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Novel oxysterols activate the Hedgehog pathway and induce osteogenesis

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ABSTRACT

Localized induction of bone formation is essential during orthopedic procedures that involve skeletal repair, such as surgical treatment of non-union bone fractures and degenerative disk disease. Herein we disclose the synthesis and biological evaluation of novel oxysterol derivatives designed as anabolic bone growth agents. Structure–activity relationship studies of oxysterol **4** have identified analogues such as **18**, **21** and **30**. These new analogues are characterized by higher potency in an osteoblast differentiation assay and/or by increased metabolic stability in human liver microsomes. Oxysterols **4**, **18** and **21** were evaluated in vivo in a rat spinal fusion model.

Oxysterols[1] are defined as oxygenated metabolites of cholesterol. Low concentrations of oxysterols occur naturally in the mammalian blood circulation and various tissues, commonly as short lived intermediates implicated in important metabolic transformations of cholesterol such as the biosynthesis of steroid hormones and bile acids. Beyond their role as passive metabolites, oxysterols can function as signaling molecules capable of modulating a range of physiological phenomena, among them transport and homeostasis of lipids as well as control over cellular states such as differentiation, inflammation and apoptosis.[2] Oxysterols have also been cited to play a role in the pathogenesis of human diseases (for example, atherosclerosis, Alzheimer's disease, and diabetes mellitus) and their occurrence and distribution in the body may be characteristically altered by age and disease.[3] To account for their wide ranging biological effects, oxysterols bind complementary protein targets that often serve as physiological sensors.[4] For example, liver X receptors (LXRα and β) and sterol regulatory element binding proteins (SREBPs) are involved in the mechanisms by which animal cells maintain the proper levels of intracellular lipids and cholesterol.[5]

Numerous reports have noted a role for oxysterols as mediators of cellular differentiation, such as the differentiation of multipotent mesenchymal stem cells (MSCs).[1,6] Specific oxysterols such as 22(*S*)-hydroxycholesterol can induce the differentiation of MSCs into cells expressing the osteoblast markers, alkaline phosphatase and osteocalcin, while inhibiting their differentiation into adipocytes.[7] This process was shown to be mediated by the Hedgehog (Hh)-pathway, a signaling pathway linked to bone metabolism not only during embryonic development but also in postnatal maintenance of bone integrity and function.[8,9] Decreased anabolic mineralization of adult bone tissue can lead to osteoporosis and impaired healing during skeletal injury, both serious health issues affecting elderly populations.[10] Small molecule agonists of Hh- signaling[11,12] may therefore be useful therapeutic agents in orthopedic medicine.[13]

Localized induction of bone formation is of critical importance in orthopedic procedures that involve skeletal repair, for example, during surgical intervention in non-union bone fractures and degenerative disk disease. Various applications and devices containing bone morphogenetic protein (BMP-2 or BMP-7)[14] have demonstrated clinical efficacies comparable to autogenous bone grafts; however, adverse events have raised safety concerns regarding the widespread use of expensive, recombinant BMP protein. Thus motivated in the quest for small molecule osteoinductive substances that are safe and cost effective, we have studied the use of naturally occurring oxysterols, 22(*R*)-hydroxycholesterol (**1**), 20(*S*)-hydroxycholesterol (**2**), and 22(*S*)-hydroxycholesterol (**3**), depicted in Scheme 1, as potential osteogenic agents.[15]

An inflection point was reached in our studies[16] when a more potent oxysterol analog was identified with lead compound **4**, which differs from 20(*S*)-hydroxycholesterol (**2**) in the additional α-hydroxyl group at C-6 (Scheme 1). Compound **4**, to the best of our knowledge, is not a naturally occurring oxysterol, unlike **1**–**3**,

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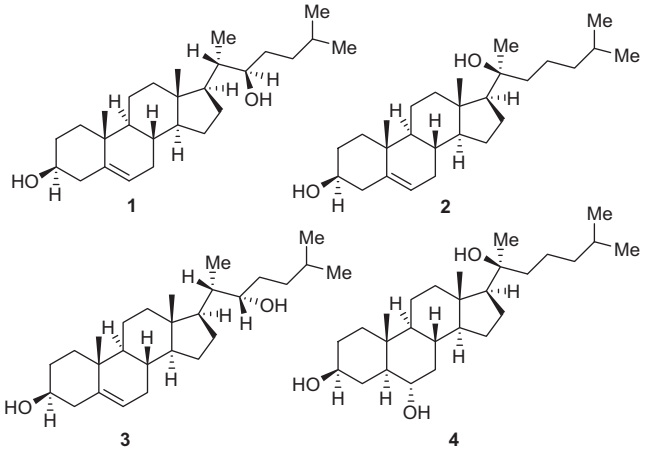
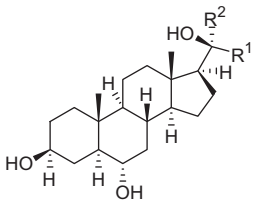


Scheme 1. Potential oxysterol osteogenic agents.

