and the HSP90 inhibitor geldanamycin). This may explain why cAMP promoted APL progression also in mice not treated with DNR, and suggests that a natural increase of bone marrow cAMP, as may occur as a consequence of inflammation-induced PGE2 production, can affect APL cell survival also in the absence of specific treatment.
The protective effect of cAMP appeared to be mediated by PKA rather than Epac, based on the efficiency of cAMP analogs stimulating selectively either PKA or Epac. Although the two PKA isoforms (PKA-I and PKA-II) share the same catalytic subunit, they may serve distinct functions due to differential cAMP sensitivity and cellular localization.24,25 The strictly perinuclear PKA-II may have a localized role not directly related to NB4 cell survival, as PKA-II-directed cAMP analog combinations failed to protect against DNR, and cAMP was fully protective in PKA-II-depleted cells (Figures 5 and 6). In contrast, cAMP analog pairs synergizing for PKA-I activation also synergized for protection against DNR, and the protection afforded by a general PKA-activating cAMP analog was counteracted by the PKA-I-directed compound Rp-8-Br-cAMPS. That PKA-I protects against death is not unprecedented. A PKA isozyme switch from PKA-II to PKA-I is associated with both cellular hypertrophy and the malignant state (see Deeskleland et al.23 for review), and activation of PKA-I can protect mature neutrophils against death.19 The possibility that PKA-I modulates mTOR phosphorylation and thereby its activity14 appears unlikely as the mTOR substrates p70 S6-kinase and 4E-BP1 were unaffected by cAMP in the NB4 cells (Supplementary Figure S6).

A search for prosurvival effects of PKA activation in the cAMP-protected NB4 and APL blast points to increased phosphorylation of S118Bad and S133CREB, both of which are recognized PKA substrate sites.29,31 PKA can thereby turn off the Bcl-2/Bcl-Xl sequestering pro-apoptotic function of Bad45 and activate the AML oncogene and drug resistance factor CREB.33 In HL60 and Mv4-11 cells, where cAMP did not confer protection, the pro-apoptotic action of Bad appeared to be constitutively turned off, as the level of PS118 Bad was high and not modulated further by cAMP elevation. A number of other factors with potential to modulate cell death were studied, but none was regulated by cAMP or correlated with cAMP protection against DNR. Thus, cAMP-stimulated NB4 cells showed unaltered phosphorylation of ERK, STAT family members and mTOR kinase substrates.35,39 In contrast, phosphorylation and CREB activation 27,29 help explain the broad related molecules, like Flt-3, p53 wild type and NPM1 expression by the PRKA-RII-targeting sequence (hairpin loop in small letters) GAGAGCAGgaccaggaccagCTGCTCTGGGTGT ATTAGCTAACAGGCC-3′ inserted in the retroviral vector RRI-Green/L071 (provided by Dr. D. Micklem and Dr. J. Lorenz, University of Bergen, Norway) downstream of a modified human U6 promoter. The one mismatch (underlined) in the nonspecific strand served to improve the stability and efficacy of the hairpin. L071/shRNA/PKARIIB and L071/empty constructs were transfected into the Phoenix retroviral packaging cell line to produce viral supernatants harvested 2 days after transfection. Infection of NB4 and NB4/lKO/1/TRC cells was in the presence of 4 µg/ml of protamine sulfate. Infected cells were sorted 3 days after infection based on their GFP fluorescence.

Materials and Methods

Materials. The following substances were from Sigma-Aldrich (Inc., St Louis, MO, USA): DNR, IDA, cycloheximide, RCV, 2-deoxyglucose, geldanamycin, rotenone, ATRA, PGE2, the BH3 mimetic HA14-1, and the PDE inhibitors IBMX, rolipram and milrinone. Bortezomib was from Millenium Pharmaceuticals, Inc (Cambridge, MA, USA). The stabilized PGE2 analog dmPGE2 was from Cayman Chemical (Ann Arbor, MI, USA). The PDE inhibitor theophylline (i.e., its precursor aminophylline) was from Nycomed (Asker, Norway). The cAMP analogs N6-benzoyl-cAMP, (N5-Bz-cAMP), Rp-8-Br-cAMPS, 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (8-CPT-2′Me-cAMP), Sp-5,6-DCl-cBIMPS, N6-mono-tert-butylcarbamoyl-cAMP (N6-MBC-cAMP), 8-NH2CH2-cAMP, N6-MB-cAMP, 8-PIP-cAMP, 8-PIP-cAMP and N6-benzoyl-8-piperidino-cAMP (N6-Bz-8-PIP-cAMP) were from Biolog Life Science Institute (Bremen, Germany). The synthesis of the new cAMP analog 2C-8-hexylaminocarboxamido-2C-(2-C8-HE-CA) was performed as previously described for the synthesis of the related 2C-8-HE-CA.13

