

# A2B adenosine receptor agonist induces cell cycle arrest and apoptosis in breast cancer stem cells via ERK1/2 phosphorylation

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## Abstract

**Purpose** It has been reported that cancer stem cells (CSCs) may play a crucial role in the development, recurrence and metastasis of breast cancer. Targeting signaling pathways in CSCs is considered to be a promising strategy for the treatment of cancer. Here, we investigated the role of the A2B adenosine receptor (A2BAR) and its associated signaling pathways in governing the proliferation and viability of breast cancer cell line derived CSCs.

**Methods** CSCs were isolated from the breast cancer cell lines MCF-7 and MDA-MB-231 using a mammosphere assay. The effect of the A2BAR agonist BAY606583 on cell proliferation was evaluated using XTT and mammosphere formation assays, respectively. Apoptosis was assessed using Annexin-V staining and cell cycle analyses were performed using flow cytometry. The expression levels of Bax, Bcl-2, cyclin-D1, CDK-4 and (phosphorylated) ERK1/2 were assessed using Western blotting.

**Results** Our data revealed that the breast cancer cell line derived mammospheres were enriched for CSCs. We also found

that A2BAR stimulation with its agonist BAY606583 inhibited mammosphere formation and CSC viability. In addition, we found that the application of BAY606583 led to CSC cell cycle arrest and apoptosis through the cyclin-D1/Cdk-4 and Bax/Bcl-2 pathways, respectively. Notably, we found that BAY606583 significantly down-regulated ERK1/2 phosphorylation in the breast cancer cell line derived CSCs. **Conclusions** From our results we conclude that A2BAR induces breast CSC cell cycle arrest and apoptosis through downregulation of the ERK1/2 cascade. As such, A2BAR may be considered as a novel target for the treatment of breast cancer.

**Keywords** Cancer stem cell · A2B adenosine receptor · Cell cycle · Apoptosis · ERK1/2

## 1 Introduction

Breast cancer stem cells (CSCs) constitute a rare population of cells that are thought to be responsible for tumor initiation, drug resistance and metastasis [1, 2]. The limited success of current treatment strategies may be due to the fact that they primarily target the bulk of the tumor cells while leaving the CSCs unaffected [3]. For obtaining a more effective treatment it may, therefore, be necessary to eradicate the CSCs [4]. In the case of breast cancer, a subpopulation of CD44<sup>+</sup>/CD24<sup>-</sup> cells has been recognized as being CSCs. These cells can grow under anchorage-independent conditions, thereby forming so called mammospheres [5]. Van Phuc et al. [6] reported that inhibition of CD44 may decrease the chemo-resistance of these CD44<sup>+</sup>/CD24<sup>-</sup> CSCs.

It has also been reported that signaling pathways play a crucial role in the maintenance of the cellular and molecular properties of breast CSCs such as growth, survival, self-renewal and

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chemo-resistance [7, 8]. Therefore, one strategy for the elimination of breast CSCs may be the targeting of its critical signaling pathways. Several studies have shown that the extracellular-signal regulated kinase (ERK1/2) pathway is implicated in the pathogenesis of breast cancer [9]. ERK1/2 is a major member of the mitogen-activated protein kinase superfamily, which plays a crucial role in regulating cellular growth and apoptosis in response to various stimuli in cancer [9, 10]. Recently, attention has shifted to the role of ERK1/2 in CSCs [11, 12] and it has been found that ERK1/2 signaling plays a crucial role in the self-renewal and proliferation maintenance capacities of CSCs [13]. Importantly, it has been found that ERK1/2 expression is increased in CSCs derived from human tumors [14]. In case of breast cancer, several studies have shown that ERK1/2 inhibition may reduce cell viability and spheroid formation, and induce apoptosis in CSCs [11, 15].

It has also been suggested that adenosine receptors (ARs) may play a role in the regulation ERK1/2 signaling [16]. ARs are members of a family of purinergic receptors and are divided into four subtypes: A1, A2A, A2B and A3 [17]. ARs are widely expressed and it has been reported that they play a critical role in the development and progression of many types of cancer [18]. The major signaling pathway of ARs involves G proteins that regulate the activity of adenylyl cyclase, thereby altering cAMP levels [19]. Previously, inconsistent results have been reported on the function of the A2 receptor A2BAR in breast cancer cells as also on its molecular mode of action. One study has reported that A2BAR agonists may reduce breast cancer cell proliferation [20], whereas another study has shown that A2BAR antagonists may inhibit the proliferation of breast cancer cells [21]. Since breast CSCs may play a pivotal role in breast cancer development and therapy resistance, there is a critical need to assess the role of A2BAR in breast CSCs. Recently, Daniele et al. [22] reported that A2BAR stimulation may have anti-proliferative and pro-apoptotic effects on glioblastoma CSCs.

The aim of the current study was to assess the role of A2BAR and its molecular mode of action in CSCs derived from MCF-7 and MDA-MB-231 breast cancer cells using mammosphere formation and cell viability assays, as well as cell cycle and apoptosis assays. In addition we assessed the effect of A2BAR on ERK1/2 signaling.

## 2 Materials and methods

### 2.1 Chemicals and antibodies

BAY606583 and PSB 603 were obtained from Tocris Bioscience (Bristol, UK), whereas XTT sodium salt and phenazine methosulfate were purchased from Sigma Aldrich (St. Louis, MO, USA). The anti-CD44 antibody (FITC) and anti-CD24 antibody (PE) used and Annexin-V were purchased

from Abcam (Cambridge, MA, USA). Mouse monoclonal antibodies directed against OCT-4, SOX-2, A2BAR, Bax, Bcl-2, CDK-4, cyclin-D1, GAPDH and ERK1/2, and goat anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A Cyclic AMP XP<sup>®</sup> Assay Kit was purchased from Cell Signaling Technology (Danvers, MA, USA), whereas propidium iodide (PI) was purchased from Sigma Aldrich. Basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and B-27 supplement were purchased from Invitrogen Co (Grand Island, NY, USA).

### 2.2 Cell lines and culture conditions

The human breast cancer-derived cell lines MCF-7 (IBRC C10082) and MDA-MB-231 (IBRC C10684) were obtained from the Iranian Biological Resource Center (IBRC). The cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin and cultured in a humidified incubator at 37 °C and 5% CO<sub>2</sub>.

### 2.3 Mammosphere formation assay

In order to obtain mammospheres, breast cancer-derived cells were suspended in serum-free DMEM containing 1% (v/v) B-27 supplement, 10 ng/ml bFGF and 20 ng/ml EGF and seeded in 6-well ultra-low attachment plates at a density of 5000 cells/well. When the cells were grown under these culture conditions, mammospheres appeared after 7 days. For serial passage (i.e., the formation of secondary and tertiary mammospheres), primary mammospheres were trypsinized to generate single cell suspensions and re-seeded in 6-well plates to form new mammospheres.

