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The role of small molecules in musculoskeletal regeneration

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Abstract

The uses of bone morphogenetic proteins and parathyroid hormone therapeutics are fraught with several fundamental problems, such as cost, protein stability, immunogenicity, contamination and supraphysiological dosage. These downsides may effectively limit their more universal use. Therefore, there is a clear need for alternative forms of biofactors to obviate the drawbacks of protein-based inductive factors for bone repair and regeneration. Our group has studied small molecules with the capacity to regulate osteoblast differentiation and mineralization because their inherent physical properties minimize limitations observed in protein growth factors. For instance, in general, small molecule inducers are usually more stable, highly soluble, nonimmunogenic, more affordable and require lower dosages. Small molecules with the ability to induce osteoblastic differentiation may represent the next generation of bone regenerative medicine. This review describes efforts to develop small molecule-based biofactors for induction, paying specific attention to their novel roles in bone regeneration.

Keywords

bone regeneration; growth factor; orthopedic; osteogenesis; small molecule; tissue engineering

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Bone growth factors

Bone growth factors are usually proteins secreted by cells that provide a driving force for osteoblast functions including proliferation and maturation. The mechanism of action of bone protein growth factors is to interact with a membrane receptor on a target cell and trigger an intracellular signal transduction system that ultimately induces the expression of bone-specific genes in the nucleus and protein production in the cytoplasm (Figure 1A) [1].

Stimulation of bone formation using bone protein growth factors is an established approach to treating musculoskeletal problems due to trauma, congenital deformities and specific bone diseases such as osteoporosis [1]. Particularly, bone morphogenetic proteins (BMPs) have been extensively used and studied due to their osteoinductive capacity – that is, the ability to induce ectopic bone formation [2,3]. BMP-2 and BMP-7 have obtained US FDA clearance for specific clinical applications such as spinal fusions and long-bone nonunions [4–6]. More recently, PTH 1–34 was approved by the FDA as an anabolic drug for the treatment of osteoporosis [7–10]. While successful, the uses of protein-based therapeutics have their drawbacks. The current recombinant BMPs and PTH medications are produced by *Escherichia coli* using recombinant DNA technology [8,11]. One of the major concerns of this technology is that the recombinant protein produced from *E. coli* is not glycosylated, as is normal in the body, and thus may present reduced stability and biological activity [12]. Additionally, since trace amounts of biologically active impurities from *E. coli* can jeopardize therapeutic applications, contamination can be another issue [13]. Additionally, the costly processes of recombinant protein production and purification make protein-based therapy very expensive [8,14,15]. Furthermore, the immunogenicity of PTH and BMPs have been reported as potentially eliciting undesirable immune responses in patients [12,16–18]. Although other protein growth factors such as FGF, IGFs, TGF- β and PDGF have shown potential for use in bone repair and regeneration [1,19–22], they too have specific drawbacks. In general, high manufacturing cost, protein instability, unwanted immune response and contamination are the common limitations in protein-based therapeutic strategies (Table 1) [23–25]. Therefore, there is clear need for an alternative and effective therapeutic strategy for treating bone diseases.

Small molecules with the ability to induce osteoblast differentiation are appealing because their inherent properties can minimize or even overcome the drawbacks observed with protein-based growth factors (Table 1) [24,25]. For example, small molecules are unlikely to have any issues with immunogenicity because they are too small in size to stimulate an immune response [26]. Moreover, unlike protein growth factors, the bioactivities of small molecules usually do not require structural integrity [23,27–29]. Interestingly, many small molecule drugs can be taken through the oral route, and therefore avoiding painful multiple injections [30]. More importantly, compared with recombinant proteins, many small molecule drugs are inexpensive organic compounds [15,31]. Considering a growing number of small molecules have been recently discovered with osteoblastic differentiation capability, they may represent the next generation of bone regenerative medicine. This review article will focus on recent developments and the prospective future of small molecules associated with bone regeneration.