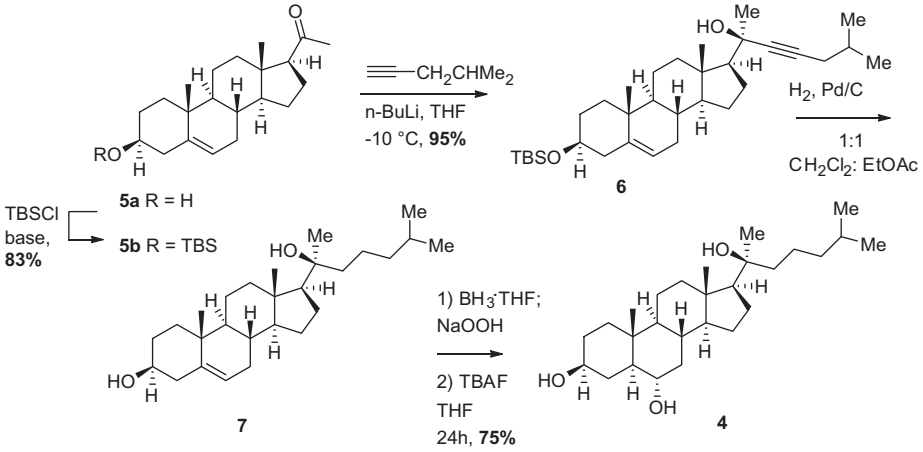
Table 1
SAR of the side-chain



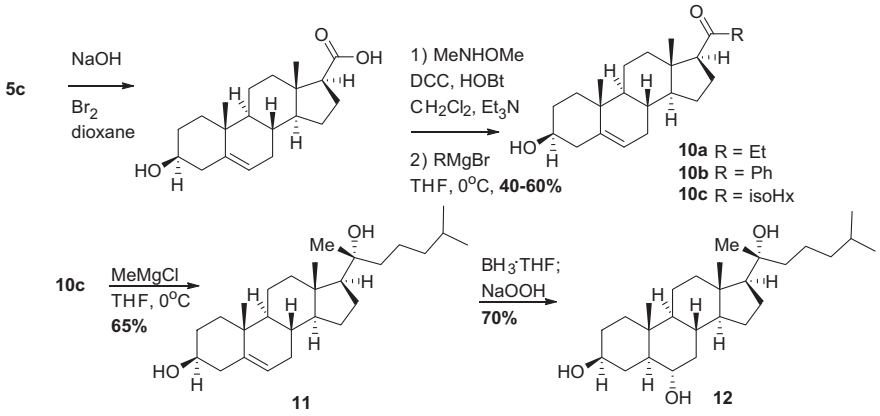
Compd	R^1	R^2	Hh light 2 EC_{50}[a] (μM)	OCN fold induct. at[b] 3.3 μM	HLM stab. % left at 1 h
4	IsoHx	Me	0.8	15	3
21	*n*-Hx	Me	0.4	36	2
22	*n*-Heptyl	Me	0.5	17	—
23	*n*-Pentyl	Me	6.6	4	—
24	$(CH_2)_3Ph$	Me	2.5	2	1
25	$(CH_2)_3$-3-Pyridyl	Me	1.2	—	—
26	$(CH_2)_3$-4-Pyridyl	Me	>5	2	—
27	*n*-Hx	Et	2.9	—	9
28	*n*-Hx	Ph	>10	—	69
12	Me	IsoHx	>10	—	—
29	$(CH_2)_4OMe$	Me	3.1	3	35
18	$(CD_2)_2CH_2CH(CH_3)_2$	Me	0.8	17	100
30	$(CD_2)_2(CH_2)_3CH_3$	Me	0.3	35	100

[a] Average values ($n > 2$).
[b] Measured after 10 days at 0.3, 1.1, and 3.3 μM.

