The effect of cannabinoid receptor agonists on breast cancer and its interaction with bone cells

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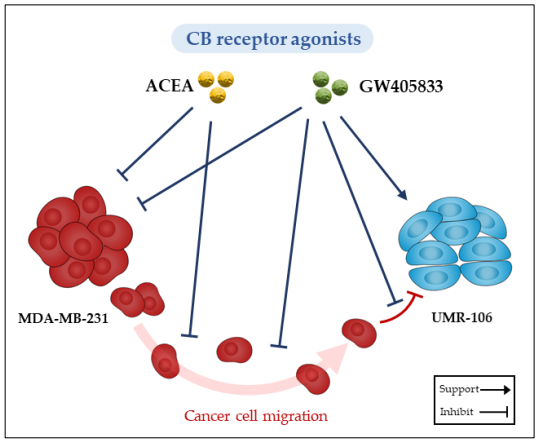
Abstract: Bone metastasis is a serious problem worldwide especially in advanced breast cancer patients. Unfortunately, the effective treatment for cancer bone metastasis is limited. Cannabinoids (CBs) are compounds that have been shown to possess diverse advantages in medical treatment including cancers. Therefore, this research aims to investigate the role of CBs on breast cancer and cancer and bone interaction by using CB receptor agonists including Arachidonyl-2'-chloroethylamide hydrate (ACEA) and GW405833 which are specific for CB1 and CB2 receptor, respectively. The results from cell viability assay revealed that both CB agonists suppressed MDA-MB-231 cell viability in a concentration dependent manner. Interestingly, ACEA and GW405833 also suppressed MDA-MB-231 cell migration at 24 hours after treatment. For the interaction between MDA-MB-231 and UMR-106, our results demonstrated that UMR-106 cell viability decreased by 25.7% when culturing with MDA-MB-231-derived conditioned media. Nevertheless, the pretreatment of GW405833 on MDA-MB-231 significantly improved UMR-106 cell viability by 18.5%. The activation of CB receptor function in breast cancer not only inhibited cancer cell survival and migration ability, but it also reduced the cytotoxic effect from breast cancer cells on bone cells. Therefore, this study provided a crucial information for the application of CB agonists as novel therapeutic agents for breast cancer bone metastasis.

Graphical abstract:



Keywords: Cannabinoids, CB receptor agonists, Triple-negative breast cancer, Osteoblast, Bone metastases

1. Introduction

Breast cancer is one of the most common cancer all over the world [1]. Seventy percent of the patients who develop metastatic breast cancer are diagnosed with bone metastasis [2]. More importantly, bone metastasis leads to several complications including muscle weakness, bone destruction, bone pain and reducing survival rate [3]. The loss of bone homeostasis is the crucial factor causing abnormal bone lesions [4]. Bone homeostasis is primarily controlled by the functions of the bone forming osteoblast cells and bone degrading osteoclast cells [5, 6]. When the cancer cells migrate and establish the new colony at bone tissue, they induce the irregular crosstalk between bone cells, especially osteoblast and osteoclast cells [7]. For instance, cancer cells induced the overexpression of receptor activator for nuclear factor κ B ligand (RANKL) from osteoblast to promote osteoclast differentiation and osteolytic lesion resulting in cancer-induced bone loss [8]. Thereby, bone metastasis is considered to be a serious problem, and the effective therapeutic strategy is urgently needed. Despite many studies attempted to improve bone metastasis treatment, there is still no efficient method to prevent or cure this disease. According to patients fail or temporal respond to the treatments especially patients with overt bone lesions which generally have re-growth of tumor and bone metastases after treatment [9, 10].