Small molecules with osteoinductive potential

A small molecule is a low molecular weight organic compound (i.e., molecular weight is usually less than 1000 Da) that can diffuse across the cell membrane to reach intracellular targets, and in certain cases, act as a signaling molecule in a number of cell signaling pathways [15,32]. Typically, the small molecules trigger intracellular signaling pathways by specifically binding to a protein receptor in the cytoplasm, which in turn leads to particular gene transcription and eventually protein expression, including osteoblast-associated genes (Figure 1b) [23,33]. Signal transduction systems such as cAMP/PKA [23,34], Hh [35,36], BMP/Smad [37], MAPK [38,39] and Wnt have well-known roles in regulating osteogenesis [40,41]. Therefore, identification of the small molecules that regulate these signaling cascades is the current drug discovery strategy in the area of bone repair and regeneration [15]. Using high-throughput screens and/or cell-based functional assays, a large number of small molecules have been recently discovered based on their capability of regulating osteoblast-associated gene expression and inducing the osteogenic phenotype through the activity of the osteogenic-related signaling pathways. Table 2 summarizes these small molecules and their target signaling mechanisms.

Osteoinduction was originally proposed by Friedenstein in the late 1960s as “the induction of undifferentiated inducible osteoprogenitor cells that are not yet committed to the osteogenic lineage to form osteoprogenitor cells” [42]. More recently, there has been generally accepted conclusive evidence that osteoinduction can only be claimed by ectopic bone formation *in vivo*, or in other words, “bone formation in the tissues or organs where bone does not naturally grow” [43,44]. Therefore, the term ‘osteoinductive small molecule’ would be used when the small molecule gives rise to ectopic bone formation after implantation in an animal. Preferably, the implantation site is subcutaneous or intramuscular [3,45–47].

Statins are classified as osteoinductive small molecules because they were able to induce ectopic bone formation in a rat subcutaneous implantation model [48]. To date, statins are the most studied small molecules for bone regeneration because a number of studies have robustly demonstrated their bone regenerative capability in various cell and animal models. For instance, studies showed that cerivastatin, fluvastatin, lovastatin and simvastatin induced osteogenesis *in vitro* and *in vivo* by increasing the expression of BMP-2 through the BMP/Smad signaling mechanism [49–57]. It is also worth noting that statins are the FDA-approved drugs for treating cardiovascular disease and they have been safely used in patients for decades [58]. This facilitated entering statins into clinical studies as a new bone regenerative therapeutic for treating various bone disorders [59].

Similar to statins, several small molecules have been discovered with osteogenic activity through the BMP/Smad signaling mechanism [20]. These include a helioxanthin derivative (TH) [60], tacrolimus hydrate (FK506) [61–65], icariin [66–68], rapamycin [69,70] and phenamil [71]. TH and icariin promoted osteoblastic differentiation in mouse osteoblast-like MC3T3-E1 cells, mouse primary osteoblasts and mouse embryonic stem cells [60,66]. TH’s *in vivo* osteogenic potential was further evaluated by implanting a sustained-release carrier containing TH into mouse calvarial defects, where new bone formation occurred within 4

weeks [72]. FK506 and rapamycin are immunosuppressants that have been used for reducing the activity of patients' immune systems after organ transplantations in order to lower the risk of organ rejections [73–75]. Intriguingly, these two small molecules were recently found to be osteoinductive, as evidenced by the ectopic bone formation in subcutaneous sites [76,77]. Phenamil is a derivative of the diuretic amiloride, which has been recently shown to induce osteogenesis of mouse mesenchymal stem cells (MSCs) *in vitro* [71]. However, *in vivo* data were not reported. Interestingly, the small molecule phenamil activates the BMP/Smad signaling pathway through the induction of Trb3 [71]. Trb3 is a novel inducer of the BMP signaling pathway that acts by downregulating the BMP antagonist Smurf1 [78]. The expression of Trb3 will thus stabilize the Smad family signaling transducers, which in turn induce the osteoblast-associated gene transcriptions [71]. This study also suggested that Trb3 might represent a new therapeutic target for bone repair and regeneration.

