Biochimica et Biophysica Acta 1842 (2014) 2656–2663

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Inhibition of soluble adenylyl cyclase increases the radiosensitivity of prostate cancer cells ☆

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ARTICLE INFO

Article history:
Received 8 May 2014
Received in revised form 15 September 2014
Accepted 16 September 2014
Available online 23 September 2014

Keywords:
Prostate cancer
Radioresistance
cAMP
EPAC
ERK1/2

ABSTRACT

Pharmacological modulation of tumor radiosensitivity is a promising strategy for enhancing the outcome of radiotherapy. cAMP signaling plays an essential role in modulating the proliferation and apoptosis of different cell types, including cancer cells. Until now, the regulation of this pathway was restricted to the transmembrane class of adenylyl cyclases. In the present study, the role of an alternative source of cAMP, the intracellular localized soluble adenylyl cyclase (sAC), in the radiosensitivity of prostate cancer cells was investigated. Pharmacological inhibition of sAC activity led to marked suppression of proliferation, lactate dehydrogenase release, and induction of apoptosis. The combination of ionizing radiation with partial suppression of sAC activity (~50%) immediately after irradiation synergistically inhibited proliferation and induced apoptosis. Overexpression of sAC in normal prostate epithelial PNT2 cells increased the cAMP content and accelerated cell proliferation under control conditions. The effects of radiation were significantly reduced in transformed PNT2 cells compared with control cells. Analysis of the underlying cellular mechanisms of sAC-induced radioresistance revealed the sAC-dependent activation of B-Raf/ERK1/2 signaling. In agreement with this finding, inhibition of ERK1/2 in prostate cancer cells enhanced the cytotoxic effect of irradiation. In conclusion, the present study suggests that sAC-dependent signaling plays an important role in the radioresistance of prostate cancer cells. This article is part of a Special Issue entitled: The role of soluble adenylyl cyclase in health and disease.

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1. Introduction

Prostate cancer remains the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in men [1]. The current standard of care consists of prostatectomy and radiation therapy, which may often be supplemented with hormonal therapies. However, nearly 30% of patients experienced relapse at the irradiation sites [2]. The resistance of prostate cancer to radiotherapy has prompted a search for new methods of treatment, such as a combination of radiotherapy with the targeting of certain molecular mechanisms responsible for the radioresistance of prostate cancer.

In particular, many anti-tumor strategies focus on suppressing the cell cycle or inducing apoptosis. Within several signaling pathways, cAMP-dependent signaling plays a substantial role in controlling cell proliferation and apoptosis. However, the specific effects of cAMP signaling on proliferation and apoptosis are controversial. For example, the elevation of cellular cAMP content by stimulating G protein-responsive transmembrane adenylyl cyclases (tmAC) or by treatment with cAMP analogs has been shown to either induce or suppress proliferation in different cell types [3–6]. Similarly, cAMP signaling has conflicting effects on apoptosis [7–10]. This discrepancy may be due to differences in cell types or experimental models. Alternatively, the lack of specificity of methods to affect the cAMP signaling, e.g. forskolin, cAMP analogs or PKA inhibitors, used in majority of studies, may also be a cause for this discrepancy. Indeed, overproduction of cAMP by tmAC due to treatment with forskolin unlikely will lead to selective activation of plasmalemma-localized targets.

Traditionally, cAMP signaling was thought to rely solely on the production of cAMP at the plasma membrane by tmAC. However, work by many groups, including our own, has changed this view. tmAC can continue to signal within the cell following internalization, thereby defining endocytic cAMP microdomains [11,12]. Furthermore, a second source of cAMP, type 10 soluble adenylyl cyclase (sAC), has been identified in mammalian cells [13]. sAC, unlike tmAC, possesses no transmembrane domains and is localized throughout the cell in the cytosol, nucleus, mitochondria, and centriole [14]. Several studies demonstrated the unique activation of sAC by bicarbonate and bivalent

☆ This article is part of a Special Issue entitled: The role of soluble adenylyl cyclase in health and disease.

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http://dx.doi.org/10.1016/j.bbadis.2014.09.008
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cations as well as the role of sAC in the function of various cells and organs, such as sperm and neutrophils as well as the brain, kidney, eye, and pancreas [15]. Furthermore, recent studies have demonstrated that sAC regulates oxidative phosphorylation in mitochondria [16] and activates the mitochondrial pathway of apoptosis [17].

The role of the sAC-dependent cAMP pool in proliferation control was unknown. Our recent study demonstrates that sAC promotes prostate cancer cell proliferation in an EPAC-dependent manner [18]. Other studies have also suggested that nuclear sAC expression is associated with the transition of keratinocytes and melanocytes from benign cells into cancers, such as squamous cell carcinoma of the skin and melanoma [19,20]. A recent report by Onodera et al. [21] also demonstrated the involvement of sAC/EPAC signaling in the proliferation of breast cancer cells. Altogether, these observations suggest that sAC plays a role in proliferation of some cancer cells and may contribute to the tumor malignancy. We therefore hypothesized that the inhibition of sAC could be effective for potentiation of the radiation-induced cytotoxicity. To test this hypothesis, we utilized human prostate carcinoma cells and investigated the anti-proliferative, cytotoxic and pro-apoptotic effects of a combined treatment including sAC inhibition and irradiation.

2. Material and methods

2.1. Cell culture

Androgen-sensitive LNCaP (ATCC-Nr. CRL-1740D) and androgen-insensitive PC3 (ATCC-Nr. CRL-1435D) human prostate carcinoma cell lines were purchased from the American Type Culture Collection, and the normal human prostate epithelial cell line PNT2 was purchased from Sigma-Aldrich (Cat. Nr. 95012613). Cells were expanded and frozen in aliquots within 4 weeks of purchase. For the experiments in this study, the cells were thawed and cultured for no more than 3 additional passages. The LNCaP and PC3 cells were cultured in Dulbecco's modified Eagle's medium that was supplemented with 5% fetal calf serum, 100 U/mL penicillin, and 100 μg/μL streptomycin. PNT2 cells were cultured in RPMI-1640 medium that was supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/μL streptomycin. The cells were seeded in culture medium containing 2% fetal calf serum (LNCaP and PC3) or 5% fetal calf serum (PNT2) 12–18 h prior to each experiment.