Cannabinoids (CBs) are active compounds that are firstly discovered in cannabis (*Cannabis sativa*) [11]. Tetrahydrocannabinol (THC) and cannabidiol (CBD) are natural extracted CB, which showed anti-tumor property in many cancer types including prostate cancer, non-small cell lung cancer, pancreatic cancer and breast cancer [12]. THC treatment decreased breast cancer cell proliferation in MCF-7 and MDA-MB-231 cells [13]. In addition, CBD treatment induced cell death via apoptosis process which reduced MDA-MB-231 viability [14]. Thus, CBs were proposed to be used as anti-tumor compound as they induced program cell death in cancer cells and inhibited cancer cell proliferation [15, 16]. Physiologically CBs act by binding to G-protein-coupled receptors (GPCR) [17]. There are 2 types of CB receptors: CB1 and CB2. Interestingly breast cancer was shown to highly correlate with CB receptor expression [16]. Therefore, the treatment of specific CB receptor agonist would provide the information about the roles of each receptor in anti-tumor effect of CBs on breast cancer development, such as growth and metastasis. However, the roles of CB receptor in controlling those characters are still unclear since THC and CBD potentially bind to both CB1 and CB2 receptor. We aimed to investigate roles of each specific CB receptor agonist breast cancer and its effects on cancer-bone interaction. Thus, we used arachidonyl-2′-chloroethylamide (ACEA) and GW405833, which are the potent synthetic CBs selective for CB1 and CB2 receptor, respectively. This research provides potential role of each type of CB receptor which could be developed into the future treatment for cancer-bone metastasis treatment.

2. Materials and Methods

2.1. Cell culture

There were 2 cell lines used in this study including triple-negative breast cancer cell line, MDA-MB-231, (ATCC, Manassas, VA, USA) and osteoblast-like cell line (UMR-106) provided by Center of Calcium and Bone research (COCAB), Faculty of Science, Mahidol University. They were cultured in Dulbecco's modified Eagle's medium, DMEM, (Gibco, Waltham, MA, USA). The cell culture medium was supplemented with 10% fetal bovine serum, FBS, (Gibco), and 1% penicillin-streptomycin (Gibco). All cell lines were cultured in the incubator at 37°C with 5% CO_2.

2.2. Cell viability assay

MDA-MB-231 cells were seeded into 96-well plate at 10,000 cells/well. However, UMR-106 cells were seeded with the seeding density of 7,500 cells/well into 96-well plate. Twenty-four hours after seedling, cells were treated with Arachidonyl-2′-

chloroethylamide hydrate, ACEA, (Sigma-Aldrich, Saint Louis, MO, USA) or GW405833 (Sigma-Aldrich) at the concentration between 1 to 100 μM. For combination treatments, ACEA was combined with GW405833 at different ratio. After treatment for 48 hours, MDA-MB-231 and UMR-106 cell viability were measured by performing MTT assay with MTT reagent (Invitrogen, Eugene, OR, USA). After 3 hours of incubation, the formazan-dissolving solution was added. The solution consists of 10% SDS (Sigma-Aldrich) and 50% N, N-dimethyl-formamide (Sigma-Aldrich). Formazan color intensity was measured by using microplate reader (Thermo, Multiskan EX) at 595 nm.

2.3. Clonogenic assay

MDA-MB-231 cells were seeded into 6-well plate (Jet Bio-Filtration, GZ, China) at 330,000 cells/well. Then, the plate was incubated overnight to allow cell attachment. Before the treatment, cell mediums will be removed and replaced with serum free medium containing ACEA and GW405833. For the positive control, MDA-MB-231 will be treated with 100 μg/ml of 5-FU (Sigma-Aldrich), which had been reported to inhibit MDA-MB-231 colony formation in previous studies [18]. After that, the trypsinization was performed to collect 800 viable cells and cultured in new 6-well plate.

After 12 days, colonies were fixed with methanol for 30 minutes at 4°C. Then, methanol was removed, and cells were stained with 0.5% crystal violet (Sigma-Aldrich) for 30 minutes. The cells were then gently washed with distilled water for 3 times, and the images of colony were taken using scanner. Colony number was counted using ImageJ software.