6-Bnz-cAMP is a PKA-specific cAMP analog that is well-validated as a molecular tool to study signaling mediated exclusively by PKA signaling [79]. Our group recently demonstrated that stimulation of the PKA signaling pathway by administering the small molecule 6-Bnz-cAMP continuously resulted *in vitro* osteogenesis in several cell types such as mouse osteoblast-like MC3T3-E1 cells [23] and human MSCs (Figure 2). We further demonstrated that phosphorylation of CREB by PKA is involved in 6-Bnz-cAMP-induced osteogenesis [23]. We are now studying *in vivo* bone regeneration by 6-Bnz-cAMP in animals including rats and rabbits. Consistent with our observation of 6-Bnz-cAMP, Miyamoto *et al.* demonstrated that the phosphodiesterase type 4 small molecule inhibitor XT-611 potentiated the osteoblastic differentiation of rodent bone marrow cells through the cAMP/PKA signaling pathway [80]. Similarly, recent data from the Babij group at Amgen Inc. suggested that the CREB pathway activity is involved in the differentiation of the MC3T3-E1 cell line in response to their osteogenic small molecules (i.e., AMG0892 [benzothiazole core] and AMG0309 [naphthyl amide core]) [81].

The small molecule purmorphamine, a purine derivative, has been shown by the Schultz and the Rosa groups to induce *in vitro* osteogenesis in mouse embryonic mesoderm fibroblasts and human osteoblasts [82,83]. The mechanism study of purmorphamine demonstrated that it functions as an agonist of Smoothened, a 7-transmembrane receptor of the Hh signaling cascade [84,85]. The Rosa group also investigated the mechanism of the previously shown osteogenic small molecule TAK-778 [86–88], a derivative of ipriflavone, and their studies revealed that small molecule TAK-778 promoted osteoblast differentiation of human bone marrow cells via an estrogen receptor-dependent signaling pathway [89].

It is a well-known fact that the Wnt signaling cascade plays a key role in osteogenesis by directly stimulating the expression of Runx2, an essential transcription factor for the regulation of osteoblast differentiation [40,90–94]. The small molecule inhibitor diphenylsulfonyl sulfonamide (WAY-316606) of the Wnt antagonist sFRP1 has been shown to activate the Wnt signaling pathway in order to enhance bone formation in an *ex vivo* organ culture assay [95–97]. While various signaling pathways have been implicated in the regulation of osteoblast differentiation, several recent studies attempted to explore the possibility of integrating multiple osteogenic signaling pathways in osteoblast differentiation

[98,99]. For instance, the small molecule oxysterols, cholesterol oxidation products, have been reported to execute their osteogenesis activity by activating both Hh and noncanonical Wnt signaling cascades [100], suggesting that signaling mechanism cross-talk between Wnt and Hh is important in osteogenesis.

It is important to point out that a large number of small molecules with osteogenic potential have been recently identified with unknown underlying signaling mechanisms. Table 3 summarizes these osteogenic small molecules. The Burdick group at the University of Pennsylvania (PA, USA) utilized a high-throughput screening of a small molecule library, and their results revealed that small molecule inducers encompass a wide range of medications including antibiotics (e.g., cephalexin), pain relievers (e.g., aspirin), psychotropic drugs (e.g., zolpidem) and steroids (e.g., cholecalciferol) [101]. Similarly, Chung's group at the University of Tokyo (Japan) developed a high-throughput screen system for osteogenic drugs and identified a novel osteogenic small molecule: glabrisoflavone (isoflavone derivative) [102]. Deng's group in China found a potent osteogenic small molecule (OIC-A006) using a high-throughput screen technology and demonstrated its osteoinductive potential *in vitro* and *in vivo* [103]. The signaling mechanisms underlying these osteogenic small molecules are currently unknown and await further investigation. It is also interesting to note that a number of research groups recently examined small molecule components extracted from herbal medicine products and claimed osteogenic effects in different cell and animal models. These osteogenic small molecules are summarized in Tables 2 & 3. Importantly, these interesting observations open a new area in bone regenerative medicine by introducing herbal medicines, including traditional Chinese medicine.