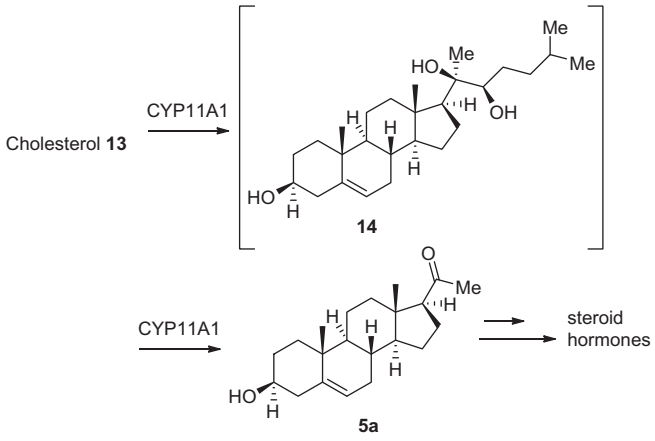
and its chemical synthesis was first reported by Djerassi et al., in 1973.[17] During biological characterization, we have shown **4** to be efficacious as a single agent both in vitro and in vivo. In M2-10 B4 marrow stromal cells, compound **4** activated the Hh-pathway and induced the expression of both early osteoblast markers, alkaline phosphatase, runx2, osterix, and markers of more mature osteoblasts, bone sialoprotein and osteocalcin. In addition, compound **4** induced robust new bone formation when dosed in an in vivo model of rat spinal fusion.[16] In this paper, we wish to discuss the synthesis and biological evaluation of additional analogues in this new class of synthetic oxysterols which can activate the Hh-pathway and induce the osteoblastic differentiation in a multipotent mouse cell line, C3H/10T1/2. In our efforts to develop structure activity relationships for **4**, we have investigated side-chain modifications in the C20, 22 region of the molecule with the aim to identify molecules with greater in vitro activity as well as improved local metabolic stability. Compound **4** displays a side-chain constellation where the C-20 hydroxyl group is flanked by a methyl-group and an isohexyl-group in the *S*-configuration. By varying the nature, size and stereochemistry of the appropriate substituents, we sought to understand the resulting consequences of these variations with respect to activation of the Hh-pathway, measured by upregulation of the mediating transcription factor, Gli, in a SHHLight2 reporter cell line.[18] The ability of test compounds to stimulate osteogenesis was measured in vitro using a murine C3H/10T1/2 cell line, tracking the transcriptional upregulation of several key markers, but especially the mature osteoblast marker, osteocalcin (OCN)[19] 10 days into the differentiation process (Table 1). As our synthetic route readily allowed for variation of the large substituent (R^1, Table 1), we turned our attention first to the isohexyl side-chain. In accordance with Djerassi's original report,[17] we found that compound **4** and its congeners can be prepared starting from commercially available pregnenolone (**5a**). The synthetic sequence involves protection of the C-3 hydroxyl group, addition of a Grignard reagent, followed by hydroboration/oxidation of the C-5,6 olefin and suitable deprotection. The diastereoselectivity of nucleophilic addition to pregnenolone derivatives (**5ab**) is governed by the sterol substrate, so that the major products of Grignard additions to the C-20 carbonyl of **5b** correspond to tertiary alcohols in the desired 20(*S*)-configuration, as predicted by the Felkin-Anh model.[20] Alternatively, alkynyl lithium species can be added to **5b** in excellent yield and diastereoselectivity. A synthesis for compound **4** is shown in Scheme 2, which was applied to most other analogues shown in Table 1, which summarizes the results.



Scheme 2. Synthesis of oxysterol **4**.



Scheme 3. Synthesis of oxysterols **10abc** and **12**.