2.2. Experimental protocols

U0126 (Cell Signaling), KH7 (Cayman) or its inactive analog, KH7.15 (kindly provided by Dr. J. Buck, Cornell University, NY), was applied to the cells throughout the duration of the experiment as indicated. At the end of the experiments, the floating cells were collected and combined with the attached cells for further analyses.

2.3. Radiation treatment

The cells were irradiated with 250 keV X-rays (16 mA) produced by a Müller RT250 (Philips Industrial X-Ray) at a dose rate of 1.5 Gy/min. The cells were returned to an incubator after the irradiation procedure and maintained at 37 °C and 5% CO_2 until further use.

2.4. sAC overexpression

The cloning of the untargeted expression of sAC, i.e., predominantly in the cytosol, was performed as previously described [22]. Briefly, the human influenza hemagglutinin (HA)-tagged rat sAC gene constructs were generated by PCR, digested with AgeI and NotI, and cloned into the eukaryotic expression vector pTurbo-G418 (Evrogen-Axxora). The plasmids encoding sAC or control vector (donated by Dr. Giovanni Manfredi, Weill Cornell Medical College, NY) were transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) and then incubated for 24 h. sAC expression in the transfected cells was analyzed using western blotting and antibodies against sAC.

2.5. Western blotting

The cells were lysed in Laemmli buffer containing 2% SDS, 10% glycerol, 0.1% 2-mercaptoethanol, 0.002% bromophenol blue, and 0.0625 mol/L Tris–HCl. The protein concentrations were determined using the Pierce 660 nm Protein Assay Reagent combined with the Ionic Detergent Compatibility Reagent (Thermo Scientific). Equal amounts of total proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The utilized primary antibodies were as follows: sAC (clone R21, kindly provided by Dr. J. Buck, Cornell University, NY), actin (Chemicon International), cleaved caspase-3, phos-B-Raf, B-Raf, phos-ERK, and ERK1/2 (Cell Signaling). After incubation with peroxidase-linked and horseradish peroxidase-labeled secondary antibodies, specific bands were visualized by chemiluminescence using the ECLPlus Kit (Amersham Pharmacia). Equivalent sample loading (10–30 μg/well) was confirmed by stripping the membranes with Restore Western Blot Stripping Buffer (Pierce) prior to incubating the membranes with antibodies against actin.

2.6. Rap1 activity assay

The level of activated Rap1 was determined using a pull-down assay kit (Jena Bioscience, Jena, Germany), which is based on glutathione S-transferase fusion to the Ras-binding domain of RalGDS, following the manufacturer's instructions. The amounts of GTP-bound Rap1 and total Rap1 were determined by western blotting followed by staining with an anti-Rap1A antibody.

2.7. Analysis of cell number

After staining with 4% trypan blue (Gibco), trypan blue-negative cells were counted using a Neubauer hemocytometer (depth: 0.1 mm).

2.8. Cellular cAMP analysis

Analysis of the total cellular cAMP content was performed using the cAMP (direct) enzyme immunoassay kit (Assay Designs). The preparation of cell extracts and cAMP measurements were performed following the manufacturer's protocol. The absorbance measured at 405 nm was used to calculate the concentration of cAMP by applying a calibration curve.

2.9. FACS-based subG1-population analysis

The cells were fixed with 70% alcohol, stained with propidium iodide and treated with RNase (BD Biosciences, Heidelberg, Germany), which was followed by DNA content analysis using a FACSCalibur flow cytometer (BD Biosciences).

2.10. Analysis of lactate dehydrogenase in the culture medium

Lactate dehydrogenase (LDH) activity in the cell culture medium was used as an indicator of necrosis and was determined using the Cytotoxicity Detection Kit (Roche Applied Science). After each experiment, the culture medium was centrifuged at 500 ×*g* for 5 min at 4 °C, and the supernatant was subjected to LDH analysis using an ELISA reader.

2.11. Statistical analysis

The data are presented as the mean ± SEM. Comparisons of the means of the groups were performed using one-way analysis of variance followed by the post hoc Bonferroni test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of irradiation on proliferation and cell death

To examine the resistance to irradiation, two human prostate carcinoma cell lines were initially utilized: LNCaP and PC3. First, the dose- and time-dependent effects of irradiation on proliferation and cell death were investigated. We found that irradiation of cells with a total dose of 10 Gy (1.5 Gy/min) led to suppression of proliferation within 24 h accompanied by a significant increase in the subG1 population (apoptosis), cleavage of caspase-3, and LDH release (necrosis) after 48 and 60 h (Supplementary Fig. 1). The effects observed at 48 h after irradiation were dose-dependent; i.e., a gradual suppression of proliferation and increase in LDH release and apoptosis were noted as the irradiation dose increased from 5 to 15 Gy (Supplementary Fig. 2). A comparison of the irradiation effects between LNCaP and PC3 cells revealed the higher radioresistance of LNCaP cells, especially in respect to irradiation-induced cytotoxicity and apoptosis. Therefore, this cell line was used for further analyses. Examination of cellular cAMP content in LNCaP cells did not reveal any significant alteration after irradiation (mean ± SEM, n = 5, pmol/mg proteins): 5.81 ± 0.41 at 0 Gy, 5.23 ± 0.33 at 5 Gy, 7.02 ± 0.31 at 10 Gy and 6.02 ± 0.43 at 15 Gy.

3.2. Effect of sAC inhibition

To examine the role of sAC in proliferation and cell death, LNCaP cells were treated for 48 h with the specific sAC inhibitor KH7 at doses of 10, 20, and 30 μmol/L. Previous studies demonstrated that KH7 inhibits sAC in various cell types over a concentration range of 10–30 μmol/L, whereas it has no effect on tmAC and soluble guanylyl cyclase at concentrations up to 100 μmol/L [23]. We found that KH7 dose-dependently suppressed the cellular cAMP content with a maximal effect at 20 μmol/L (Fig. 1A). KH7 treatment had a similar dose-dependent effect on cell proliferation and death (Fig. 1B–D). To further rule out possible nonspecific, sAC-independent effects of KH7, an inactive analog of KH7, KH7.15 [24], was utilized in these experiments. In contrast to KH7, KH7.15 did not affect the cellular cAMP content, proliferation, or cell death (Fig. 1).