2.4. Wound healing assay

MDA-MB-231 cells were seeded into 24-well plate (Corning, Glendale, AZ, USA) at the seeding density of 4×10^5 cells/well. Twenty-four hours after seedling, culture medium was replaced with the complete media containing CB receptor agonists treatment. Then, the scratching wound was made by using P200 pipette tip. The images of the wound area were taken at the same position every 12 hours. The percentage of wound closure was determined by image analysis software, ImageJ.

2.5. MDA-MB-231 conditioned media

MDA-MB-231 cells were seeded into 6-well plate (Jet Bio-Filtration) at 5×10^5 cells. After culturing in the incubator at 37°C and 5% CO_2 overnight. The culture medium was removed and replaced with serum free medium with or without CB receptor agonists for 48 hours. After pre-treatment, the cells were washed with PBS twice, and the medium was replaced with serum free medium for 48 hours. Next, the MDA-MB-231 conditioned medium was harvested and filtered with 0.2 μm filter (Sartorius, Göttingen, Germany). The MDA-MB-231 conditioned medium was kept at -80°C before use.

2.6. Statistical analysis

The results were shown as mean ± standard deviation (SD). The mean values were from at two biological replicates with at least internal duplicates. The treatment groups were analyzed by the multiple comparison of one-way analysis of variance (ANOVA). The difference between pairs of means was analyzed by Fisher's Least Significant Difference (LSD) all pair comparison. The level of significance for all statistical tests was $P < 0.05$.

3. Results

3.1. Both CB1 and CB2 receptor agonists inhibited MDA-MB-231 in a concentration-dependent manner

To investigate the effect of CB receptor agonists, CB1 agonist (ACEA) and CB2 agonist (GW405833) were used. The triple-negative breast cancer cells (MDA-MB-231) were treated with either ACEA or GW405833 between 0-100 μM. MDA-MB-231 cell viability was examined by performing MTT assay after 48 hours of treatment. ACEA decreased MDA-MB-231 cell viability in a concentration dependent manner (IC_{50} = 40.89 μM) as shown in Figure 1. This effect can also be observed when MDA-MB-231 was treated with GW405833. The results indicated that treatment of GW405833 also shown cytotoxic effect on breast cancer cell at concentration dependent manner (IC_{50} = 23.46 μM) (Figure 1). Furthermore, our results suggested that GW405833 likely to show higher cytotoxic effect on MDA-MB-231 than ACEA.

3.2. Pretreatment of CB agonists did not affect MDA-MB-231 colony formation

We next studied the ability of cancer colony formation on 6-well cell culture plate. MDA-MB-231 cells were pre-treated with either ACEA or GW405833 for 48 hours before viable cells were collected for colony formation assay. After 12 days of culture, the results illustrated that pretreatment of ACEA and GW405833 did not affect MDA-MB-231 colony formation as compared to control (Figure 2a). Therefore, CB receptor agonists did not show long term inhibitory effect on MDA-MB-231 cell growth. This can be indicated that at low concentration below IC_{50}, CB receptor agonists cannot suppress MDA-MB-231 cell proliferation and colony formation.

3.3. CB receptor agonists suppressed MDA-MB-231 cell migration

Cancer cell migration is one of the important characteristics for metastatic cancer cells. We investigated the efficacy of using CB receptor agonists to inhibit cancer cell migration by wound healing assay. We decided to use IC_{50} at 72 hours to avoid cell death from CB receptor agonist treatment on MDA-MB-231. The IC_{50} of ACEA and GW405833 at 72 hours after treatment were 30 μM and 15 μM, respectively. There was no difference in percentage of wound closure between treated and untreated groups at 12 hours after treatment. However, at 24 hours after treatment, our results showed significant reduction of percent wound closure in both CB receptor agonist treatment as compared to vehicle control (Figure 2b and 2C). This suggested that at 30 μM ACEA and 15 μM GW405833 suppressed migration ability of MDA-MB-231 cells.

