A Novel Cell Permeable Target Specific Cyclic Adenosine Monophosphate Analogue for Bone Tissue Engineering

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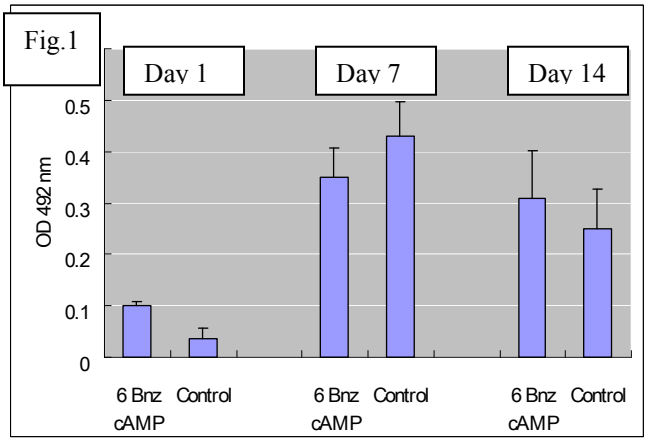
Statement of Purpose: Bone tissue engineering can be defined as the application of biological, chemical, and engineering principles toward the repair, restoration, or regeneration of living tissue using biomaterials, cells, and factors alone or in combination. Of these, bioactive factors for tissue engineering have been extensively investigated worldwide because it is so important for bone tissue formation (osteoinductive). Bone Morphogenetic Protein 2 (BMP-2) is one of the well studied biofactor for tissue engineering. Because of its osteoinductive feature, it has been approved by FDA for clinical purposes. However, there are a number of potential disadvantages of using BMP-2. (1) It is very expensive; (2) It may elicit unwanted immune response to host; (3) It is quite unstable in both thermal and storage conditions; (4) It requires super-physiological concentration that may create side effects to host. Thus, it is in need to find an alternative molecule to overcome the problems. N^6-Benzoyladenosine-3′, 5′- cyclic monophosphate (6-Bnz-cAMP) is a novel target specific analogue of natural cAMP that has been previously shown to bind and activate Protein Kinase A (PKA) signaling cascade exclusively. It is an inexpensive and stable small molecule for cell and molecular biology research purposes. Preliminary data from our laboratory show that the 6-Bnz-cAMP promotes both proliferation (osteo-proliferation) and differentiation of mouse pre-osteoblasts (MC3T3-E1). These observation implicates that 6-Bnz-cAMP may serve as a new bioactive molecule for improving the current bone tissue engineering technology.

Methods: MC3T3E1 subtype 4 preosteoblast cell line (ATCC) was used for proliferation assay and cell differentiation study. Briefly, cells were maintained in tissue culture flask contain minimal essential medium alpha (Invitrogen) supplemented with 10% FBS and 1% of antibiotic at 37°C in a humidified incubator containing 5% carbon dioxide. Cells were grown in osteogenic medium (10 mmol/L beta-glycerophosphate, and 0.05 mmol/L L-ascorbic acid-2-phosphate) for osteoblastic differentiation study. Cells were treated with 6-Bnz cAMP analogue as indicated in the figures. For cell proliferation assay, MTS assay (Promega) was performed according to the manufacturer's instructions. We performed Western Blot analysis for osteoblast marker expression as an indication of differentiation of MC3T3E1 cells. Briefly, treated cells were collected and lysed in 2X Laemmli sample buffer (Bio-Rad) for at least 5 min at room temperature. Protein in the lysates were loaded and resolved on a 12.5% SDS-PAGE (Bio-Rad). The separated proteins were then electrotransferred to a nitrocellulose membrane which in turn probed with primary antibodies as indicated in the figure. Membranes were washed few times in Tris-buffered saline and then incubated with horseradish peroxidase-conjugated secondary antibodies (KPL). Labeled bands were visualized using enhanced chemiluminescence (Pierce) according to the manufacturer's instructions.

Results: Figure 1 revealed that the 6-Bnz-cAMP promotes MC3T3E1 cell growth in the earlier day of cell culture (Day 1) and maintain the cell proliferation rate at Day 7 and Day 14. Error bars represent SD. It is of interests to point out that this is the first report to show the osteo-proliferative effect of a cAMP analogue, while other commonly used cAMP analogs i.e 8-Br-cAMP and db-cAMP have been shown to inhibit cell proliferation including osteoblasts (our unpublished data).

Fig.1



6 Bnz-cAMP induces the expression of Runx2 in the absence of osteogenic medium Samples were collected at day 21 (Fig.2). It is thought that Runx2 serves as a gatekeeper to promote osteogenesis, thus it is one of the most important marker for osteoblastic differentiation. In the control panel, consistent with the reported data in literature, Runx2 was detected in the presence of osteogenic medium but remain undetectable level without the osteogenic medium. Note that the addition of osteogenic medium to the 6-Bnz-cAMP setting further enhanced the expression of Runx2. The blot tubulin serves as loading control for each sample loading.

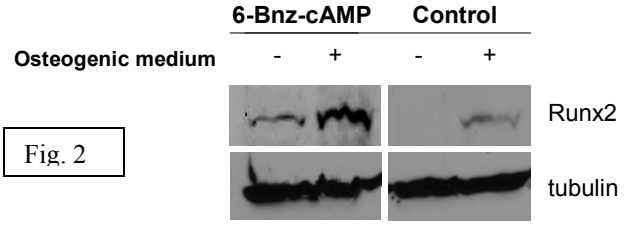


Fig. 2

Conclusion: A more comprehensive *in vitro* cell differentiation study approach is required to evaluate the osteo-inductivity of 6-Bnz-cAMP. Moreover, better cell model system i.e human stem cells will be used to address the question. Nevertheless, this is the first report on the positive effects of a target-specific cAMP analog on osteoblast's proliferation and differentiation.