### 2.4 Flow cytometry

For the analysis of surface markers, tertiary mammospheres were trypsinized to obtain single cell suspensions and washed with phosphate-buffered saline (PBS). Next, the cells were incubated with anti-CD44 and anti-CD24 antibodies in the dark for 30 min. Following this incubation, the cells were washed twice with PBS to remove unbound antibody and analyzed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). The gates were set based on the isotype-matched controls. The data were analyzed using Flow Jo software version 7.6.1 (Tristar, CA, USA).

### 2.5 Quantitative RT-PCR

Total RNA was extracted from cell pellets using RNX-plus (Cinnagen, Tehran, Iran) according to the manufacturer's

instructions, after which cDNA was generated using a PrimeScript-RT Master Mix (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. The expression of GAPDH (control housekeeping gene) and A2BAR was assessed using SYBR green-based real-time PCR (Ampliqon, Herlev, Denmark). Relative mRNA expression levels were determined using the  $2^{-\Delta\Delta C_t}$  method. The following primers were used: A2BAR forward 5'-CTG TGT CCC GCT CAG GTA TAA-3'; A2BAR reverse 5'-CAG CAG CTT TCA TTC GTG GTT-3' and GAPDH forward, 5'-CTC CCG CTT CGC TCT CTG-3'; GAPDH reverse, 5'-TCC GTT GAC TCC GAC CTT C-3'.

## 2.6 Cyclic AMP measurement

cAMP accumulation was evaluated using a competitive ELISA kit (Cell Signaling Technology, Danvers, MA, USA). Briefly, cells were seeded in 6-well plates and cultured for 24 h. Then, the cells were washed twice with warm PBS and incubated in serum-free medium containing ADA (1 U/ml) for 30 min at 37 °C. After this, the cells were treated with different concentrations of BAY606583, (0.1, 1, 10 and 100  $\mu$ M) in the presence or absence of PSB 603 (A2BAR antagonist; 1  $\mu$ M) for 30 min. After lysis of the cells with the buffer available in the kit, the amount of cAMP in the cell lysate was measured according to the manufacturer's instructions.

## 2.7 XTT Cell Viability assay

Cell viability was quantified using a XTT-based assay (Sigma, St. Louis, MO, USA). Briefly, 3000 cells/well from disaggregated secondary mammospheres were seeded in 96-well plates and cultured for 7 days to re-form spheres. Next, the cells were treated with various concentrations of BAY606583 (0.1, 1, 10 and 100  $\mu$ M) in the presence or absence of PSB 603 (1  $\mu$ M) for 48 h. Finally, 125  $\mu$ M XTT (sodium 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) and 25  $\mu$ M PMS (phenazine methosulphate) were added to each well and incubated at 37 °C for 4 h, after which the soluble formazan product was measured at a wavelength of 450 nm using a multiplate reader (Synergy H1 Hybrid Multimode-BioTek).

## 2.8 Cell cycle analysis

Cell cycle analyses were carried out using flow cytometry as reported before [23, 24]. Briefly, CSCs were harvested after treatment with various concentrations of BAY606583 (0.1, 1, 10  $\mu$ M) for 48 h, washed with cold PBS and fixed gently (drop by drop) in 1 ml cold ethanol at 4 °C for 30 min. Next, the cells were washed and centrifuged at 1500 rpm for 5 min, after which the cell pellets were

resuspended in PBS containing 20 mg per ml propidium iodide (PI), 0.1% Triton X-100 and 100 mg/ml RNase. After incubation for 30 min in the dark on ice, the cells were analyzed for DNA content using a FACS Calibur flow cytometer. The cell cycle distribution of cells was quantified using Flow Jo software version 7.6.1 (Treestar Inc., Ashland, OR, USA).

## 2.9 Apoptosis assay

CSCs from disaggregated secondary-mammospheres were seeded at a concentration of  $5 \times 10^4$  cells/well in 6-well plates and incubated for 7 days to allow sphere re-formation. Next, the cells were treated with various concentrations of BAY606583 (0.1, 1, 10 and 100  $\mu$ M) in the presence or absence of PSB 603 (1  $\mu$ M) and, after 48 h incubation, collected by centrifugation. The resulting cell pellets were washed twice with PBS, resuspended in 195  $\mu$ l binding buffer and incubated with 5  $\mu$ l Annexin-V-FITC for 10 min on ice in the dark, after which 1  $\mu$ l propidium iodide (PI) (50  $\mu$ g/ml) was added and flow cytometry (see above) was carried out.

## 2.10 Western blotting

Western blotting was carried out as reported before [25]. Briefly, cells were treated with various concentrations of BAY606583 (0.1, 1, 10 and 100  $\mu$ M) for 48 h. For the OCT-4, SOX-2 and A2BAR protein level assays, the cells were not treated with BAY606583. Next, the cells were washed twice with ice-cold PBS (pH 7.4) and lysed in RIPA buffer supplemented with a Complete Protease Inhibitors Cocktail (Roche Applied Science, Penzberg, Germany). After incubation on ice for 2 h, the lysates were centrifuged at 14,000 $\times$ g for 10 min at 4 °C and the supernatants were transferred to clean tubes. Equal amounts of soluble protein were subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% skim milk for 2 h and incubated with mouse anti-OCT-4, anti-SOX-2, anti-A2BAR, anti-Cyclin-D1, anti-CDK-4, anti-Bax, anti-Bcl-2, anti-ERK1/2 and anti-GAPDH antibodies at 4 °C overnight and, subsequently, with an appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Protein bands were detected using an ECL reagent (Bio-Rad, Hercules, CA, USA). The relative intensities of the protein bands were measured using ImageJ software (NIH, Bethesda, MA, USA).

## 2.11 Caspase-6 activity measurement

CSCs were treated with various concentrations of BAY606583 (0.1, 1, 10 and 100  $\mu$ M) for 48 h. In addition,

CSCs were treated with BAY606583 (20  $\mu$ M) for 0, 6, 12, 24, 48 and 60 h. Subsequently, caspase-6 activities were assessed using a Caspase Colorimetric Assay Kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). To this end, cells were harvested and lysed in lysis buffer. After incubation on ice for 10 min, the lysed cells were centrifuged after which caspase-6 substrate (VEID-pNA) was added to the supernatants in a reaction buffer and incubated at 37 °C for 2 h. The absorbance of p-nitroaniline was measured at 490 nm in a Microplate Reader (Synergy H1 Hybrid Multimode-BioTek).

### 2.12 Statistical analysis

The results are presented as mean  $\pm$  SD and statistical analyses were carried out using the nonparametric test of variance between groups (ANOVA) followed by Dunnett's post hoc test. All experiments were repeated at least three times independently and the software package SPSS version 18 was used. A difference was regarded statistically significant at  $p < 0.05$ .