3.3. Effect of irradiation in combination with sAC inhibition

To test whether inhibition of sAC may support the cytotoxic and anti-proliferative effects of irradiation, treatment with the sAC inhibitor KH7 was applied immediately after irradiation. In these experiments, KH7 was applied at 10 μmol/L, which causes only partial suppression

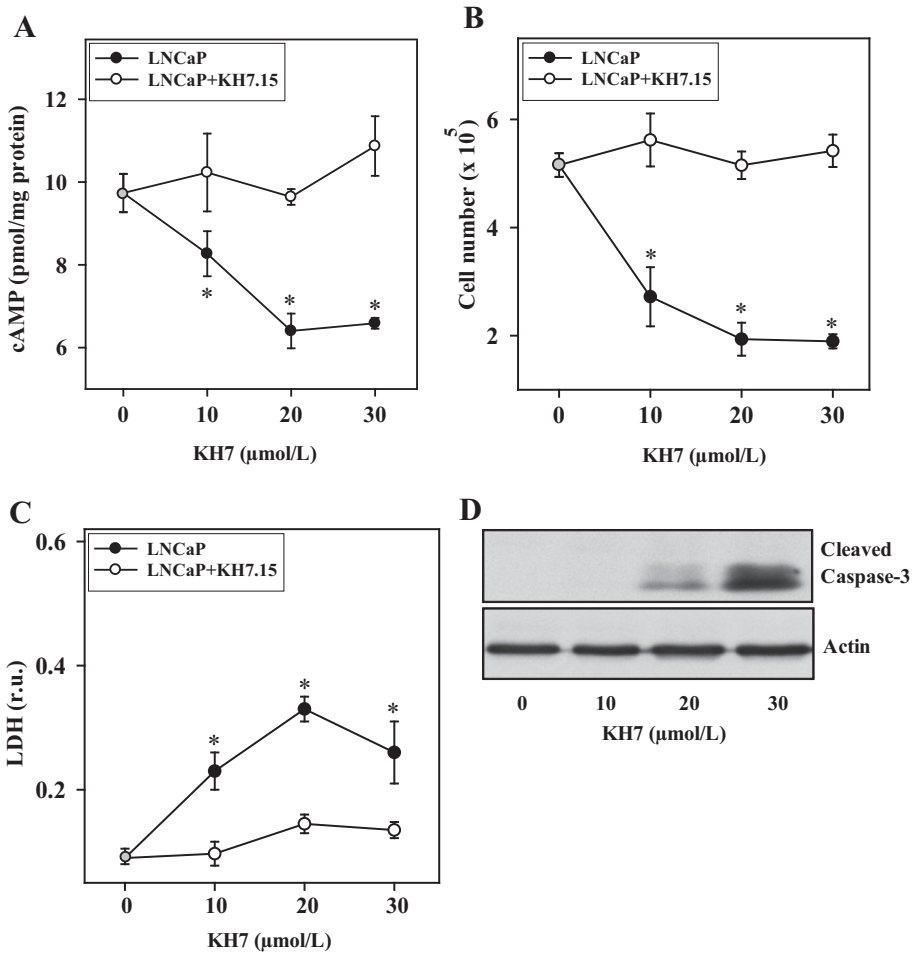


Fig. 1. Inhibition of sAC suppresses the proliferation and leads to the death of prostate cancer cells. (A–C) Statistical analyses of the cellular cAMP content (24 h after treatment), the cell number per dish, and lactate dehydrogenase (LDH) activity in the cell culture medium (presented as a ratio of the individual protein content, relative units) performed with LNCaP cells treated with the sAC inhibitor KH7 (0–30 μmol/L, black symbols) or its inactive analog KH7.15 (0–30 μmol/L, white symbols) for 48 h. Note that 150,000 cells per dish were plated at the beginning of all experiments. The values are presented as the mean ± SEM (n = 3–12). *P < 0.05 vs. 0 μmol/L. (D) Western blot analysis of cleaved caspase-3 was performed using LNCaP cell lysates. The treatment conditions are similar to those described for (A). The data are representative of four independent experiments with similar results.

of sAC activity (i.e., approximately 50% of the maximal effect) (Fig. 1A). Furthermore, a submaximal dose of irradiation, 5 Gy, was applied. We found that irradiation at a dose of 5 Gy led to suppression of proliferation and promoted a level of cytotoxicity that was comparable with that of partial sAC inhibition via treatment with 10 μmol/L KH7 (Fig. 2). Combining irradiation at 5 Gy with sAC inhibition significantly increased the anti-proliferative and cytotoxic effects of irradiation, which were significantly stronger than that of irradiation at 10 Gy (Fig. 2A, B). Similarly, the inhibition of sAC significantly enhanced the pro-apoptotic effect of irradiation (Fig. 2C). In contrast to KH7, treatment with inactive analog, i.e., KH7.15, had no effects on proliferation, LDH release and caspase-3 cleavage (Fig. 2).

3.4. Overexpression of sAC increases radioresistance

To further substantiate the role of sAC in radioresistance the overexpression of sAC was performed in PNT2 cells. This LNCaP-relevant, non-tumor prostate epithelial cell line showed markedly low expression of sAC and a low proliferation rate compared to LNCaP cells, although this difference in proliferation may not be necessary due to differences of sAC expression (Fig. 3A). In line with these data, overexpression of sAC significantly increased cAMP content and the proliferation rate (Fig. 3B, C). By applying irradiation at a dose of 10 Gy, we found that the sAC overexpression significantly increased the radioresistance of PNT2 cells, i.e., a higher cell number and lower LDH release were found 48 h after irradiation in sAC-overexpressed cells compared with the control (Fig. 3C, D). In fact, irradiation of cells transfected with sAC vector reduced the cell number to 76 ± 7%, whereas in cells transfected with control vector the cell number was reduced to 55 ± 5% ($P < 0.05$). Furthermore, sAC expression suppressed irradiation-induced caspase-3 cleavage (Fig. 3E).

To additionally prove the role of sAC in radioresistance in sAC overexpressing PNT2 cells, inhibition of sAC in these cells with KH7 has been applied. The sAC inhibition abolished the effect of sAC overexpression (Fig. 3C, D, E).