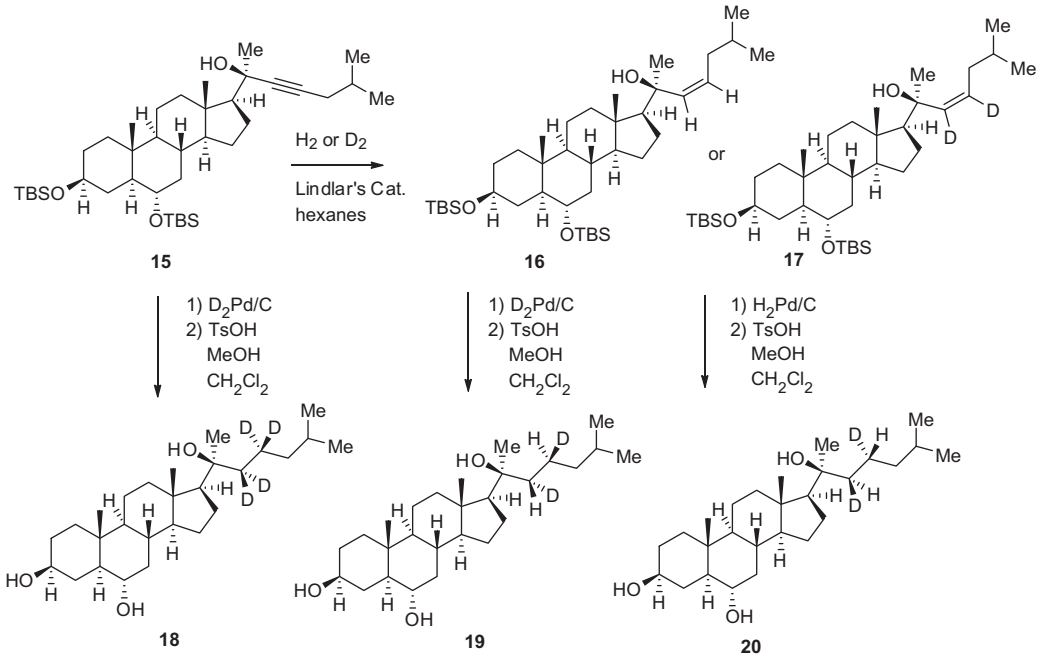


Scheme 4. Enzymatic degradation of oxysterols, for example, cholesterol.

Generally speaking, excessive polarity is not tolerated in the side-chain region (R^1/R^2) of the molecule, with the notable exception of some heterocyclic substitutions (compounds **25** and **26**). Differences in activity can be discerned for substituted alkyl derivatives with a slight preference for the *n*-hexyl (compounds **21** and **30**) when compared to the isohexyl (compound **4**) and phenpropyl derivatives (compound **24**). Next, we addressed the role of the C-21 methyl-group which is in close proximity to the C-20 hydroxyl group. Although, chain extension to the ethyl group was tolerated at C-21, the introduction of larger groups such as phenyl was not (compounds **27** and **28**), whereas the C-20-*R* tertiary alcohol was inactive (compound **12**). Compounds **27**, **28** and **12** were prepared according to chemistry depicted in Scheme 3 starting with the corresponding substituted ketones, **10a–c** (Scheme 3).

The latter were available from pregnenolone acetate, (**5c**), in 3 steps via a haloform reaction,[21] Weinreb-amidation and Grignard addition, as shown in Scheme 3. The synthesis of the C-20-*R* tertiary alcohol **12** was accomplished via Grignard reaction of the ketone **10c**, followed by hydroboration.

Stability data in human liver microsomes (HLM)[22] obtained for some of the compounds listed in Table 1 indicates that metabolic stability is lacking for lead compound **4** and close analogues such as compound **21** (3% and 2% remaining after 1 h incubation). The



Scheme 5. Synthesis of deuterated analogues.

increasing HLM stability exhibited with compounds **27** and **28** suggests that side-chain-substitution can affect metabolic stability, designating this region of the molecule as a possible site for cytochrome P450 (CYP)-metabolism. Among the multitude of CYP enzymes, CYP11A1,[23] is known to specialize in the tissue specific conversion of cholesterol (**13**) to pregnenolone (**5a**), via a side-chain cleavage reaction illustrated in Scheme 4. The rate limiting step in this process is the formation of intermediate **14**, 20(α), 22(*R*)-dihydroxycholesterol. The oxidation is believed to occur in a stepwise manner, first at C-22, then at C-20 followed by rapid cleavage of the glycol, releasing pregnenolone (**5a**) and isocaproic aldehyde.[23] Given the structural analogy of intermediate **14** and the lead compound **4**, we surmised that the presence of the 20(*S*)-hydroxyl group in **4** could lower the activation barrier toward similar metabolic degradation (via a putative intermediate analogous to **14**), resulting in low HLM stability of **4**. In this context, the synthesis of deuterated analogs of **4** was explored, given the ready access to various alkyne intermediates (Schemes 2 and 5). Remarkably, compound **18**, the C-22, C-23-tetradeuterated analog of **4**, displayed significantly increased HLM stability while retaining all potency in the Light2 and C3H10T1/2 assays (Table 1). Similar results were obtained for **30**, the tetradeuterated version of **21** (Table 1). In order to examine the stabilizing kinetic isotope effect[24] of the deuterium substitution in more detail, analogs of compound **4** were prepared in which deuterium atoms were introduced stepwise in a stereo-selective manner, as depicted in Scheme 5 with the synthesis of compounds **18**, **19** and **20**. Alkyne **15** was fully deuterated to afford **18**, or subjected to a partial reduction with Lindlar's catalyst using either hydrogen or deuterium gas. The partially reduced products **16** and **17** were then further reduced using hydrogen or deuterium gas in reverse order, to afford, after deprotection, **19** and **20**.