## 3 Results

### 3.1 Identification of breast cancer cell derived CSCs

Previously, it has been reported that CD44 and CD24 may act as specific cell surface markers for the identification of human breast CSCs [26]. Using flow cytometry, we found that tertiary mammospheres derived from MCF-7 and MDA-MB-231 breast cancer cells were enriched for up to 60.8% and 85.1% in CD44<sup>+</sup>/CD24<sup>-</sup> CSCs, respectively (Fig. 1). OCT4 and SOX-2 are well-known key regulators of self-renewal in normal stem cells and, in addition, have previously been used as biomarkers for the identification of CSCs [27, 28]. In order to assess whether the MCF-7 and MDA-MB-231 derived CSCs have stem cell properties, we examined their OCT-4 and SOX-2 expression levels relative to those of the parental cell lines. Using Western blot analysis, we indeed found that the expression of OCT-4 and SOX-2 was increased in the MCF-7 and MDA-MB-231 derived CSCs (Fig. 2a).

### 3.2 A2BAR expression is increased in breast cancer cell derived CSCs

Next, we set out to assess the expression level of the A2B adenosine receptor A2BAR in MCF-7 and MDA-MB cells and in CSCs derived from them. Using Western blot analysis we found that the expression of A2BAR in the breast cancer cell derived CSCs was higher than in its parental cell lines (Fig. 2a). Using qRT-PCR, we also found that the A2BAR mRNA expression levels were 2.8 fold (MCF-7

derived CSCs) and 2.2-fold (MDA-MB-231 derived CSCs) higher compared to the parental cell lines, respectively ( $p < 0.05$ ; Fig. 2b).

### 3.3 A2BAR is functional in breast cancer cell derived CSCs

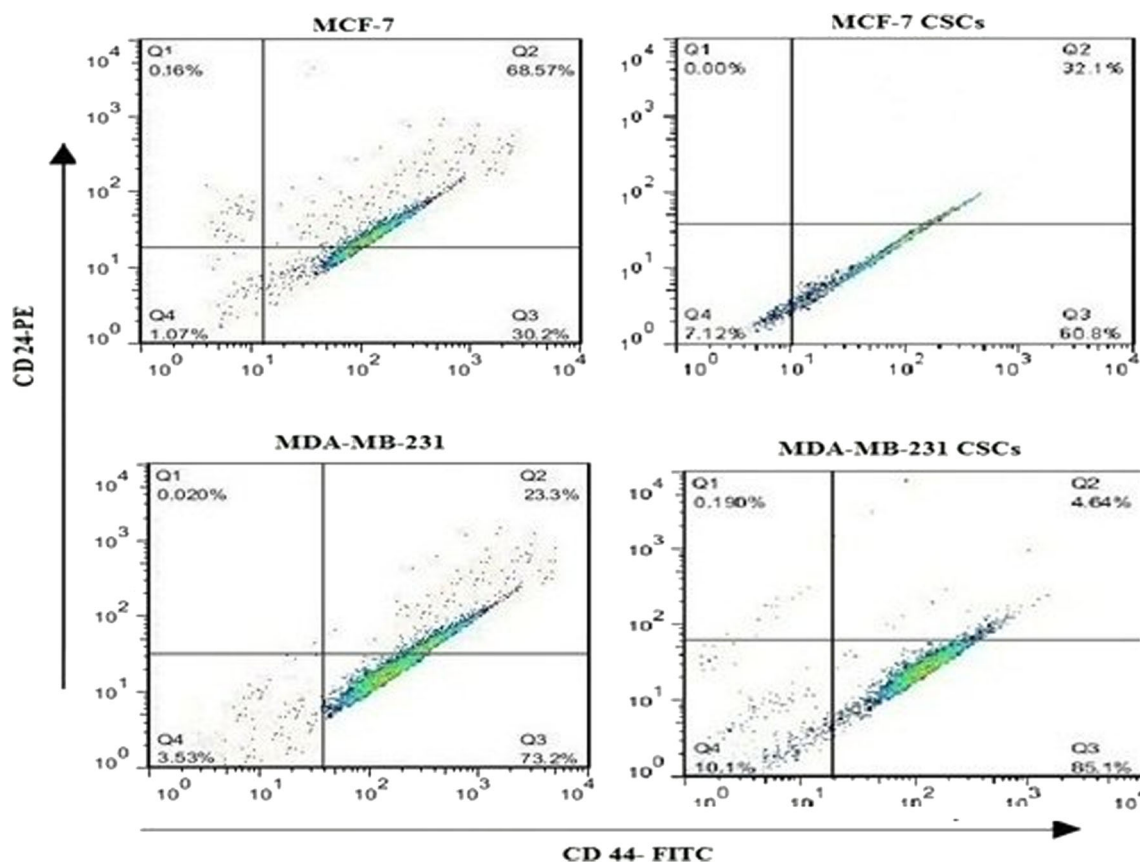
To verify the functional responsiveness of A2BAR in MCF-7 and MDA-MB cells and its derived CSCs, its cAMP levels were examined [29]. After treatment of the respective cells with various concentrations of the A2BAR agonist BAY606583 we found that the breast cancer cell derived CSCs exhibited increased cAMP levels in a concentration-dependent manner, corresponding to EC50 values of 8.1 and 9.23 for the MCF-7 and MDA-MB 231 derived CSCs, respectively (Fig. 2c). We also assessed the effect of the A2BAR agonist BAY606583 in the presence of its antagonist PSB 603 (1  $\mu$ M). By doing so, we found that PSB 603 antagonized the BAY606583-induced cAMP levels in both breast cancer cell derived CSCs (Fig. 2c).

### 3.4 A2BAR agonist BAY606583 inhibits mammosphere formation

To evaluate whether A2BAR agonism inhibits mammosphere formation, we accumulated breast CSCs using sphere medium and assessed the sphere forming efficiency of MCF-7 and MDA-MB-231 cells after BAY606583 treatment. We found that this BAY606583 treatment reduced the formation of mammospheres in both MCF-7 and MDA-MB231 cells (Fig. 3a, c). To confirm the specificity of A2BAR inhibition, the respective CSCs were pretreated with the A2BAR antagonist PSB 603 (1  $\mu$ M). By doing so, we found that the inhibitory effects of BAY606583 on mammosphere formation were blocked (Fig. 3a, c).

### 3.5 A2BAR agonist BAY606583 has anti-proliferative effects on breast cancer cell derived CSCs

To examine the effects of the A2BAR agonist BAY606583 on breast cancer cell derived CSCs, changes in cell viability were measured using a XTT assay. We found that treatment of the CSCs with BAY606583 resulted in a dose-dependent reduction in cell viability compared to that of the untreated controls (Fig. 3b, d). At a concentration of 0.1  $\mu$ M BAY606583 had a significant inhibitory effect on the viability of the CSCs ( $p < 0.05$ ), whereas at 100  $\mu$ M the effect reached a maximum in both the MCF-7 and MDA-MB-231 derived CSCs ( $p < 0.01$ ). To confirm the specificity of A2BAR on the inhibition of cell viability, the CSCs were pretreated with the A2BAR antagonist PSB 603 (1  $\mu$ M). By doing so, we found that the inhibitory effect of BAY606583 was significantly blocked ( $p < 0.05$ ; Fig. 3b, d).