3.5. Importance of sAC/B-Raf/ERK signaling in radioresistance

Because the EPAC/Rap1 pathway was found to be responsible for the control of sAC-dependent proliferation [18], we examined whether the anti-proliferative effect of sAC inhibition in the present study was due to downregulation of MAP kinase signaling, which is downstream of the EPAC/Rap-1 pathway. An analysis of B-Raf and ERK1/2 demonstrated that the phosphorylated forms of these proteins were downregulated after 12 h of sAC inhibition and were associated with the downregulation of GTP-bound Rap1 (Fig. 4A). In contrast, treatment with an inactive analog of KH7, i.e., with KH7.15 had no effects on ERK1/2 phosphorylation (Supplementary Fig. 3). Because the ERK1/2 signaling supports radioresistance of various tumors [25,26], we hypothesized that this signaling may be a primary downstream pathway responsible for the sAC-dependent radioresistance of LNCaP cells. To prove this hypothesis, we examined the effects of ERK1/2 on the irradiation effects in LNCaP cells. Treatment with U0126, a highly selective inhibitor of upstream MEK1/2, suppressed ERK1/2 phosphorylation/activation in control as well as irradiated cells (Fig. 4B). In support of our hypothesis, inhibition of ERK1/2 significantly enhanced the anti-proliferative and cytotoxic effects of irradiation (Fig. 4C).

4. Discussion

The purpose of the present study was to determine whether sAC plays a role in the resistance of prostate carcinoma cells to irradiation. The main findings are as follows: (i) Inhibition of sAC enhanced the radiosensitivity of LNCaP tumor cells. In contrast, the overexpression of sAC in non-tumor PNT2 cells promotes radioresistance. (ii) ERK1/2

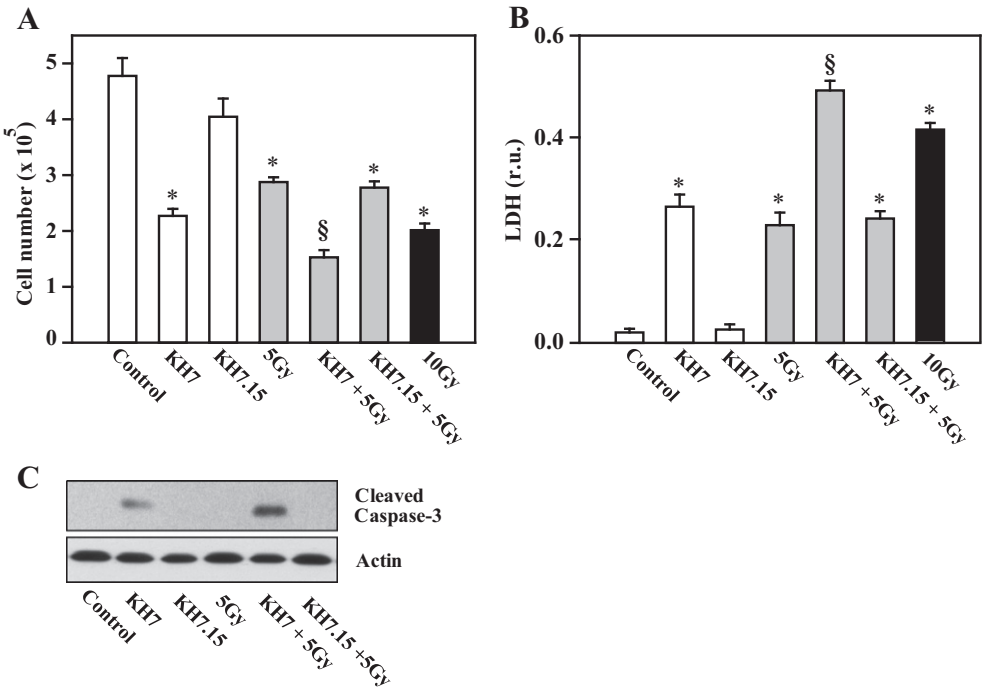


Fig. 2. Inhibition of sAC increases the radiosensitivity of prostate cancer cells. (A, B) Statistical analyses of the number of cells per dish and lactate dehydrogenase (LDH) activity in the cell culture medium (presented as a ratio of the individual protein content, relative units) performed with control LNCaP cells and cells treated with gamma radiation (5 or 10 Gy) followed by incubation of cells without or with the sAC inhibitor KH7 (10 μmol/L) or its inactive analog KH7.15 (10 μmol/L) for 48 h. The values are presented as the mean ± SEM (n = 4–7). *$P < 0.05$ vs. Control. §$P < 0.05$ vs. KH7, 5 Gy and 10 Gy. (C) Western blot analysis of cleaved caspase-3 was performed using LNCaP cell lysates. The treatment conditions were similar to those described for (A) and (B). The data are representative of three independent experiments with similar results.