Origin of the cells used. NB4, NB4-LR1 and NB4-LR2 cells were isolated and grown as described.45,47 NB4 cells were grown in RPMI 1640 (Sigma-Aldrich) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), L-glutamine (2 mM), streptomycin (5 µg/ml) and penicillin (5 U/ml). Mv4-11 and HL60 cells were from ATCC. Mv4-11 cells were grown in Iscove’s modified Dulbecco’s media (Sigma-Aldrich Inc.) and HL60 in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco), L-glutamine (2 mM), streptomycin (5 µg/ml) and penicillin (5 U/ml). The collection of patient cells was approved by the Regional Research Ethics Committee (Health Region III, Bergen, Norway) and conducted in accordance with the Declaration of Helsinki Principles. For details on patients, see Supplementary Table S1. Samples were collected from patients shortly after the diagnosis of APL or AML, with informed consent, and stored in bio-banks approved by the Norwegian Ministry of Health and the Directorate for Health and Social Affairs. Primary peripheral blood mononuclear cells were isolated by density gradient separation (Ficoll-Hypaque; NyCoMed, Oslo, Norway); the percentage of leukemic cells after gradient separation exceeded 95% for all patients. Cells were stored frozen in liquid nitrogen.46 After thawing, the blasts were suspended in StemSpan medium (Stem Cell Technologies, Vancouver, BC, Canada) before incubation with agents to be tested for death induction.

Viral knockdown of PKA-RIs/II and PKA-RI/I. Knockdown of PKA-RI/II. NB4 cells were transduced with a lentiviral plasmid (pLKO.1/shRNA/PKAR2A, Sigma, CHSGLYC-TRCN0000033806) driving the expression of a short hairpin RNA targeting the sequence 5′-GCTGAGACCTA TAACCTCGAT-3′. The empty vector pLKO.1/TRC, given by D. E. Root (MIT, Cambridge, MA, USA)45 was used as control (NB4V0 cells). Lentiviral particle production, NB4 cell infection and selection of transduced NB4 cells were as described.45

Knockdown of PKA-RI/I. NB4sh and the NB4/lKO.1/shRNA/PKAR2A subclone with most robust PKA-RIs protein knockdown were silenced for PKA-RI/I expression by the PRKA-RII targeting sequence (hairpin loop in small letters) 5′-GGCTTAAATGTACAATACACCGAAGAGCGacctgctcggtgt ATTAGCTAACAGGCC-3′ inserted in the retroviral vector RRI-Green/L071 (provided by Dr. D. Micklem and Dr. J. Lorenz, University of Bergen, Norway) downstream of a modified human U6 promoter. The one mismatch (underlined) in the nonspecific strand served to improve the stability and efficacy of the hairpin. L071/shRNA/PKARIIB and L071/empty constructs were transfected into the Phoenix retroviral packaging cell line to produce viral supernatants harvested 2 days after transfection. Infection of NB4 and NB4/lKO/1/TRC cells was in the presence of 4 µg/ml of protamine sulfate. Infected cells were sorted 3 days after infection based on their GFP fluorescence.

The NB4 orthotropic xenograft model of APL. Male NOD-scid IL2rnull (NSG) breeding mice were a generous gift from Dr. L. D. Shultz (Jackson Laboratories, Bar Harbor, Maine, USA). The animals (7- to 8-week old) received 5 × 106 NB4 cells (Day 0) via the tail vein. On days 4, 5 and 6 post-transplantation, each animal received one intravenous (0.1 ml i.v.) and two intraperitoneal (0.2 ml i.p.) injections, one 5 min before and one 40 min after the i.v. injection. Group 1 (Ctrl, 5 animals) received phosphate-buffered saline (PBS) both i.v. and i.p. Group 2 (cAMP, 6 animals) received PBS i.v. and a mixture of dmPGE2 (to 125 µg/kg) and aminophylline (to 20 mg/kg) i.p. Group 3 (DNR, 6 animals) received DNR (diluted in physiological saline to give 2.5 mg/kg) i.v. and PBS i.p. Group 4 (DNR + cAMP, 6 animals) received both DNR and the dmPGE2/aminophylline mixture. The health status and weight of the mice was monitored and moribund animals were humanely euthanized. The animal experiments were approved by The Norwegian Animal Research Authority and performed in accordance with The Cell Death and Disease
European Convention for the Protection of Vertebrates Used for Scientific Purposes. The survival data are presented according to Kaplan and Meier and survival distributions analyzed by the Mantel-Cox log rank statistics, using PASW Statistics (version 18.0).