**Fig. 1** CD44 and C244 expression in breast cancer cell derived CSCs and parental cell lines. Flow cytometry analysis of CD24 and CD44 expression in MCF-7, MCF-7 derived CSCs, MDA-MB-231 and MDA-MB-231 derived CSCs

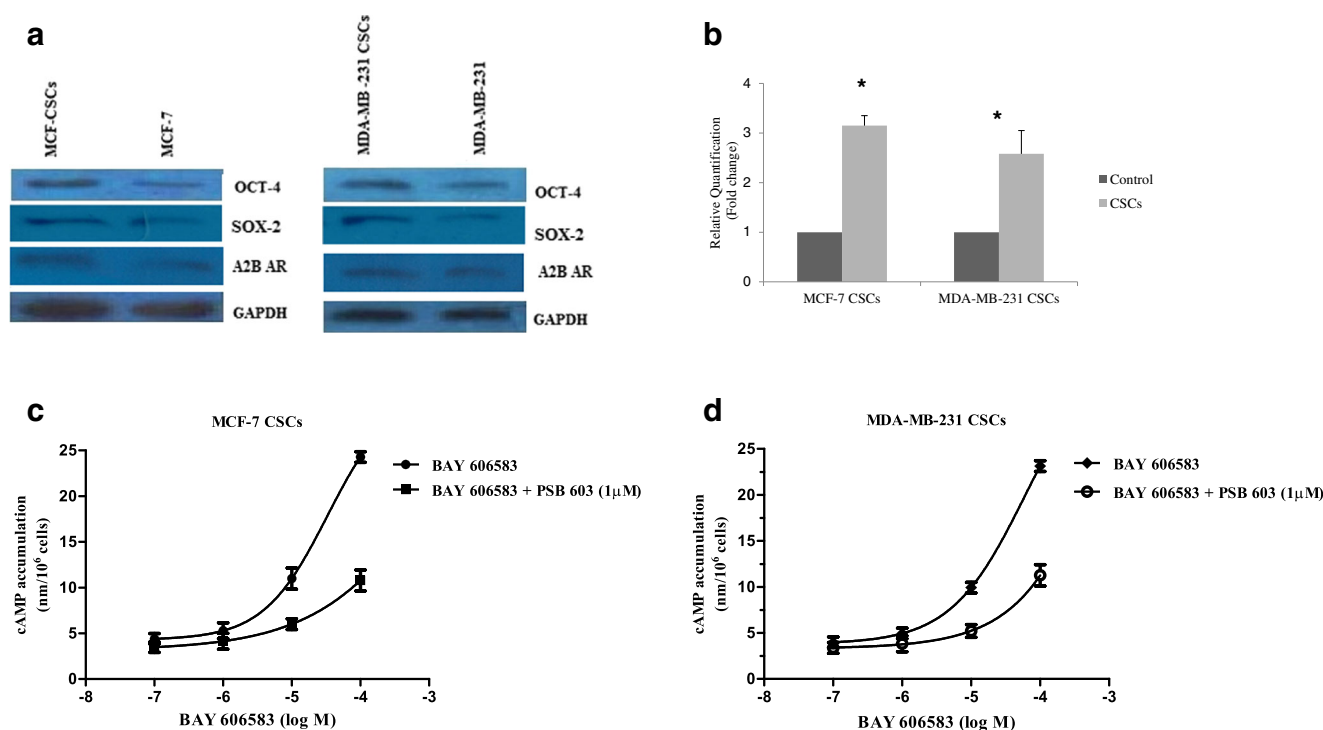
### 3.6 A2BAR agonist BAY606583 induces cell cycle arrest in breast cancer cell derived CSCs

In order to evaluate whether the A2BAR agonist BAY606583 can induce cell cycle arrest in breast cancer cell derived CSCs, the respective cells were treated with both low concentrations of BAY606583 (0.1, 1  $\mu$ M) and a high concentration of BAY606583 (10  $\mu$ M) for 48 h. After treatment of the CSCs with 0.1 and 1  $\mu$ M BAY606583 an apparent dose-dependent accumulation of cells in the G1 phase was observed ( $p < 0.05$ ; Fig. 4a). At the concentration of 10  $\mu$ M an apparent accumulation of cells in the subG1 phase was observed in both CSC groups ( $p < 0.05$ ). After 48 h the percentages of MCF-7 derived CSCs in the G1 phase were 65.01%, 69.48%, 71.53% and 60.45% for the untreated, and 0.1  $\mu$ M, 1  $\mu$ M and 10  $\mu$ M treated cells, respectively, whereas in the MDA-MB-231 derived CSCs these percentages were 57.45%, 60.43%, 66.56% and 60.14% for the untreated and 0.1  $\mu$ M, 1  $\mu$ M and 10  $\mu$ M treated cells, respectively. To determine the molecular mechanism underlying the BAY606583-induced G1 arrest in the breast cancer cell derived CSCs, the effect of BAY606583 on the expression of the G1 cell cycle regulatory proteins Cdk-4 and cyclin-D1 was assessed by Western blotting. We found that BAY606583 inhibited the expression levels of both cyclin-D1

and CDK-4, which may explain the observed G1 cell cycle arrest in both the MCF-7 and MDA-MB-231 derived CSCs (Fig. 4c).

### 3.7 A2BAR agonist BAY606583 induces apoptosis in breast cancer cell derived CSCs

To determine the apoptotic nature of the A2BAR agonism-induced cell death, we assessed the effect of low (1  $\mu$ M) and high (10 and 100  $\mu$ M) BAY606583 concentrations on the breast cancer cell derived CSCs using Annexin-V-FITC and PI staining in conjunction with flow cytometry (Fig. 5a). We found that after BAY606583 treatment for 48 h the percentage of apoptotic cells increased in a concentration-dependent manner ( $p < 0.05$ ; Fig. 5b). In addition, we found that PSB 603 inhibited the BAY606583-induced apoptosis (Fig. 5b), thus confirming the involvement of A2BAR in the induction of apoptosis by BAY606583. In order to substantiate this notion, we decided to determine the expression of the Bcl-2 family members Bcl-2 and Bax in the breast cancer cell derived CSCs. Using Western blotting, we observed a significant reduction in Bcl-2 expression after BAY606583 treatment. In addition, we found that BAY606583-induced apoptosis was accompanied by increases in pro-apoptotic Bax expression in



**Fig. 2** OCT-4, SOX-2 and A2BAR expression in breast cancer cell derived CSCs and parental cell lines. (a) OCT-4, SOX-2 and A2BAR protein expression assessed by Western blotting in MCF-7 and MDA-MB-231 cells and its derived CSCs. (b) Relative gene expression of A2BAR

assessed by qRT-PCR in MCF-7 and MDA-MB-231 cells and its derived CSCs. (c and d) cAMP accumulation assessed by competitive ELISA in breast cancer cell derived CSCs after treatment with various concentrations of BAY606583 in the presence or absence of PSB 603 (1 $\mu$ M)

a dose-dependent manner (Fig. 5c). Since caspase cascade activation plays a key role in apoptosis [3], we next set out to assess the catalytic activities of caspase-6 in A2BAR agonist-treated cells using colorimetric assays. By doing so, we found that, compared to the control group, BAY606583 treatment of the CSCs resulted in significant increases in caspase-6 activity in a concentration (1, 10, 20, 40, 80, 100  $\mu$ M; Fig. 6a) and incubation (12, 24, 48, 60 h; Fig. 6b) dependent manner ( $p < 0.05$ ).