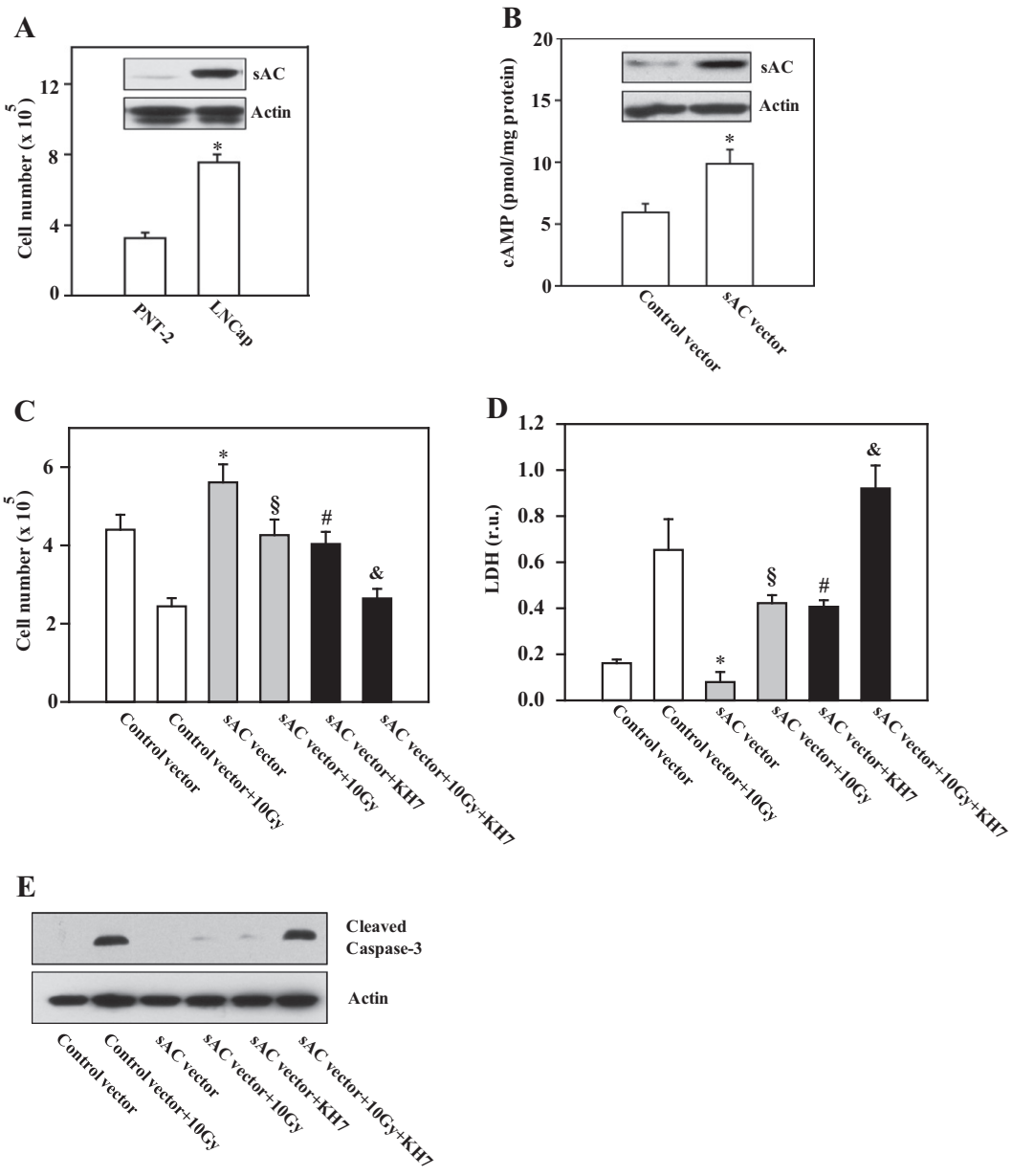


Fig. 3. sAC expression increases the radioresistance of PNT2 cells. (A) Western blot analysis of sAC and statistical analysis of the number of cells per dish performed with non-tumor prostate epithelial PNT2 cells or prostate cancer LNCaP cells cultured for 48 h. Note that 200,000 cells per dish were plated at the beginning of all experiments. The values are presented as the mean ± SEM (n = 4). *P < 0.05 vs. PNT2. (B) Western blot analysis of sAC and statistical analysis of the cellular cAMP content in PNT2 cells transfected with a control or sAC vector. The values are presented as the mean ± SEM (n = 4–6). *P < 0.05 vs. Control vector. (C, D) Statistical analyses of the cell number per dish and lactate dehydrogenase (LDH) activity in the cell culture medium (presented as a ratio of the individual protein content, relative units) performed with PNT2 cells transfected with a control or sAC vector. Cells were treated with irradiation (10 Gy) as indicated followed by culturing for 48 h without or with 20 μmol/L KH7. The values are presented as the mean ± SEM (n = 4–8). *P < 0.05 vs. Control vector. §P < 0.05 vs. Control vector + 10 Gy. #P < 0.05 vs. sAC vector. &P < 0.05 vs. sAC vector + 10 Gy. (E) Western blot analysis of cleaved caspase-3 was performed with PNT2 cell lysates. The treatment conditions are similar to those described for (C) and (D). All western blot data are representative of three to four independent experiments with similar results.

is an essential downstream target contributing to the sAC-dependent radioresistance.

cAMP signaling regulates diverse cell functions, including cell death and growth. However, traditionally, cAMP synthesis was solely attributed to the G protein-coupled tmAC; therefore, it was restricted to the plasmalemma. Our reports demonstrated that an alternative source of cAMP, intracellular sAC, plays a key role in apoptosis induced by ischemia, acidosis, or oxysterols in several cell types [17,27,28]. Furthermore, recent studies also emphasized the role of sAC in promoting the proliferation of prostate carcinoma and breast cancer cells [18,21]. These data suggest that sAC may be a promising target for improving radiotherapy outcomes.

Utilizing LNCaP cells, we examined the contribution of sAC in radioresistance by applying pharmacological inhibition of sAC immediately after irradiation. Because sAC activity plays a role in several physiological cell functions [15], complete suppression of sAC may be detrimental to non-tumor cells. Thus, in these experiments, we partially suppressed sAC (by approximately 50%). Under these conditions, the anti-proliferative, cytotoxic, and pro-apoptotic effects of irradiation at 5 Gy were significantly increased. Importantly, these effects were stronger than those observed with irradiation at the higher dose (10 Gy).

To further emphasize the contribution of sAC in radioresistance, an opposite approach, i.e., sAC overexpression, was applied in non-tumor PNT2 cells. Interestingly, these untreated cells showed a significantly