Osteogenic/osteoinductive small molecules with the potential to directly promote angiogenesis and/or inhibit osteoclast function

Successful approaches to bone regeneration and bone graft repair also require other phenomena such as neovascularization and osteoclastic bone resorption. Neovascularization is particularly important because this facilitates the transport of oxygen and nutrients to cells transplanted in the bone grafts, as well as migration from the host tissue [104]. Insufficient neovascularization of implanted bone grafts often impedes recovery and may lead to hypoxia-induced cell death [29]. Several protein growth factors have been investigated to promote angiogenesis during bone repair and regeneration [21]. For instance, VEGF has been extensively researched for this purpose [105]. While promising, as discussed in the previous section, protein-based therapeutic strategies are generally limited in their universal use (Table 1) [15,24,25,106]. The Botchwey group has proposed that small molecules with angiogenic potential would serve as options to combat the issues with protein-based factors [29,106,107].

Osteoclasts are well known for bone resorption, which is associated with many bone-related disorders such as osteoporosis. Therapeutic strategies have targeted this cell type for developing antiresorptive agents [15]. A large number of small molecule drugs have been discovered and developed with the capability of inhibiting osteoclast activity [15]. For instance, bisphosphonate (BPP) is a synthetic small molecule that is one of the major

antiresorptive drugs currently available on the market to treat osteoporosis and related bone diseases [108]. However, similar to most anti-resorptive drugs, BPP may not meet the demand for osteoporosis because it is unable to promote the regeneration of damaged osteoporotic bone tissue. In order to regenerate and repair bone efficiently, novel therapeutic approaches should be developed in order to promote osteogenesis, promote angiogenesis and inhibit osteoclastogenesis. More specifically, multifunctional small molecules, in which one functionality is capable of inducing osteogenesis and angiogenesis while another functionality is capable of inhibiting osteoclast formation, should be investigated. In this review, in addition to focusing on osteo-inductive potential, we also summarized a number of osteogenic/osteoinductive small molecules with the potential to promote angiogenesis (Table 4) and/or inhibit osteoclast function (Table 5). According to Tables 2–5, it is interesting to note that statins, icariin and prostaglandins are the small molecules that contain all three properties that are important for bone regeneration.

Future perspective & challenges

Strategies employing protein-based growth factors have been successfully used in bone repair and regeneration; however, as discussed, they have had a number of drawbacks. The incidence of skeletal complications will continue to grow as the life expectancy of the population increases. Therefore, there is a clear need for an alternative, yet effective, therapeutic strategy. It is believed that using bone regenerative small molecules is an attractive alternative strategy. In addition, recent advances in phenotypic high-throughput screens of small molecule libraries play a remarkable role in accelerating the process of drug discovery. It is expected that the value of small molecules for musculoskeletal complications over the next 5–10 years is enormous.

While promising, it is important to point out that a common issue with small molecule drugs is their off-target cell effects (Table 1) [24]. To overcome the aforementioned issues, small molecules with osteoinductive potential can be delivered to bone defect sites locally via controlled release systems [24,109]. Biodegradable polymeric-based matrix systems have already shown great promise as novel drug delivery vehicles in the controlled delivery of vaccines, chemotherapeutics and various growth factors [24,110–115]. In fact, research studies have shown that small molecules can be easily incorporated into biodegradable polymer systems by a variety of methods for bone regenerative engineering applications [29,116,117]. Therefore, polymeric controlled release systems have great potential to be utilized in the delivery of osteoinductive small molecules for treating musculoskeletal complications through bone regenerative engineering methods [24,118]. In addition, it is worth noting that despite the significant evidence of potential for bone regeneration in various cell types and animal models, further investigation will be needed to define variables such as dosage, specificity and general toxicity.

Specificity & dosage

Although oral administration is feasible and effective for many small molecule drugs due to their ease of absorption into the bloodstream by the digestive system, the bioavailability of small molecule drugs is low for bone tissue, thus the same effectiveness is not expected for treating bone disorders [119]. Interestingly, specificity and effectiveness can be also