### 3.8 ERK1/2 may act as mediator of the anti-apoptotic A2BAR effects

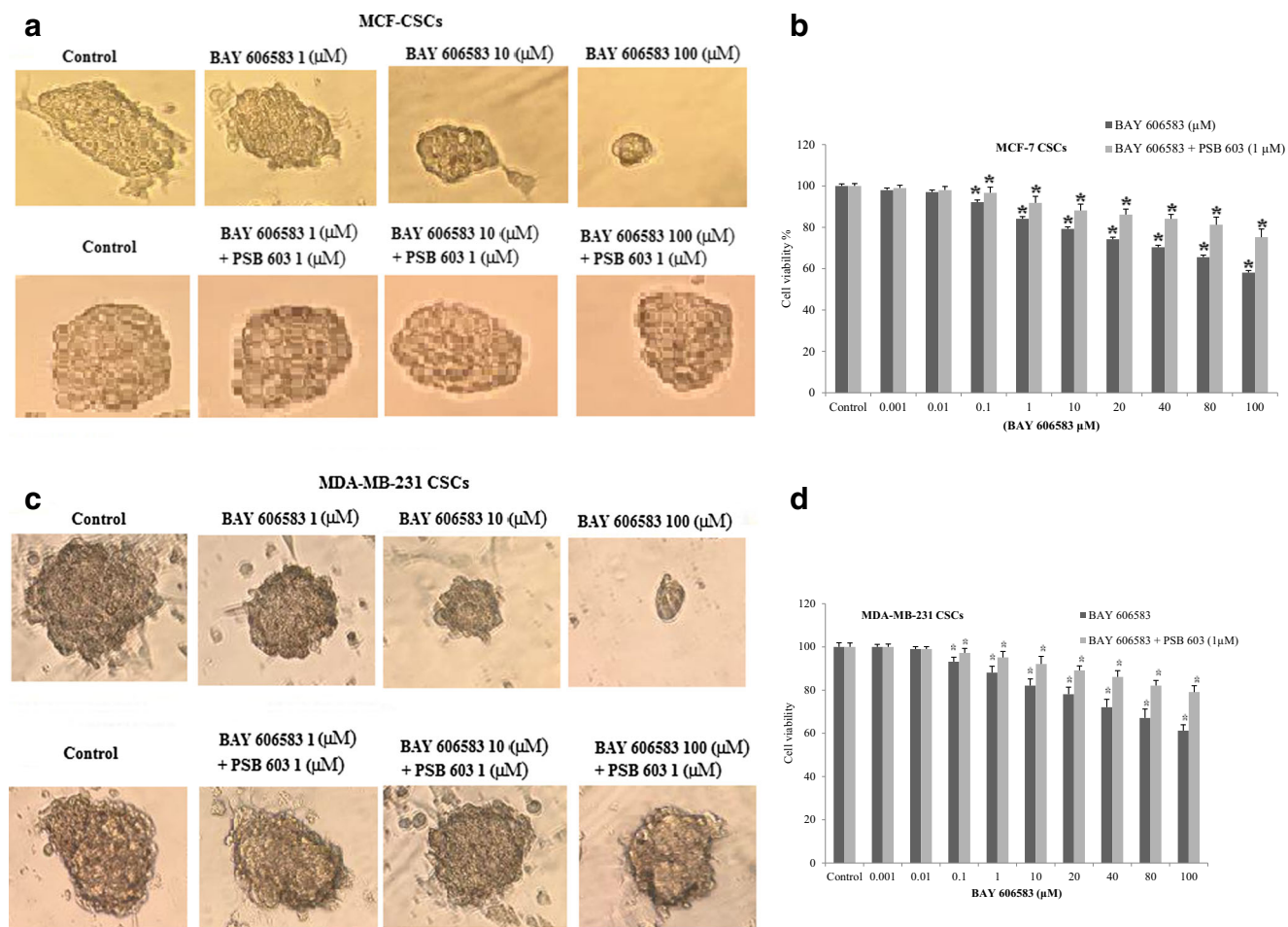
Several signaling pathways have been shown to be involved in the growth and survival of CSCs, including the ERK1/2 pathway [14]. Therefore, we decided to evaluate the effects of low concentrations (1  $\mu$ M) and high concentrations (10 and 100  $\mu$ M) of BAY606583 on the ERK1/2 signaling pathway in breast cancer cell derived CSCs. We found that the A2BAR agonist did not change the total ERK protein level, but down-regulated the p-ERK1/2 level at both the low and high concentrations of BAY606583 tested in MCF-7 and MDA-MB-231 derived CSCs after 12 h incubation ( $p < 0.05$ ; Fig. 7). This result suggests a regulatory role of A2BAR on the ERK1/2 pathway.

## 4 Discussion

Previously, it has been reported that CSCs may be involved in the recurrence and metastasis of cancer, including breast cancer [29]. It has also been reported that CSC targeting may be an effective way to treat cancer and that the identification of signaling pathways governing CSC biology may be instrumental for this [3]. Here, we report for the first time that A2BAR may play a pivotal role in the regulation of Erk1/2 signaling in breast cancer cell derived CSCs.

The role of A2BAR and its associated signaling pathways have been evaluated in several human tumor types [30] and it has for instance been found that A2BAR can induce apoptosis in colon and ovarian cancer cells [31, 32]. Mittal et al. [33] reported that A2BAR may play a role in promoting tumor metastasis and, concordantly, it has been found that A2BAR blockade may inhibit the growth and metastasis of prostate cancer and melanoma cells [34, 35]. As of yet, a role of A2BAR in breast cancer has not been documented, including a putative role in the biology of breast cancer derived CSCs.