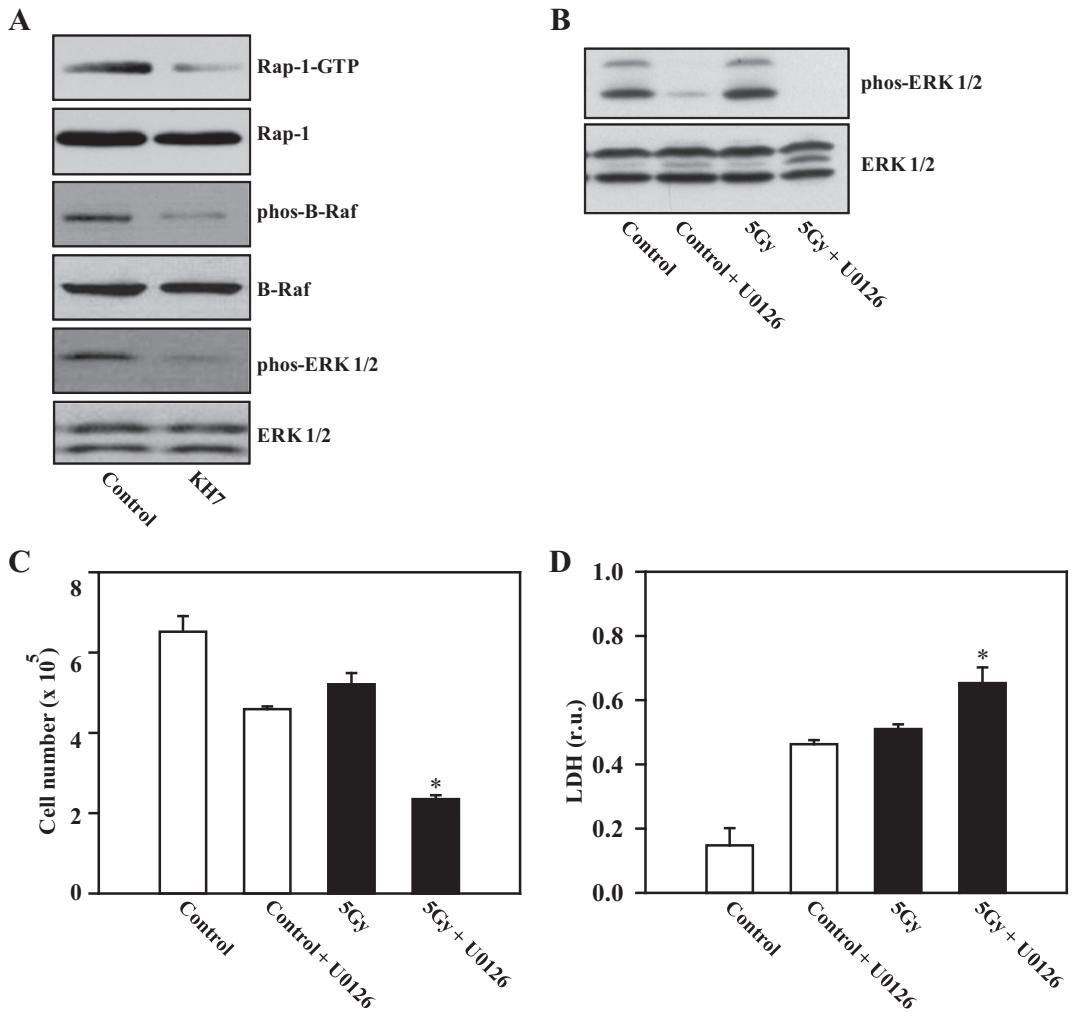


Fig. 4. ERK1/2 is a key downstream target responsible for the sAC-dependent radioresistance of LNCaP cells. (A) Western blot analysis of the active form of Rap-1 (Rap-1-GTP) and the phosphorylated forms of B-Raf and ERK1/2 performed with lysates of control LNCaP cells or cells treated for 12 h with 20 μmol/L KH7. Western blot data are representative of three to four independent experiments with similar results. (B) Western blot analysis of the phosphorylated form of ERK1/2 performed with lysates of control LNCaP cells or cells treated with irradiation (5 Gy) followed by treatment for 48 h with 10 μmol/L MEK1/2 inhibitor (U0126) as indicated. Western blot data are representative of three to four independent experiments with similar results. (C) Statistical analyses of the cell number per dish and lactate dehydrogenase (LDH) activity in the cell culture medium (presented as a ratio of the individual protein content, relative units) performed with LNCaP cells. The treatment conditions are similar to those described for (B).

slower proliferation rate compared to LNCaP tumor cells. However, when targeted expression of sAC was applied, a significant rise in the proliferation rate could be observed that was comparable with that of LNCaP cells. Furthermore, these transformed cells showed a significantly higher radioresistance compared to the scrambled control. Therefore, both approaches, which manipulate sAC signaling, argue for a key role of sAC in radioresistance.

The mechanisms responsible for the anti-proliferative, cytotoxic, and pro-apoptotic effects of sAC inhibition appear to be different from those induced by ionizing radiation. Indeed, radiation suppresses cell growth due to DNA damage by indirect (formation of oxygen radicals) or direct (double-strand breaks followed by the DNA damage response and cell cycle arrest) actions [29,30]. In a previous study, we found that the inhibition of sAC suppresses proliferation due to downregulation of the EPAC/Rap-1 pathway [18]. In contrast to non-tumor PNT2 cells, sAC is overexpressed in tumor LNCaP cells (Fig. 3A); similarly, sAC is overexpressed in human prostate carcinoma compared with benign hyperplasia [18]. Such overexpression upregulates EPAC/Rap-1 signaling and may lead to overactivation of the downstream MAP kinase pathway. Indeed, the present study revealed that suppression of sAC reduced the phosphorylation of B-Raf and ERK1/2 kinases, which are downstream of the EPAC/Rap-1 axis. The activity of MAP kinases, particularly of the ERK1/2 kinases, has been shown to be responsible for the radioresistance of various cancer cells [25,26]. In agreement with these studies, suppression of ERK1/2 phosphorylation in our study significantly enhanced the radiosensitivity of prostate cancer cells. Therefore, ERK1/2 seems to be a key downstream target responsible for sAC-dependent radioresistance. Although the role of EPAC in proliferation and oncogenesis has been demonstrated in various models [3–5,21], the possible contribution of EPAC signaling in the radioresistance of tumor cells remains weakly understood. One recent study [31] suggests the inhibitory role of EPAC in the repair of irradiation-induced DNA damage. However, only the contribution of the membrane-bound adenylyl cyclase (forskolin treatment) was examined in this study.

In contrast to EPAC, numerous reports have emphasized the role of PKA, the second major target for cAMP, in the radioresistance of cancer cells. Indeed, PKA has been shown to support the radioresistance of prostate cancer cells [32]. Moreover, PKA overexpression is correlated with prostate cancer patient outcomes after radiotherapy [33]. In contrast to these reports, PKA has also been shown to promote apoptosis due to phosphorylation of the prostate apoptosis response-4 protein [34]. Similarly, activation of PKA by treatment with a specific agonist enhances the radiosensitivity of prostate cancer cells [35]. Although the

role of PKA in the radiosensitivity of prostate cancer cells is still controversial, PKA significantly contributes in radiation outcome. Nevertheless, PKA is unlikely to play a role in sAC-dependent radioresistance in our study. Indeed, our previous reports demonstrated that the sAC/PKA axis promotes apoptosis rather than proliferation [27,28]. Additionally, the direct inhibition or activation of PKA in LNCaP cells has no effect on proliferation [18].