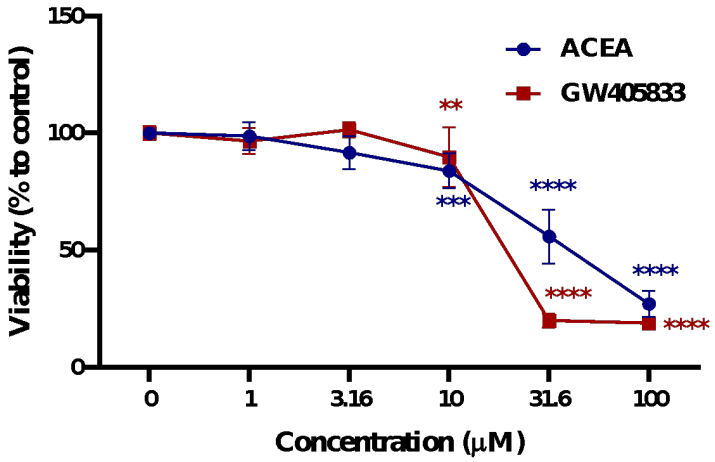


Figure 1. MDA-MB-231 cell viability under CB receptor agonist treatment was examined; ACEA (blue) and GW405833 (red). Both CB receptor agonists showed their cytotoxic effects on MDA-MB-231 in a concentration dependent manner. The data was shown as mean ± SD from two biological replicates with internal triplicates each. The statistical analysis was tested among the same CB receptor agonist treatment by one-way ANOVA. **P < 0.01, ***P < 0.001, ****P < 0.0001 as compared to control group (0 μM).

3.4. High concentration of CB receptor agonists negatively affected UMR-106 cell viability.

Effects of CB receptor agonists on osteoblast cell viability was examined in this experiment. Osteoblast-like cell line (UMR-106) was used in this study. UMR-106 cells were treated with CB receptor agonists for 48 hours before cell viability assay by MTT assay. Our results showed that ACEA treated group was shown to suppress UMR-106 cell viability in a concentration dependent manner (IC_{50} = 25.94 μM) as shown in Figure 3a. Interestingly, the treatment of low concentration CB2 receptor agonist (GW405833) significantly increased osteoblast cell viability, but the high concentration of GW405833 could also inhibit UMR-106 cell survival (Figure 3a). The IC_{50} of GW405833 on UMR-106 was 75.53 μM which was approximately 3-fold higher than that for MDA-MB-231. These results suggested that osteoblast is less sensitive to CB2 agonist than MDA-MB-231 (Figure 1 and 3a).