First, we enriched CSCs from breast cancer cell line MCF-7 and MDA-MB-231 derived mammospheres using serum-free medium. We found that these cells exhibit a CD44<sup>+</sup>/CD24<sup>-</sup> breast CSC phenotype and express the stem cell marker OCT-4. In agreement with this finding, de la Mare et al. [36] recently reported an increase in the CD44<sup>+</sup>/CD24<sup>-</sup> population



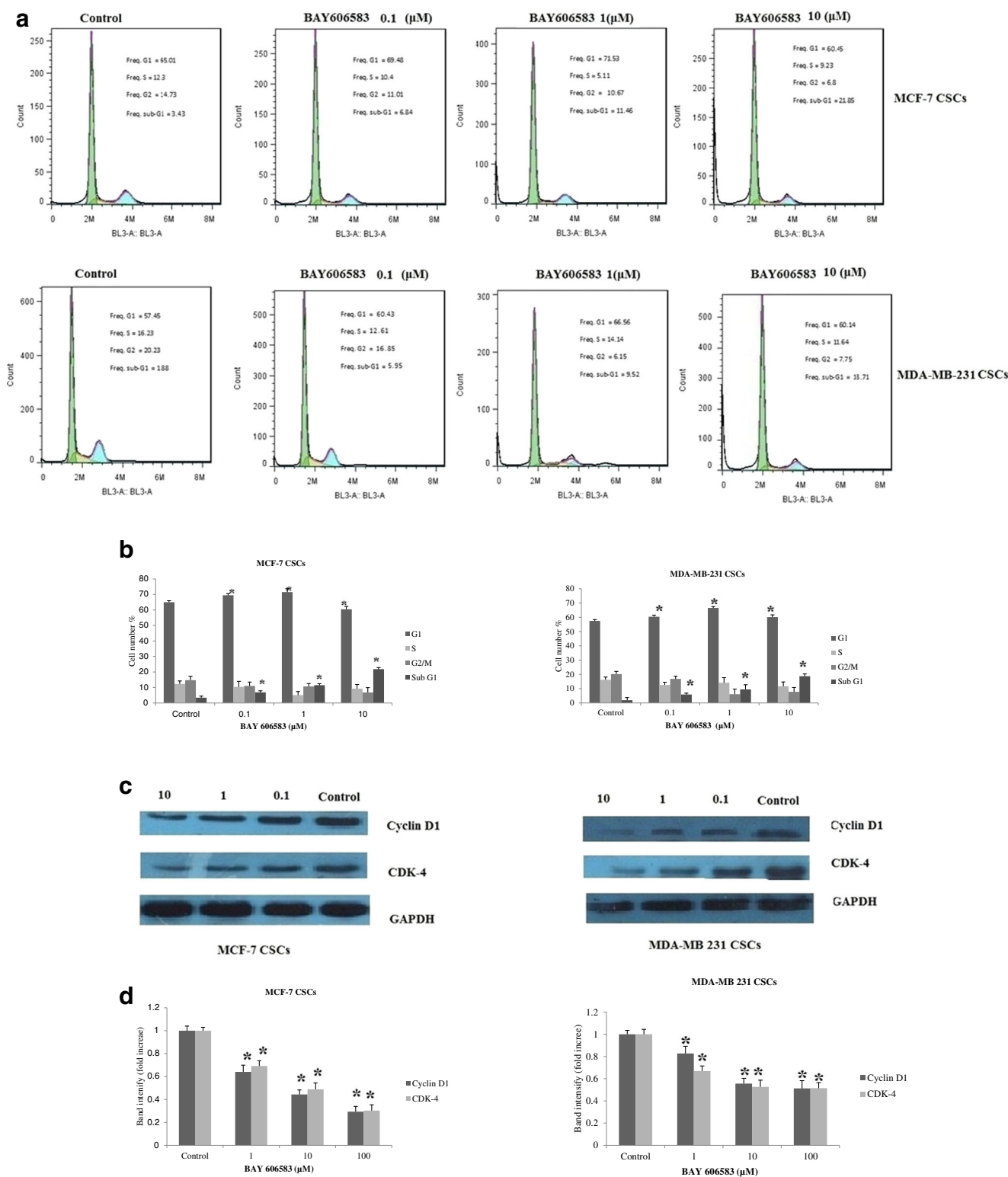
**Fig. 3** Effect of A2BAR agonist BAY606583 on mammosphere formation and cell viability. Cells were cultured in sphere medium and treated with various concentration of BAY606583 (1, 10, and 100 μM) in the presences or absence of PSB 603 (1 μM). **(a and c)** Decreased mammosphere sizes after treatment with BAY606583 compared to controls (MCF-7, MDA-MB-231). The inhibitory effect of BAY606583

on mammosphere formation is blocked by PSB 603. **(b and d)** BAY606583 treatment of CSCs results in dose-dependent reductions in cell viability compared to controls (MCF-7, MDA-MB-231). The inhibitory effects of BAY606583 on cell viability are blocked by PSB 603. Data are representative mean values ± S.E.M. of three independent experiments. \**p* < 0.05

and in the expression of OCT-4 in mammospheres derived from MCF-7 cells, and Huang et al. [27] similarly found that mammospheres derived from MDA-MB231 cells are enriched for CD44<sup>+</sup>/CD24<sup>-</sup> cells and exhibit a high level of OCT-4 expression. We also found that A2BAR was expressed at higher levels in the breast cancer cell derived CSCs compared to the parental cells and that A2BAR is functionally active in these CSCs. Previously, it has been found that the expression of A2BAR is increased in glioma CSCs compared to the bulk of glioma tumor cells [22]. Here, XTT and mammosphere formation assays were used to evaluate the effect of the A2BAR inhibitor BAY606583 on the growth of breast cancer cell derived CSCs. From our results, we conclude that A2BAR mediates an inhibitory effect on the growth of these CSCs in vitro. This result is consistent with a previous report showing that BAY606583 can inhibit the proliferation of glioblastoma derived CSCs [22].

Modulation of cell cycle progression is considered to be a promising approach to control tumor growth [37]. We found that BAY606583 at low concentrations (0.1 and 1 μM) induces G1 cell cycle arrest and at a higher concentration (10 μM) induces cell death with an apparent accumulation of CSCs in the subG1 phase. The control over cell cycle G1/S phase transition is driven by a complex formed by Cdk-4 and cyclin-D1 [38]. We found that A2BAR induces G1 cell cycle arrest through the down-regulation of cyclin-D1 and CDK-4. Previously, Lamb et al. [39] showed that cyclin-D1 and CDK-4 inhibition leads to a decrease in mammosphere formation [38], whereas another study has indicated that CDK-4 may regulate CSC properties in breast cancer. Based on these observations, cyclin-D1 and CDK-4 may be considered as targets for the treatment of breast cancer.