In conclusion, the present study describes a novel signaling that contributes to the radioresistance of prostate cancer cells. Suppression of the sAC-specific cAMP pool, leading to inhibition of B-Raf/ERK signaling, significantly enhances the anti-proliferative and cytotoxic effects of irradiation. Further analysis of the sAC-dependent pathways in other radioresistant tumors may have a potential clinical significance. Particularly, one recent study [19] supposes the role of sAC in squamous cell carcinoma, which belongs to radioresistant tumors.

Funding

This work was supported by Grant LA 1159/7-1 of the Deutsche Forschungsgemeinschaft and by Forum Grants F690-2010 and F719-2011 from the Ruhr-University Bochum.

Abbreviations

EPAC	exchange protein activated by cAMP
ERK	extracellular signal-regulated kinase 1/2
LDH	lactate dehydrogenase
MAP	mitogen-activated protein kinase
PKA	protein kinase A
sAC	soluble adenylyl cyclase
tmAC	transmembrane adenylyl cyclases

Acknowledgements

We thank Dr. Lonny Levin and Dr. Jochen Buck (Weill Medical College of Cornell University, New York) for providing the R21 antibody and KH7.15. We also thank the RUBION Team at the Ruhr-University Bochum for technical assistance with the irradiation of cells. The technical assistance of G. Scheibel and K. Rezny is also gratefully acknowledged.

This work was a part of the master's thesis of Hanna Flacke that was submitted in fulfillment of the requirements for the degree of a Master's in Biology at the Ruhr-University Bochum (Germany).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.09.008.

