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Small molecules with potent osteogenic-inducing activity in osteoblast cells

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ABSTRACT

A chemical screen of 45,000 compounds from a diverse collection led to the identification of two series of small molecules with potent osteogenic activity in mouse MC3T3-E1 osteoblast cells. The first chemical group was characterized by an amino benzothiazole core (AMG0892 series) and the second group by a naphthyl amide core (AMG0309 series). Using alkaline phosphatase (ALP), osteocalcin (OCL) and calcium as markers of osteoblast differentiation and mineralization, both chemical series showed EC_{50}s in the 0.01–0.2 μM range and were consistent for all three markers. Compounds inhibited cell proliferation, had no effect on apoptosis and showed evidence for CREB pathway activity. The present compounds represent some of the most potent osteogenic small molecules reported to date and provide new tools for elucidating signaling mechanisms in osteoblasts.

The identification of small molecule bone anabolic agents capable of increasing bone mass represents a major unmet medical need in the area of osteoporosis and bone disease. Several small molecules with osteogenic activity have been described but few if any showed EC_{50}s in the sub-micromolar range. Previously it was reported that the osteogenic compound purmorphamine was active at 1 μM in C3H10T1/2 mesenchymal cells.[1] Here we describe the identification of two potent chemical series with osteogenic activity and cellular EC_{50}s in the 0.01–0.2 μM range. The first was characterized by an amino benzothiazole core (AMG0892 series) and the second by a naphthyl amide core (AMG0309 series).

Bone formation is driven by the activity of osteoblasts and the production of a mineralized matrix. However, in bone diseases such as osteoporosis, the rate of bone resorption by osteoclast cells exceeds the rate of formation by osteoblasts resulting in a net loss of bone. Thus there is a major interest in the identification of therapeutic small molecules that can enhance osteoblast activity and result in a net increase in skeletal bone formation.

A chemical screening approach was used previously to identify the compound purmorphamine that increased alkaline phosphatase (ALP) activity in C3H10T1/2 cells.[1] Expression of Runx2 which is essential for bone formation[2] was also increased but less so in the more differentiated osteoblast progenitor cell line MC3T3-E1. Purmorphamine in the 1–3 μM range was also shown to increase ALP and bone nodule formation in human osteoblasts derived from bone marrow mesenchymal cells.[3] Subsequent work on the mechanism of action of purmorphamine revealed its role as a smoothened agonist in the hedgehog pathway.[4,5]

ALP expression is a marker that typically correlates with the early phase of osteoblast differentiation. To search for compounds that also potentially affected osteoblast function, we designed a screen in mouse MC3T3-E1 osteoblast cells that also examined the mineralization response. A chemically diverse collection of approximately 45,000 compounds was screened measuring both ALP activity and calcein fluorescence that was used as a surrogate for calcium incorporation into mineralizing cultures. The screen yielded 558 hits of which 2% were positive for both ALP and calcein fluorescence. Analogs (>98% purity) of selected hits were subsequently tested in secondary assays that measured ALP, osteocalcin (OCL) a marker of late osteoblast differentiation and calcium directly for evidence of mineralization.

As shown in Figure 1, after 3 weeks in culture, several compounds from two chemical classes stimulated osteogenesis with EC_{50}s for ALP, OCL and calcium in the 0.01–0.2 μM range. Effects were evident within 1 week of treatment (data not shown). The structures of the benzothiazole and naphthyl amide series are shown in Figure 2. Preliminary structure–activity relationships (SAR) of examples from both series are shown in Table 1. For the benzothiazole analogs, fluorine substitution on the phenyl group of the benzyl amine was well tolerated (AMG0895), while conversion of the amine to an amide (AMG9594) resulted in a significant