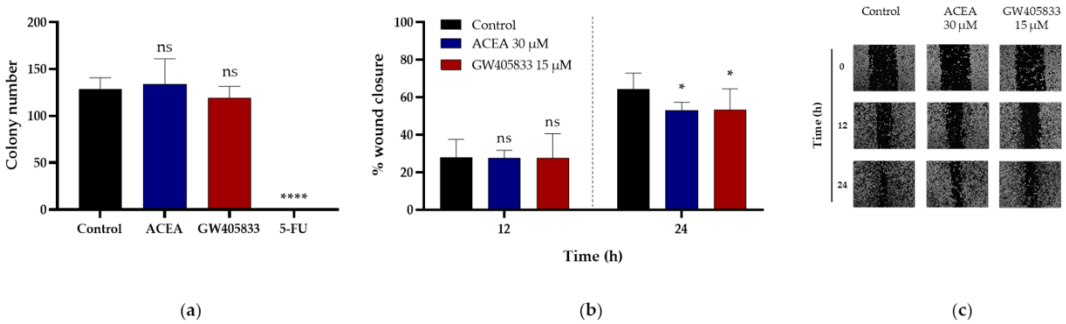


Figure 2. ACEA and GW405833 exhibited inhibitory effect on MDA-MB-231 cell migration but not on colony formation. **(a)** Pre-treatment of 10 μM ACEA and 10 μM GW405833 did not significantly affect MDA-MB-231 colony formation **(b)** Both CB receptor agonists significantly suppressed MDA-MB-231 cell migration after 24 hours of treatment. The percentage of wound closure was compared with their own initial wound area. Bars represented mean ± SD from two independent biological replicates each with internal triplicates, and the statistical analysis was tested by one-way ANOVA. ns; not significant, *P < 0.05, ****P < 0.0001 as compared to control groups (0 μM). **(c)** The images represent wound area at different time point.

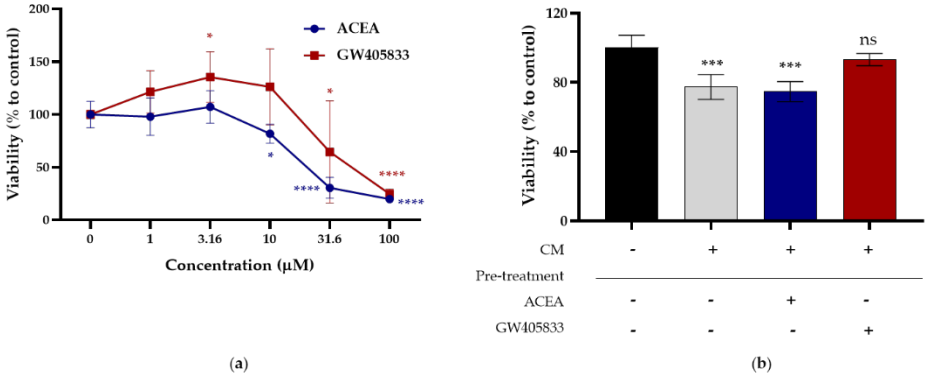


Figure 3. UMR-106 cell viability after 48 hours of treatment of CB agonists. Graphs are plotted as mean ± SD **(a)** At low concentration of GW405833 enhanced UMR-106 cell viability, but high concentration of both CB receptor agonists suppressed UMR-106 cell viability. Data show means ± SD from two biological replicates with internal triplicates each. **(b)** MDA-MB-231 conditioned-media (CM) significantly reduced UMR-106 cell viability represented in grey bar, while pretreatment of GW405833 on MDA-MB-231 before collecting conditioned-media significantly rescued UMR-106 cell viability represented in red bar as compared to untreated CB conditioned-media. Data show means ± SD from two biological replicates with internal duplicates each. The statistical analysis was tested by one-way ANOVA. ns; not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 as compared to control group.

3.5. MDA-MB-231-derived conditioned media suppressed osteoblast cell viability, but CB2 agonist could recover this effect.

We next investigated the effect of indirect interaction between breast cancer and osteoblast cell by culturing UMR-106 in the presence of MDA-MB-231-derived conditioned media. MDA-MB-231 conditioned media significantly reduced UMR-106 cell viability by 25.7% as compared to untreated groups (Figure 3b). Further, the pre-treatment on breast cancer cells with both types of CB receptor agonists at the same concentration with wound healing assay before collecting conditioned media was performed. When MDA-MB-231 was pre-treated with 30 μM ACEA, this did not affect UMR-106 growth suppression by the conditioned media (Figure 3b). Interestingly, pretreatment of 15 μM GW405833 on MDA-MB-231 before collecting the conditioned media significantly rescued the osteoblast cell viability by 18.5% as compared to untreated CB agonist conditioned media. This suggested the potential role of CB2 agonist in breast cancer and bone interaction.