References

[1] W. Fu, E. Madan, M. Yee, H. Zhang, Progress of molecular targeted therapies for prostate cancers, Biochim. Biophys. Acta 1825 (2012) 140–152.
[2] J. Lovey, D. Nie, J. Tovari, I. Kenessey, J. Timar, M. Kandouz, K.V. Honn, Radiosensitivity of human prostate cancer cells can be modulated by inhibition of 12-lipoxygenase, Cancer Lett. 335 (2013) 495–501.
[3] D. Hochbaum, K. Hong, G. Barila, F. Ribeiro-Neto, D.L. Altschuler, Epac, in synergy with cAMP-dependent protein kinase (PKA), is required for cAMP-mediated mitogenesis, J. Biol. Chem. 283 (2008) 4464–4468.
[4] U.K. Misra, S.V. Pizzo, Epac1-induced cellular proliferation in prostate cancer cells is mediated by B-Raf/ERK and mTOR signaling cascades, J. Cell. Biochem. 108 (2009) 998–1011.
[5] R.C. Hewer, G.B. Sala-Newby, Y.J. Wu, A.C. Newby, M. Bond, PKA and Epac synergistically inhibit smooth muscle cell proliferation, J. Mol. Cell. Cardiol. 50 (2011) 87–98.
[6] S. Lucchi, D. Calebiro, T. de Filippis, E.S. Grassi, M.O. Borghi, L. Persani, 8-Chloro-cyclic AMP and protein kinase A I-selective cyclic AMP analogs inhibit cancer cell growth through different mechanisms, PLoS One 6 (2011) e20785.
[7] V. Leone, A. di Palma, P. Ricchi, F. Acquaviva, M. Giannouli, A.M. Di Prisco, F. Iuliano, A.M. Acquaviva, PGE2 inhibits apoptosis in human adenocarcinoma Caco-2 cell line through Ras–PI3K association and cAMP-dependent kinase A activation, Am. J. Physiol. Gastrointest. Liver Physiol. 293 (2007) G673–G681.
[8] J.A. Rudolph, J.L. Poccia, M.B. Cohen, Cyclic AMP activation of the extracellular signal-regulated kinases 1 and 2: implications for intestinal cell survival through the transient inhibition of apoptosis, J. Biol. Chem. 279 (2004) 14828–14834.
[9] P.G. Smith, F. Wang, K.N. Wilkinson, K.J. Savage, U. Klein, D.S. Neuberg, G. Bollag, M.A. Shipp, R.C. Aguiar, The phosphodiesterase PDE4B limits cAMP-associated PI3K/AKT-dependent apoptosis in diffuse large B-cell lymphoma, Blood 105 (2005) 308–316.
[10] L. Zhang, P.A. Insel, The pro-apoptotic protein Bim is a convergence point for cAMP/protein kinase A- and glucocorticoid-promoted apoptosis of lymphoid cells, J. Biol. Chem. 279 (2004) 20858–20865.
[11] D. Calebiro, V.O. Nikolaev, M.C. Gagliani, T. de Filippis, C. Dees, C. Tacchetti, L. Persani, M.J. Lohse, Persistent cAMP-signals triggered by internalized G-protein-coupled receptors, PLoS Biol. 7 (2009) e1000172.
[12] S. Ferrandon, T.N. Feinstein, M. Castro, B. Wang, R. Bouley, J.T. Potts, T.J. Gardella, J.P. Vilardaga, Sustained cyclic AMP production by parathyroid hormone receptor endocytosis, Nat. Chem. Biol. 5 (2009) 734–742.
[13] Y. Chen, M.J. Cann, T.N. Litvin, V. Iourgenko, M.L. Sinclair, L.R. Levin, J. Buck, Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor, Science 289 (2000) 625–628.
[14] J.H. Zippin, Y. Chen, P. Nahirney, M. Kamenetsky, M.S. Wuttke, D.A. Fischman, L.R. Levin, J. Buck, Compartmentalization of bicarbonate-sensitive adenylyl cyclase in distinct signaling microdomains, FASEB J. 17 (2003) 82–84.
[15] M. Tresguerres, L.R. Levin, J. Buck, Intracellular cAMP signaling by soluble adenylyl cyclase, Kidney Int. 79 (2011) 1277–1288.
[16] R. Acin-Perez, E. Salazar, M. Kamenetsky, J. Buck, L.R. Levin, G. Manfredi, Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation, Cell Metab. 9 (2009) 265–276.
[17] S. Kumar, S. Kostin, J.P. Flacke, H.P. Reusch, Y. Ladilov, Soluble adenylyl cyclase controls mitochondria-dependent apoptosis in coronary endothelial cells, J. Biol. Chem. 284 (2009) 14760–14768.
[18] J.P. Flacke, H. Flacke, A. Appukuttan, R.J. Palisaar, J. Noldus, B.D. Robinson, H.P. Reusch, J.H. Zippin, Y. Ladilov, Type 10 soluble adenylyl cyclase is overexpressed in prostate carcinoma and controls proliferation of prostate cancer cells, J. Biol. Chem. 288 (2013) 3126–3135.
[19] J.H. Zippin, P.A. Chadwick, L.R. Levin, J. Buck, C.M. Magro, Soluble adenylyl cyclase defines a nuclear cAMP microdomain in keratinocyte hyperproliferative skin diseases, J. Invest. Dermatol. 130 (2010) 1279–1287.
[20] C.M. Magro, A.N. Crowson, G. Desman, J.H. Zippin, Soluble adenylyl cyclase antibody profile as a diagnostic adjunct in the assessment of pigmented lesions, Arch. Dermatol. 148 (2012) 335–344.
[21] Y. Onodera, J.M. Nam, M.J. Bissell, Increased sugar uptake promotes oncogenesis via EPAC/RAP1 and O-GlcNAc pathways, J. Clin. Invest. 124 (2014) 367–384.
[22] R. Acin-Perez, E. Salazar, S. Brosel, H. Yang, E.A. Schon, G. Manfredi, Modulation of mitochondrial protein phosphorylation by soluble adenylyl cyclase ameliorates cytochrome oxidase defects, EMBO Mol. Med. 1 (2009) 392–406.
[23] K.C. Hess, B.H. Jones, B. Marquez, Y. Chen, T.S. Ord, M. Kamenetsky, C. Miyamoto, J.H. Zippin, G.S. Kopf, S.S. Suarez, L.R. Levin, C.J. Williams, J. Buck, S.B. Moss, The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization, Dev. Cell 9 (2005) 249–259.
[24] J.J. Young, A. Mehdi, L.L. Stohl, L.R. Levin, J. Buck, J.A. Wagner, A.M. Stessin, "Soluble" adenylyl cyclase-generated cyclic adenosine monophosphate promotes fast migration in PC12 cells, J. Neurosci. Res. 86 (2008) 118–124.
[25] T. Shonai, M. Adachi, K. Sakata, M. Takekawa, T. Endo, K. Imai, M. Hareyama, MEK/ERK pathway protects ionizing radiation-induced loss of mitochondrial membrane potential and cell death in lymphocytic leukemia cells, Cell Death Differ. 9 (2002) 963–971.
[26] S. Jalal Hosseinimehr, O. Inanami, T. Hamasu, M. Takahashi, I. Kashiwakura, T. Asanuma, M. Kuwabara, Activation of c-kit by stem cell factor induces radioresistance to apoptosis through ERK-dependent expression of survivin in HL60 cells, J. Radiat. Res. 45 (2004) 557–561.
[27] A. Appukuttan, S.A. Kasseckert, M. Micoogullari, J.P. Flacke, S. Kumar, A. Woste, Y. Abdallah, L. Pott, H.P. Reusch, Y. Ladilov, Type 10 adenylyl cyclase mediates mitochondrial Bax translocation and apoptosis of adult rat cardiomyocytes under simulated ischaemia/reperfusion, Cardiovasc. Res. 93 (2012) 340–349.
[28] A. Appukuttan, S.A. Kasseckert, S. Kumar, H.P. Reusch, Y. Ladilov, Oxysterol-induced apoptosis of smooth muscle cells is under the control of a soluble adenylyl cyclase, Cardiovasc. Res. 99 (2013) 734–742.
[29] T. Hayashi, I. Hayashi, T. Shinohara, Y. Morishita, H. Nagamura, Y. Kusunoki, S. Kyoizumi, T. Seyama, K. Nakachi, Radiation-induced apoptosis of stem/progenitor cells in human umbilical cord blood is associated with alterations in reactive oxygen and intracellular pH, Mutat. Res. 556 (2004) 83–91.
[30] J. Cmielova, R. Havelek, A. Jiroutova, R. Kohlerova, M. Seifrtova, D. Muthna, J. Vavrova, M. Rezacova, DNA damage caused by ionizing radiation in embryonic diploid fibroblasts WI-38 induces both apoptosis and senescence, Physiol. Res. 60 (2011) 667–677.
[31] E.A. Cho, Y.S. Juhnn, The cAMP signaling system inhibits the repair of gamma-ray-induced DNA damage by promoting Epac1-mediated proteasomal degradation of XRCC1 protein in human lung cancer cells, Biochem. Biophys. Res. Commun. 422 (2012) 256–262.
[32] H.H. Hensley, J.M. Hannoun-Levi, P. Hachem, Z. Mu, R. Stoyanova, L.Y. Khor, S. Agrawal, A. Pollack, PKA knockdown enhances cell killing in response to radiation and androgen deprivation, Int. J. Cancer 128 (2011) 962–973.

[33] L.Y. Khor, K. Bae, T. Al-Saleem, E.H. Hammond, D.J. Grignon, W.T. Sause, M.V. Pilepich, P.P. Okunieff, H.M. Sandler, A. Pollack, Protein kinase A RI-alpha predicts for prostate cancer outcome: analysis of radiation therapy oncology group trial 86-10, Int. J. Radiat. Oncol. Biol. Phys. 71 (2008) 1309–1315.
[34] S. Gurumurthy, A. Goswami, K.M. Vasudevan, V.M. Rangnekar, Phosphorylation of Par-4 by protein kinase A is critical for apoptosis, Mol. Cell. Biol. 25 (2005) 1146–1161.
[35] V. Vucic, A. Niciforovic, M. Adzic, M.B. Radojcic, S. Ruzdijic, The combination of gamma ionizing radiation and 8-Cl-cAMP induces synergistic cell growth inhibition and induction of apoptosis in human prostate cancer cells, Invest. New Drugs 26 (2008) 309–317.