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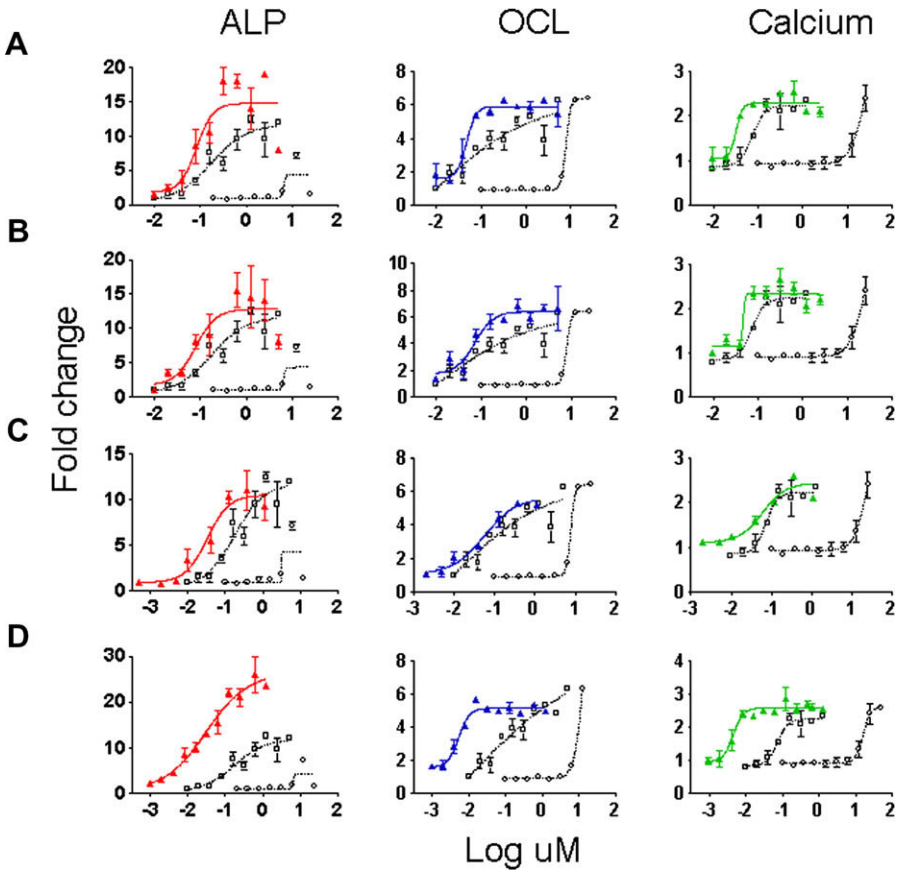


Figure 1. Induction of osteogenesis by (A) AMG0892 and (B) AMG0895 (benzothiazole series) and, (C) AMG6168 and (D) AMG0309 (naphthyl amide series). Original hit (AMG2888, open black circles) and intermediate analog (AMG5581, open black squares).

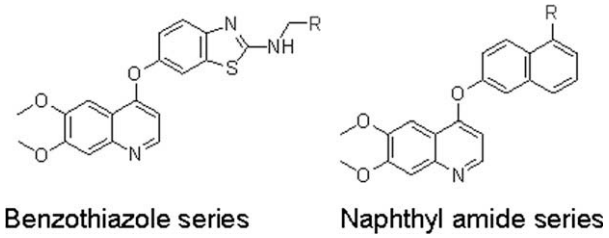


Figure 2. Chemical structures of the benzothiazole and naphthyl amide series.

drop in activity. In the naphthyl amide series, incorporation of other aromatic amines (AMG6168) as well as the corresponding 'reverse amide' (AMG4500) resulted in a slight drop in activity relative to the potent compound AMG0309. Both AMG1051 and AMG6610 showed little or no activity in the cellular assays (Details on compound synthesis are shown in Supplementary material). Alizarin Red and von Kossa staining were also increased in MC3T3-E1 cultures providing further qualitative evidence for enhanced mineralization (data not shown). Compounds were also active in 1- to 3-day-old primary rodent calvarial cells that contain a mixture of osteoprogenitor cells and differentiated osteoblasts (Supplementary material).