The stereochemical outcome can be rationalized based on conformational preferences ('inside alkoxy effect') of partially reduced products **16** and **17**,[25] which direct the second reduction, as suggested in Figure 1. In this way, the atom that is added to C-22 during the Lindlar reduction of the alkyne winds up on the re-face of the fully reduced molecule and determines the HLM stability of the product: hydrogen, in the case of **19**, and, deuterium, in the case of **20**. As depicted in Table 2, compounds **4**, **18**, **19** and **20** performed similarly in the Light2 assay, but displayed significant differences in the rates of intrinsic clearance in HLM. The increased stability of compounds **18** and **20** relative to compounds **4** and **19** supports the notion that a stereoselective C–H bond cleavage at C-22 could be involved during a rate limiting step of HLM clearance.

While HLM stability measurements are meant to model hepatic clearance, very little can be inferred from this data regarding metabolism in the local bone environment. Cholesterol and steroid metabolism is prominently associated with non-bone tissues (e.g., liver, adrenal gland); however, osteoblasts are known for their localized production of steroid hormones and their precursors.[26] Recently, Teplyuk, et al., have reported that in differentiating and mature osteoblasts the osteogenic marker Runx2 robustly stimulates the expression of a non-mitochodrial isoform of CYP11A1, expressed in the cytosol and nucleus.[27] The activity of this enzyme could not only stimulate localized production of steroid hormones, but also account for degradation of oxysterols in the bone

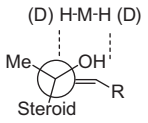
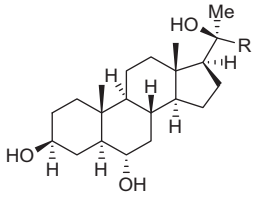
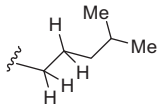
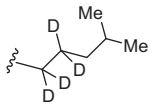
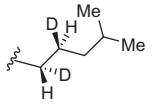
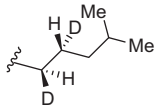


Figure 1. Inside-alkoxy effect for addition to steroidal alkenes.

Table 2
Deuteration of the side-chain and HLM stability



Compd	R	Hh light 2 EC_{50} (μM)	Intrinsic clearance in HLM $t1/2$ (min)
4		0.8	4[a]
18		1.2	>120[b]
19		1.1	5[a]
20		1.0	>120[b]

[a] Measured after 0, 15, 30, 45 and 60 min.
[b] Measured after 0, 30, 60, 90 and 120 min. Pooled liver microsomes with NADPH, pH 7.4, 37 °C.

environment. Conceivably, deuterated oxysterol analogues such as compound **18** and **30** may attenuate this process, potentially resulting in improved in vivo performance. In order to correlate in vitro properties such as potency and metabolic stability with in vivo efficacy, compounds **4**, **18**, and **21** were selected further study. Compared to lead compound **4**, deuterated analogue **18** displays greater HLM stability, whereas compound **21** is more a potent inducer of osteocalcin production (Table 1).

The in vivo effects of compounds **4**, **18**, and **21** were evaluated locally in a spinal fusion model in rats.[28] Each compound was dissolved in DMSO to a concentration of 50 mg/mL, and 40 μL of the dissolved compound was added to a collagen sponge. The compound/sponge was incubated at room temperature for 1–2 h before implantation. Sprague Dawley rats were implanted bilaterally between the L4 and L5 segments after decorticating transverse processes at these segments. Decorticating provides bone marrow MSCs in the fusion site. Eight weeks after implantation, the

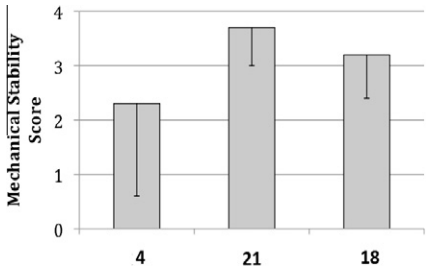


Figure 2. Mechanical stability of treated lumbar vertebral segments assessed by manual palpation. The positive control, **4**, and the test compounds, **21** and **18**, all induced partial vertebral fusion (mean ± SD, n = 10/treatment group).