4. Discussion

Our study provided the data regarding the effects of CB receptor agonists, ACEA and GW405833, on MDA-MB-231 cell growth and migration as well as on the interaction between cancer and bone. Our results showed that CB agonists reduced MDA-MB-231 cell viability in a concentration dependent manner. The cytotoxic effect might happen by the activation of program cell death including apoptosis and autophagy. This had been showed in previous study of cannabidiol increased cleavage of PARP and decreased Pro-PARP which are the marker for apoptosis in breast cancer [14]. The comparative cytotoxic effects between CB1 and CB2 receptors agonists showed that breast cancer cells exhibited higher sensitivity to CB2 over CB1 receptor agonist. This corresponded to previous study reported a high CB2 receptor expression in breast cancer [19]. Nevertheless, further study of binding kinetics is also required. Moreover, our results revealed that both CB receptor agonists significantly suppressed breast cancer cell migration. Stimulation of CB1 receptor by ACEA might affect the adhesion molecules as described in previous study that activating of the endocannabinoid (eCB) system in breast cancer by anandamide (AEA) can reduce the expression of FAK and Src, which are important molecules for cell migration [20]. Moreover, the binding of GW405833 with CB2 receptor may disrupt heterodimerization between CB2 receptor and CXCR4 receptor, which potentially inhibit Ga13/RhoA-mediated cancer migration [21]. However, more studies are needed to explore the mechanism behind CB agonist-mediated breast cancer suppression. In addition, our results showed that breast cancer conditioned media caused decreasing osteoblast survival, while the exposure of CB agonist could recover this effect. Therefore, this leads to our future direction to identify the exact factor in MDA-MB-231 conditioned media that induced osteoblast cell death as well as the alteration upon GW405833 pretreatment. Furthermore, the data about direct effects of CB receptor agonists on osteoblast cell function are also crucial. The well-known markers for osteoblast functions including alkaline phosphatase activity, mineralization, osteopontin (OPN) and osteocalcin (OCN) expression should be investigated. These findings are necessary for the future development of the CB receptor agonists as the potential cancer-bone metastasis therapy.

5. Conclusions

In this study, ACEA and GW405833 were utilized CB receptor agonists for CB1 and CB2, respectively. We demonstrated that both CB receptor agonists suppressed triple-negative breast cancer (MDA-MB-231) cell viability in dose-dependent manner with the higher sensitivity for GW405833. Moreover, CB receptor agonists could also suppress MDA-MB-231 cell migration. Nevertheless, at low concentration of CB receptor agonists did not show a long-term suppression in cancer cell colony formation. We also observed

the effect of breast cancer and bone interaction by culturing osteoblast cells (UMR-106) in the presence of MDA-MB-231 conditioned media. We showed that conditioned media induced UMR-106 cell death by 25.7% as compared to control. Nevertheless, pre-treatment of GW405833 on breast cancer cells before collecting the conditioned media could rescue osteoblast cell viability by 18.5% as compared to control condition media. Therefore, CB receptor agonists possessed the anti-tumor effect on breast cancer as well as the therapeutic potential for cancer bone metastasis.