The consistency shown by the compounds in having potent effects on three independent markers suggests a robust effect on osteoblast differentiation. Other small molecules in addition to purmorphamine, have been reported to increase osteogenesis but in most cases at much lower potencies. For example, TAK-778 was active at 1–10 μM,[6] statins at 0.1–5 μM,[7,8] 91E2 at 5 μM,[9] PGE-551609 at 10–100 μM,[10] TH at 1 μM[11] and OIC-A006 at 6 μM.[12] However other studies have shown more potent effects with the immunosuppressants Rapamycin (50 nM),[13] FK506 (10-100 nM),[14,15] and cyclosporin A (10–500 nM).[16] In the latter example, the effects of cyclosporin A were biphasic, showing osteogenic effects only at lower concentrations.

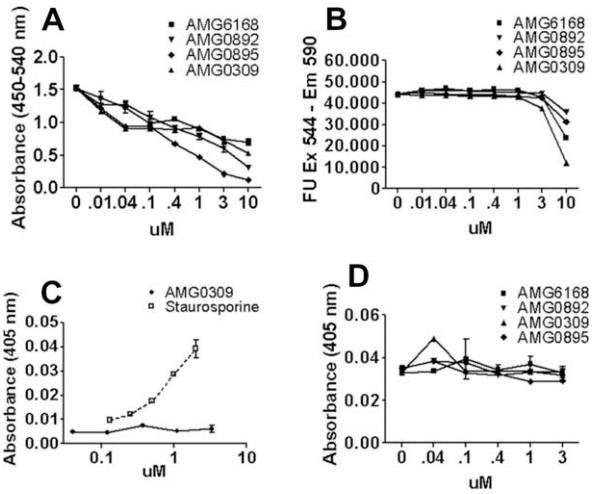
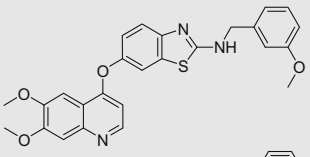
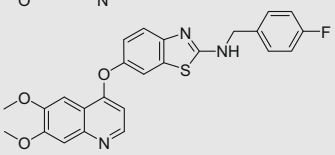
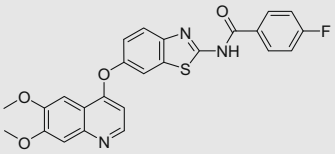
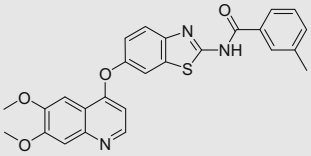
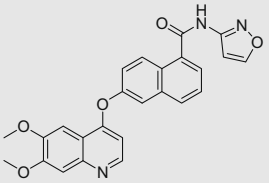
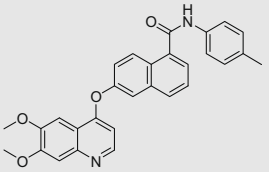
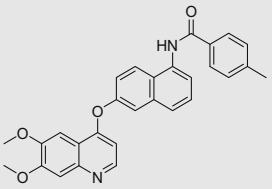
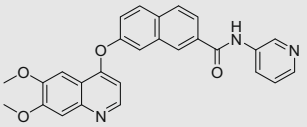


Figure 3. Compounds dose-dependently inhibit cell proliferation (A), show no cytotoxicity except at the highest concentration (B), do not increase apoptosis (C) and do not inhibit apoptosis in Staurosporine-induced MC3T3-E1 cells (D).

Table 1
Structure–activity relationship of osteogenic compounds on markers measured in MC3T3-E1 cells at 3 weeks

Compound	Structure	ALP (μM)	OCL (μM)	Calcium (μM)
AMG0892		0.09	0.06	0.09
AMG0895		0.15	0.09	0.21
AMG9594		0.52	0.41	0.51
AMG1051		>20	>20	>20
AMG0309		0.04	0.03	0.03
AMG6168		0.09	0.03	0.05
AMG4500		0.12	0.10	0.15
AMG6610		5.0	>20	>20