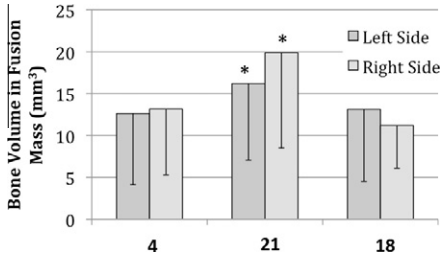


Figure 3. New bone formation at the site of implant. Comppound **21**-treatment induced more bone formation than the positive control, **4** (*$p < 0.05$, Dunnett's multiple comparison test, mean ± SD, $n = 10$/treatment group).

lumbar vertebral segment of each animal was excised and evaluated. For mechanical stability testing, two investigators evaluated the extent of fusion in freshly isolated samples by manual palpation, and scored the extent of fusion as described by Qiu, et al.[29] In this scoring system, no detectable new bone is scored as 0, and complete fusion is scored as 4, with grades of partial fusion scored as 1, 2 or 3. After fixation in 10% neutral buffered formalin, the excised tissue was assessed for new bone formation by microCT. The samples were scanned at 18 μM resolution. In this experiment, **21** and **18** were tested in parallel with **4** at 4 mg/kg. We had previously observed that this dose of **4** is submaximal in the rat spinal fusion model,[16] and therefore provides a window for measuring improved efficacy relative to this positive control. As shown in Figure 2, mechanical stability testing of treated vertebral segments did not reveal a difference between **21** or **18** and **4**. A trend towards higher scores for the **21** and **18** groups compared to **4** was noted, but did not reach statistical significance when analyzed by Kruskal-Wallis testing,[30] a nonparametric analysis of variance.

MicroCT analysis revealed a larger bone volume in the callus of **21**-treated rats compared to **4**, while **18** treated animals were comparable to **4**-treated rats (Figure 3).

In conclusion, a tentative correlation of in vitro potency (Hh-activity and OCN induction, Table 1) and a single in vivo efficacy outcome (Bone Volume, Figure 3) was observed with oxysterol analogues **4** and **21**, affirming the importance of potency optimization. Dosed at 4 mg/kg in a rat spinal fusion model, deuterated analogue **18** did not produce a significantly enhanced response compared to parent compound **4**, suggesting that in vivo efficacy may not be exclusively limited by metabolic stability. These results also suggest that in vivo efficacy of **4** may not be a consequence of metabolic activation involving the side-chain at C-22 and C-23. We will report additional data on compounds **21**[31] and **30** in due time.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.07.073.

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18. SHHLight2 assay: Activation of the Hedgehog pathway was measured using the SHHLight2 reporter cell line. This reporter line contains a concatemer of 8 Gli response elements upstream of a minimal promoter driving the expression of firefly luciferase. The cells constitutively express Renilla luciferase for normalization of the firefly luciferase signal. SHHLight2 cells were plated at 10,000 cells/well in 384 well plates with 20 μL/well of DMEM/10% FCS. 24 h after plating, cells were treated 20 μL/well of test compound in DMEM/0.5% FCS. Triplicate wells were used for each treatment. After 24 h of treatment, firefly luciferase and Renilla luciferase were measured with Promega's DualGlo reagent. The firefly signal in each well was divided by the Renilla signal in that well to normalize for cell number, and results are expressed relative to cells treated with the DMSO solvent control.
19. C3H/10T1/2 Osteogenesis assay: The ability of test compounds to stimulate osteogenesis was measured in vitro using the C3H/10T1/2 cell line. This multipotential cell line is a model of mesenchymal stem cells, and can be differentiated into osteoblasts, chondrocytes, myoblasts and adipocytes. C3H/10T1/2 cells were plated in 24 well plates and cultured to confluence in DMEM/10% FCS. After reaching confluence, duplicate wells of cells were treated 3 times per week for 2 weeks with test compound in fresh DMEM/10% FCS. Total RNA was prepared using the Qiagen RNeasy Plus kit, and cDNA was created from total RNA using invitrogen superscript III kit. Quantitative RT-PCR was performed on cDNA samples using ABI Taqman assays, with the ΔΔCt method, to measure the expression levels of osteoblast genes, BGLAP and IBSP. Results were normalized to GAPDH and expressed relative to cells treated with the DMSO solvent control: Dworetzky, S. I.; Fey, E. G.; Penman, S.; Lian, J. B.; Stein, J. L.; Stein, G. S. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 4605.
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