Author Contributions: Conceptualization, K.L.; methodology, K.L., T.K. and C.C.; investigation, K.L., T.K. and C.C.; data analysis, C.C.; writing, review and edit, K.L. and C.C..; supervision, K.L. and N.C.; funding acquisition K.L. and N.C. All authors have read and agreed to publish.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1 Siegel, R.L., Miller, K.D., and Jemal, A. Cancer statistics, 2019, CA Cancer J Clin, 2019, 69, (1), pp. 7-34
2 Akhtari, M., Mansuri, J., Newman, K.A., Guise, T.M., and Seth, P. Biology of breast cancer bone metastasis, Cancer Biol Ther, 2008, 7, (1), pp. 3-9
3 Burr, D. Basic and applied bone biology (Elsevier, 2019, 2nd edition. edn. 2019)
4 Kozlow, W., and Guise, T.A. Breast cancer metastasis to bone: mechanisms of osteolysis and implications for therapy, J Mammary Gland Biol Neoplasia, 2005, 10, (2), pp. 169-180
5 Nakahama, K. Cellular communications in bone homeostasis and repair, Cell Mol Life Sci, 2010, 67, (23), pp. 4001-4009
6 Fornetti, J., Welm, A.L., and Stewart, S.A. Understanding the Bone in Cancer Metastasis, J Bone Miner Res, 2018, 33, (12), pp. 2099-2113
7 Yoneda, T., and Hiraga, T. Crosstalk between cancer cells and bone microenvironment in bone metastasis, Biochem Biophys Res Commun, 2005, 328, (3), pp. 679-687
8 Chen, Y.C., Sosnoski, D.M., and Mastro, A.M. Breast cancer metastasis to the bone: mechanisms of bone loss, Breast Cancer Res, 2010, 12, (6), pp. 215
9 Buijs, J.T., Kuijpers, C.C., and van der Pluijm, G. Targeted therapy options for treatment of bone metastases; beyond bisphosphonates, Curr Pharm Des, 2010, 16, (27), pp. 3015-3027
10 Schmid-Alliana, A., Schmid-Antomarchi, H., Al-Sahlanee, R., Lagadec, P., Scimeca, J.C., and Verron, E. Understanding the Progression of Bone Metastases to Identify Novel Therapeutic Targets, Int J Mol Sci, 2018
11 Grotenhermen, F. Cannabinoids, Curr Drug Targets CNS Neurol Disord, 2005, 4, (5), pp. 507-530
12 Daris, B., Tancer Verboten, M., Knez, Z., and Ferk, P. Cannabinoids in cancer treatment: Therapeutic potential and legislation, Bosn J Basic Med Sci, 2019, 19, (1), pp. 14-23
13 Cridge, B.J., and Rosengren, R.J. Critical appraisal of the potential use of cannabinoids in cancer management, Cancer Manag Res, 2013, 5, pp. 301-313
14 Shrivastava, A., Kuzontkoski, P.M., Groopman, J.E., and Prasad, A. Cannabidiol induces programmed cell death in breast cancer cells by coordinating the cross-talk between apoptosis and autophagy, Mol Cancer Ther, 2011, 10, (7), pp. 1161-1172
15 Guzman, M. Cannabinoids: potential anticancer agents, Nat Rev Cancer, 2003, 3, (10), pp. 745-755
16 Qamri, Z., Preet, A., Nasser, M.W., Bass, C.E., Leone, G., Barsky, S.H., and Ganju, R.K. Synthetic cannabinoid receptor agonists inhibit tumor growth and metastasis of breast cancer, Mol Cancer Ther, 2009, 8, (11), pp. 3117-3129
17 Kiskova, T., Mungenast, F., Suvakova, M., Jager, W., and Thalhammer, T. Future Aspects for Cannabinoids in Breast Cancer Therapy, Int J Mol Sci, 2019
18 Berger, D.P., Henss, H., Winterhalter, B.R., and Fiebig, H.H. The clonogenic assay with human tumor xenografts: evaluation, predictive value and application for drug screening, Ann Oncol, 1990, 1, (5), pp. 333-341
19 Hermanson, D.J., and Marnett, L.J. Cannabinoids, endocannabinoids, and cancer, Cancer Metastasis Rev, 2011, 30, (3-4), pp. 599-612
20 Grimaldi, C., Pisanti, S., Laezza, C., Malfitano, A.M., Santoro, A., Vitale, M., Caruso, M.G., Notarnicola, M., Iacuzzo, I., Portella, G., Di Marzo, V., and Bifulco, M.: Anandamide inhibits adhesion and migration of breast cancer cells, Exp Cell Res, 2006, 312, (4), pp. 363-373
21 Scarlett, K.A., White, E.Z., Coke, C.J., Carter, J.R., Bryant, L.K., and Hinton, C.V. Agonist-induced CXCR4 and CB2 Heterodimerization Inhibits Galpha13/RhoA-mediated Migration, Mol Cancer Res, 2018, 16, (4), pp. 728-739