To confirm the molecular basis for the osteogenic effects, several RNA markers (Mouse Osteogenesis RT^2 Profiler PCR Array, SABiosciences, Frederick, MD) were evaluated after MC3T3-E1 cells were treated with AMG0892 and AMG0309 at 0.75 μM or with BMP2 at 50 ng/ml as a positive control for up to 21 days. In addition to ALP, several genes involved in osteoblast differentiation including BMP3, Dmp1, Phex, and Sclerostin were significantly changed in a manner that resembled the changes seen for the potent osteogenic molecule BMP2 (Supplementary material). Furthermore, there was a transient 4- to 5-fold increase in expression of the key osteogenic gene Runx2 which was similar to the upregulation observed with purmorphamime in C3H10T1/2 cells.[1]

As an initial step in characterizing the mechanism underlying the osteogenic effects, we examined the ability of the compounds to regulate cell proliferation and apoptosis. The results in Figure 3 showed that both series of compounds strongly inhibited cell proliferation measured by BrdU incorporation and did not alter cell viability measured by Alamar Blue labeling. In addition, the compounds had no effect on apoptosis measured either by an increase in caspase-3 activity or a decrease in caspase-3 activity following Staurosporine-induced apoptosis. This finding thus suggests a link between inhibition of cell growth and enhancement of osteoblast differentiation.

Since it is known that BMP and Wnt signaling are important for osteoblast differentiation,[17,18] we tested whether the compounds might have effects via these two pathways. Stable MC3T3-E1 cell lines expressing BMP2[19] and Wnt[20] response elements linked to luciferase were treated with compounds at doses up to 3 μM for periods up to 3 weeks. A similar Wnt reporter was used previously in a chemical screen to identify small molecule modulators of Wnt signaling.[21] Interestingly, we did not observe any effects of the compounds in any of these assays at any time point, thus raising the possibility that other pathways may be involved in the osteogenic response (data not shown).

Surprisingly, however, the osteogenic compounds were observed to activate the CREB pathway in MC3T3-E1 cells as shown using a CREB response element (Clontech, Mountain View CA) linked to luciferase (Fig. 4). A dose-dependent increase in luciferase activity was noted, in contrast to the lack of response from two negative control compounds (AMG6610 and AMG1051) that were structurally similar but possessed very low cellular activity.

The cellular activities of the two chemical series provide a convenient starting point for exploration of the mechanism of action of the compounds in a manner similar to that for purmorphamine.[1] Moreover, the greater cellular potency and distinct chemical structures of the current compounds compared to purmorphamine raise the possibility that the molecular target(s) is also unique. Other work has shown that tool compounds identified via chemical genetic screens and that demonstrate functional activity can be used as the basis to identify novel targets.[22–26] Thus different compounds have been used in affinity chromatography approaches

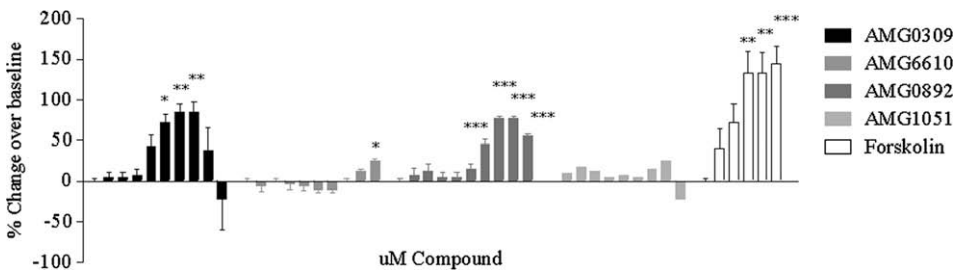


Figure 4. Osteogenic compounds activate CREB pathway. A compound dose-response at 1:3 dilution was used up to 3 μM. Negative control compounds for the benzothiazole (AMG1051) and naphthyl amide (AMG6610) series were structurally similar but showed very little cellular activity. Forskolin used as positive control at 1:2 dilution up to 1 μM. Means ± SEM for two experiments in duplicate. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control.

for target enrichment and protein sequencing by mass spectrometry. It is important to note that in the current work, the chemical hits were not optimized for osteoblast cellular activity or target specificity but instead provide a starting point for such studies.

Thus the present compounds represent some of the most potent osteogenic small molecules reported to date and provide new tools for elucidating signaling mechanisms in osteoblasts.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.025.

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