Apoptosis is a mode of cell death that is crucially involved in tumor formation. The Bcl-2-family of proteins



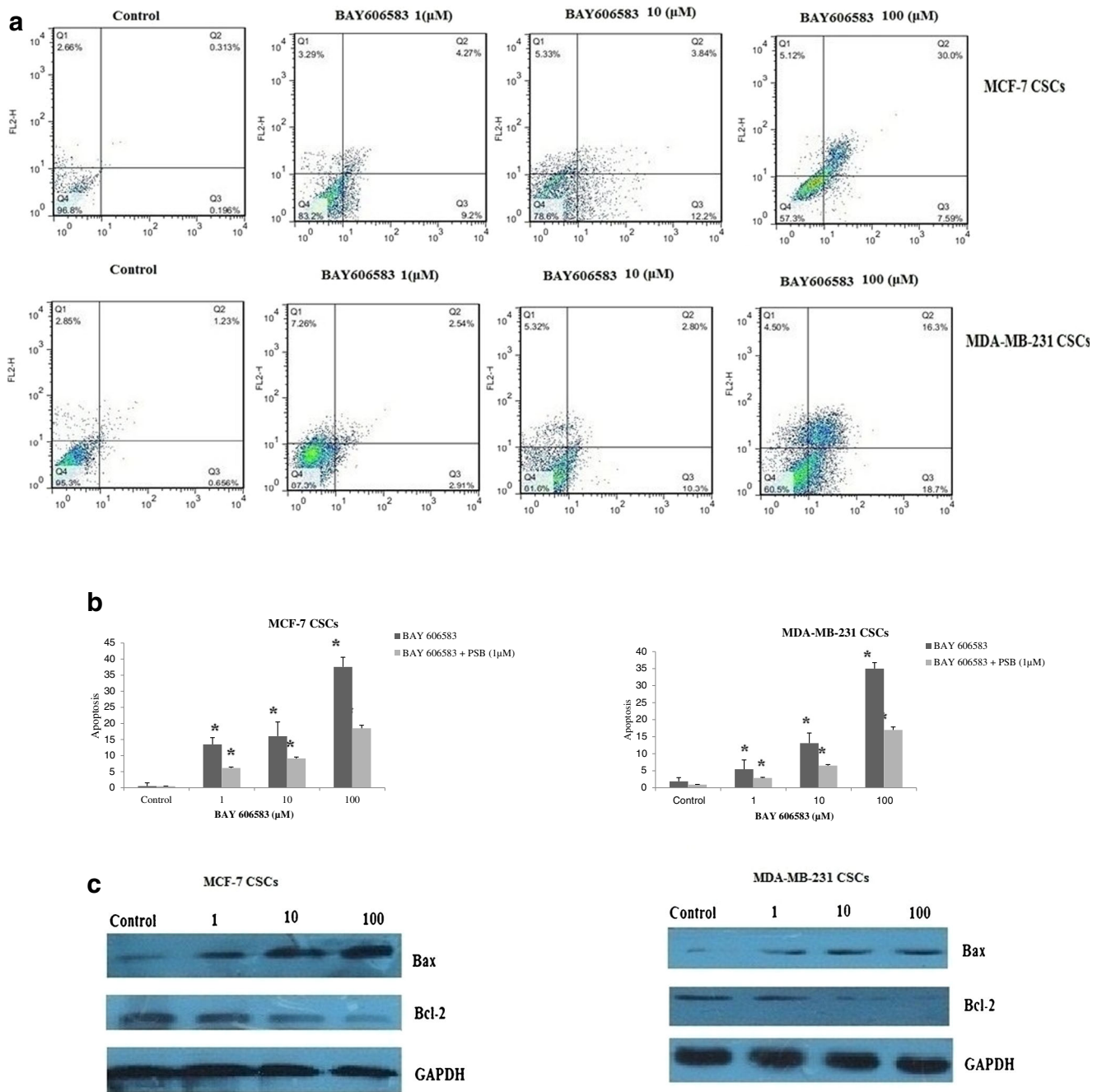
**Fig. 4** Effect of A2BAR agonist BAY606583 on CSC cell cycle distribution. **(a)** Cell cycle analysis by flow cytometry of cells treated with various concentrations of BAY606583 (0.1, 1 and 10 μM) for 48 h. **(b)** Histograms indicating the percentages of cells in each phase

of the cell cycle. **(c)** Effect of BAY606583 on G1 cell cycle regulatory proteins assessed by Western blotting. **(d)** Densitometric analysis of cyclin-D1 and CDK-4 bands using ImageJ software. Data are representative mean values ± S.E.M. of three independent experiments. \**p* < 0.05

are key regulators of apoptosis and are known to act through the mitochondrial apoptotic response pathway.

This family of proteins includes both pro-apoptotic and anti-apoptotic members [40]. Also, caspases play a





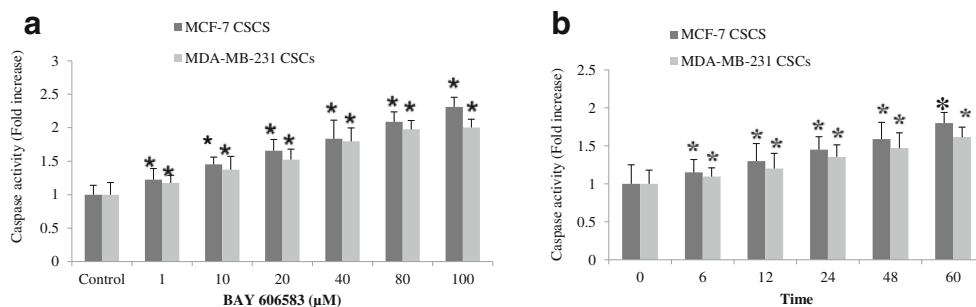
**Fig. 5** Quantification of A2BAR agonist BAY606583-induced apoptosis in breast cancer cell derived CSCs. **(a)** Flow cytometry analysis of cells treated with various concentration of BAY606583 (1, 10 and 100 μM) for 48 h. **(b)** Histograms indicating the percentages of apoptotic breast cancer cell derived CSCs after treatment with various concentrations of

BAY606583 in the presence or absence of PSB 603. **(c)** Effect of BAY606583 on Bax and Bcl-2 expression assessed by Western blotting. Data are representative mean values ± S.E.M. of three independent experiments. \**p* < 0.05

prominent role in apoptosis [41]. Caspase-6 is a key effector that becomes activated downstream of caspase-3 and caspase-7 in apoptosome-mediated apoptosis [42]. Many cancer treatment strategies are based on activation of apoptosis signal transduction pathways, mitochondrial function disturbance and proteolytic processing of caspases [43]. We found that BAY606583 increases the expression