Assessment of cell death and determination of cAMP content. Cells (at 0.5 × 10^6/ml) were exposed to various cell death-modulating agents. Apoptosis of cell lines was assessed mainly by microscopy to visualize surface budding and nuclear chromatin condensation. Upon prolonged incubation with apoptogens, some cells underwent secondary necrosis. Although swollen, such cells retained enough of the apoptotic phenotype to be recognized as ‘dead’. In most cases, the % of apoptotic + (secondarily) necrotic cells is given as % dead cells. NB4 cell lines were also studied by flow cytometry on a FACS Accuri C6 (BD Biosciences, San Jose, CA, USA, Accuri CytoFyter), after staining with Alexa Fluor 647-Annexin V (Molecular Probes, Grand Island, NY, USA) and propidium iodide (PI), and the forward and side scatter determined. At least 50,000 non-gated live cell events were collected for each sample. The determination of fluorescence from fluorescence-labeled Annexin V (binds to phosphatidyl serine) is a challenge for cells treated with DNR, which has a strong fluorescence when excited by the 488-nm blue argon laser. We eliminated the problem by using Annexin V coupled to the far-red Alexa Fluor 647 dye, which was excited with a 635-nm red diode and sampled in the FL-4 channel. PI gives a stronger signal than DNR when excited at 488 nm, particularly when sampled after a long-pass 670-nm filter (FL-3). The DNR content was determined after excitation at 488 nm and sampling in the FL-1 channel.

For primary human blasts at least 30,000 non-gated events were collected for each sample. For the two first samples studied (APL,1,2) staining was with PI, and % PI-positive cells taken as % death. For all other samples cell death was determined based on the sum of Annexin V + PI-positive cells. It should be noted that the primary blasts were more prone to (secondary) necrotic death than the cell lines and only minor differences were observed using PI-positive and Annexin V – PI-positive cells.

For study under hypoxic conditions, the medium was pre-equilibrated for 24 h in 2% O2, 5% CO2 atmosphere. For statistical analysis, Wilcoxon Paired Comparison test and Student’s T-Test were used.

The ATRA-induced differentiation of NB4 cells was evaluated by the nitroblue tetrazolium reduction assay as described.\(^7\)

Determination of cAMP: The cell medium was rapidly removed and cAMP extracted in 5% trichloroacetic acid with 0.1 M HCl. The trichloroacetic acid was extracted with acetone and the extract neutralized in phosphate-HEPES buffer before determination of cAMP by a competitive binding assay based on displacement of [H]cAMP from the RI subunit of PKA.\(^7\)

Immunoblotting and immunocytofluorescence. Immunoblotting was as described.\(^7\) Proteins were detected with antibodies against, P-Ser155Bad (murine, corresponds to human P-Ser115), P- Thr180-PKB (Santa Cruz, Biotechnology, Dallas, TX, USA), Bad, Bcl-2, PKA-RiI (BD Biosciences), P-Ser116Bad (murine, corresponds to human P-Ser75; Cell Signaling, Danvers, MA, USA), p23 (mouse monoclonal J33, generously supplied by Dr. D. O. Toft, Mayo Clinic, Rochester, MN, USA) or β-actin (Amerham Biosciences, Piscataway, NJ, USA). For immunofluorescence, cells were collected by centrifugation, fixed in 2% PFA in PBS (10 min), permeabilized in 0.1% Triton X-100 in PBS (10 min) and blocked (2% BSA in PBS, 1 h). Incubation with anti-PKA-RI and anti-PKA-RiI (in PBS-BSA) was overnight. After washing in PBS, cells were incubated 1 h with secondary antibody Alexa 488-lgG or Alexa 546-lgG (Invitrogen), washed and mounted on slides with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). The cell fluorescence was analyzed on a Leica DMRF microscope.

Intracellular phospho-epitope staining for flow cytometry. Phos- pho-flow cytometry analysis was performed as previously described.\(^{36}\) Alexa 487-conjugated anti-phospho-CREB (pS133), anti-phospho-STAT1 (pY701), anti-phospho-STAT5 (pY694), anti-phospho-STAT3 (pS277), anti-phospho-Erk1/2 (pT202/pY204) and anti-phospho-Akt (pS473) were from BD Biosciences. At least 50,000 non-gated live cell events were collected for each sample on a FACSS Accuri C6 (BD Biosciences, Franklin Lakes, NJ). Normalization of flow cytometry data was performed by comparing median fluorescence intensity values to an unstimulated control using the log10 ratio of a sample divided by the control.

Conflict of Interest
The authors declare no conflict of interest.

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Author contributions
GG, AW, and SOD designed the research, performed most experiments, analyzed data and wrote the paper. EN, FP, ML and ES-B provided the NB4-LR1 and -LR2 sub-clones and generated the NB4-RIIKD cell line, characterized their content and distribution of PKA subunits, and contributed to data interpretation and writing. UH and FS contributed to syntheses of cAMP analogs. JS, BTG and OB performed phospho-flow analysis, provided and characterized patient blasts, and helped presenting data. NR and EM designed and performed animal experiments. RK and LH contributed to data analysis.

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this scale an increment of +1 corresponds to a 10-fold increase in median fluorescence intensity.

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