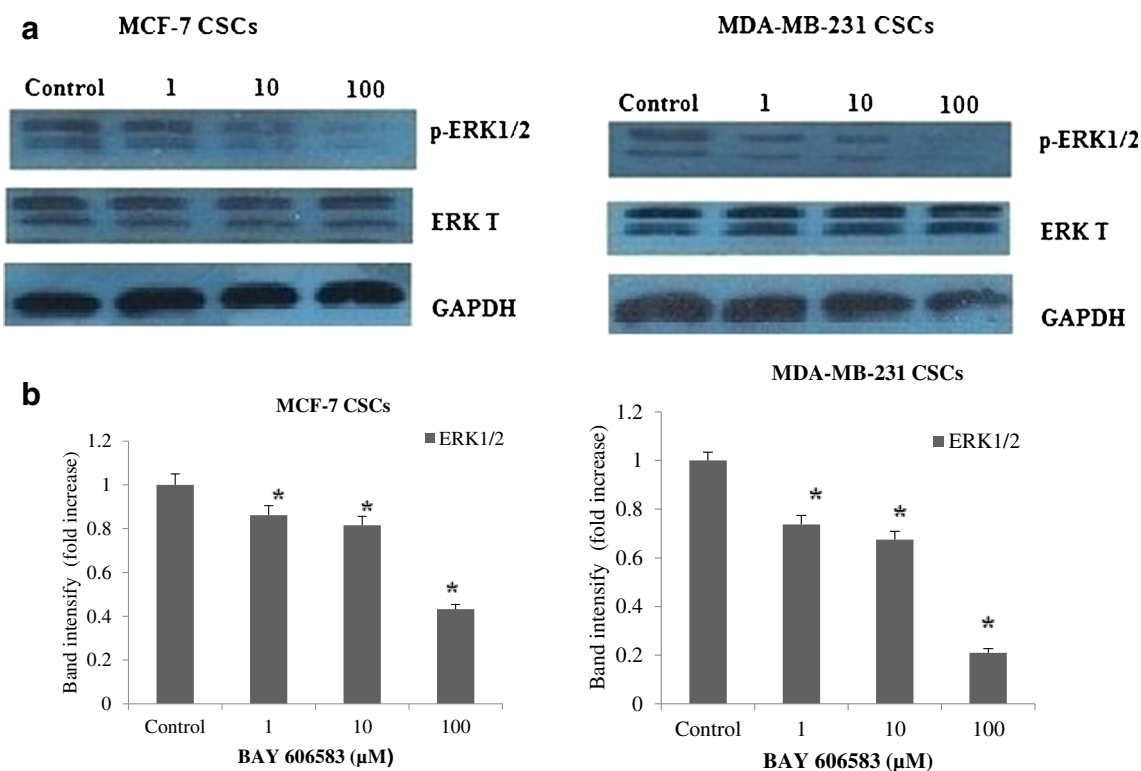
of Bax, decreases the expression of Bcl-2 and induces the activation of caspase-6. These results indicate that this A2BAR agonist up-regulates caspase-6 through an increment of the Bax/Bcl-2 ratio, thereby inducing apoptosis in breast cancer cell derived CSCs. Others have found that A2BAR stimulation can induce apoptosis via activation of caspase-3 and deregulation of the Bax/Bcl2 ratio [26],

**Fig. 6** Colorimetric assessment of caspase-6 activities after BAY606583 treatment. Caspase-6 activity assessed by colorimetric assay in cells treated (a) with various concentrations of BAY606583 (1–100  $\mu$ M) for 48 h and (b) during various periods (0–60 h) with BAY606583 (20  $\mu$ M). Data are representative mean values  $\pm$  S.E.M. of three independent experiments. \* $p$  < 0.05



and that A2BAR agonism may induce apoptosis in glioblastoma derived CSCs through caspase activation and Bax up-regulation [22]. Several studies have confirmed the involvement of A2BAR in cancer development through agonist and antagonist treatment experiments. Previously, it has for example been found that the effects of A2BAR agonists could be completely abrogated by A2BAR antagonists in glioblastoma derived CSCs, indicating that the anti-proliferative effects of A2BAR agonists are specifically mediated by A2BAR activation [22]. Another study on ovarian cancer has shown that the inhibitory effects of

A2BAR agonists on cellular growth could significantly be abrogated by A2BAR antagonists, indicating the specificity of A2BAR in the inhibition of ovarian cancer cell viability [32]. Our data also indicate that the A2BAR antagonist PSB 603 can inhibit BAY606583-induced cAMP levels in breast cancer cell derived CSCs and that PSB 603 can counteract the inhibitory effects of BAY606583 on cell proliferation and mammosphere formation. Additionally, we found that apoptosis induction by A2BAR activation was antagonized by PSB 603. Together, these results confirm the observed effects of A2BAR at high BAY606583



**Fig. 7** Effect of A2BAR agonist BAY606583 on ERK1/2 signaling. (a) Expression analysis of ERK1/2 by Western blotting in cells treated with 1, 10 and 100  $\mu$ M BAY606583 for 12 h. (b) Densitometric analysis of

protein bands using ImageJ software. Data are representative mean values  $\pm$  S.E.M. of three independent experiments. \* $p$  < 0.05

doses. Other studies have shown that BAY606583 at high doses may protect the colonic epithelial barrier during acute colitis through A2BAR activation [44] and that A2BAR activation at high BAY606583 doses may mediate hematopoiesis [45].

Several signaling cascades, such as the ERK1/2 signaling cascade, are known to be involved in cell proliferation and apoptosis [46] and evidence has been reported indicating that A2BAR may be involved in regulation of the ERK1/2 signaling pathway [16]. The ERK1/2 signaling pathway regulates a wide range of cellular activities and physiological processes [10], and it has been shown that it is critical for the maintenance of CSC properties [11]. Other studies have uncovered the importance of the ERK1/2 pathway in governing G1/S cell cycle progression and apoptosis through cyclin-D1 and Bcl-2 regulation, respectively [47, 48]. Yet another study has shown that ERK1/2 pathway activation constitutes one of the mechanisms imposing resistance to apoptosis in CSCs [49]. To uncover the role of ERK1/2 in A2BAR-mediated cell cycle arrest and apoptosis in breast cancer cell derived CSCs, we assessed the effects of BAY606583 on ERK1/2 signaling. We found that BAY606583 treatment led to a decrease in ERK1/2 phosphorylation in the CSCs. Recently, it has been found that A2BAR agonism can induce a significant inhibition of ERK1/2 phosphorylation in glioblastoma derived CSCs [22]. From these combined results, we conclude that ERK1/2 inhibition may be of interest for the design of novel anticancer therapies.

Taken together, we found that mammosphere cells derived from the MCF-7 and MDA-MB-231 breast cancer cell lines exhibit CSC properties and that these cells show an increased level of A2BAR expression. In addition, we found that the A2BAR agonist BAY606583 exhibits antiproliferative effects by reducing CSC cell viability and inhibiting mammosphere formation. We also found that BAY606583 at lower concentrations can down-regulate the expression levels of cyclin-D1 and CDK-4 via inhibition of the ERK1/2 cascade, thereby inducing cell cycle arrest. At a higher concentration BAY606583 was found to increase the Bax/Bcl-2 ratio, to up-regulate caspase-6 and to induce apoptosis in breast cancer cell derived CSCs through ERK1/2 cascade inhibition.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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