achieved by delivering small molecule drugs locally to the bone site via a controllable, sustained-release polymeric-based delivery vehicle as mentioned above [120]. Such local delivery systems are advantageous for two main reasons: they reduce systemic side effects normally caused by supraphysiological dose requirements and increase therapeutic effectiveness by providing targeted delivery and improved drug stability against chemical/enzymatic degradation [121]. Additionally, delivery vehicles can be engineered so they not only provide a matrix for local drug delivery, but also a physical substrate for cell attachment and growth. Particularly, polymeric biodegradable scaffolds have the capability to provide such support while releasing loaded agents through hydrolytic degradation of the matrix into non-toxic components. Once released, drug delivery is typically mediated by passive diffusion, but also impeded by renal clearance [122]. As depicted in Figure 3, there is an extended therapeutic window of drug efficiency when delivered from a delivery vehicle, as opposed to more traditionally systemic methods of delivery [123]. Despite the many advantages, local delivery carriers have some disadvantages. For instance, dosage cannot be adjusted after injection or implantation, and the rate of drug release typically decreases with time. Furthermore, repeated implantation may be required for long-term release and the implantation surgery is invasive [124]. While systemic drug delivery can be advantageous because repeated administration allows for dosage adjustments, the possible toxic effects due to high systemic exposure to the drug are avoidable. Since both drug delivery systems have advantages and disadvantages, the selection of the appropriate drug delivery carrier is dependent on the nature and location of the disease. However, in this section, we will focus on local delivery methods for the treatment of musculoskeletal injuries and diseases.

There are many delivery vehicle systems that have been investigated in order to achieve local delivery. For example, a recent report suggested the use of an injectable hydrogel drug delivery system to deliver statins (e.g., fluvastatin) for bone regeneration [119]. Hydrogels are cross-linked networks of insoluble hydrophilic polymers that can absorb large amounts of water. While their soft and rubbery consistency makes these constructs attractive for soft tissue applications [125], hydrogels have also been used for bone tissue engineering [126]. Small molecules entrapped within the polymeric chains of the porous matrix are distributed heterogeneously throughout the biomaterial, and as the hydrogel degrades, they diffuse from the gel to the surrounding environment [127]. As previously reviewed, several natural and synthetic polymers are used to fabricate hydrogels, and these exhibit many different properties such as charge, mechanical and structural features and physical structures, among others [128]. One large advantage of hydrogels is their ability to form matrices *in situ*, thus overcoming the aforementioned issue of intrusive implantation surgery. Such injectable hydrogels can gel via thermoresponsiveness, photopolymerization and other environmental stimuli [129]. On the other hand, hydrogels can be disadvantageous because they lack the physical integrity to bear heavy mechanical loads, and can exhibit rapid release profiles of drugs without physical and/or chemical modifications [126].

Another biomaterial frequently used for bone regeneration is calcium phosphate (CaP). Researchers have been recently investigating CaP matrices as targeted drug-delivery vehicles to deliver small molecule BPP specifically to osteoporotic bone tissue [130–132].

CaP delivery vehicles of various forms (ceramics, cements and composite coatings) have been investigated extensively in bone tissue engineering due to their marked similarities in mineral composition, properties and microarchitecture to human cancellous bone. Further basis is on the strong and specific chelation between BPP and CaP crystals. There are three main categories of CaP materials with varying chemical and thus physical properties: hydroxyapatite (HAp), β -tricalcium phosphate and biphasic CaP (combination of β -tricalcium phosphate and HAp). Of these categories, HAp is highly attractive for BPP encapsulation and release since its high affinity for BPP does not hamper BPP pharmacological ability [133]. On the other hand, BPP incorporated in CaP cements are advantageous because cements can harden *in situ*, easily matching the shape of bone cavities or defects [134]. Pristine or combinatorial uses of these biomaterials with natural or synthetic biodegradable polymers result in a wide range of applications with varying mechanical strength, bioresorbability and osteoconductivity.

Another popular class of local drug carriers is biodegradable synthetic polymers. Several types of polymeric scaffolds are used extensively in bone tissue engineering due to their biocompatibility, hydrolytic biodegradability, formability and ease of use [135]. There are a number of ways to deliver small molecules from biodegradable polymers, but they are typically either physically encapsulated within the polymeric matrix or immobilized to the surface, in addition to undergoing ionic, hydrophobic and/or hydrogen bonding interactions with the polymer. Our group is currently engineering biodegradable poly(lactide-co-glycolic) acid (PLGA)-based microsphere scaffolds encapsulated with 6-Bnz-cAMP for bone regenerative engineering applications. Other than its demonstrated biocompatibility, PLGA has been shown to degrade into nontoxic components, and has thus been FDA approved for several clinical applications [25].

In addition to employing local delivery vehicles, lowering the dosages of the drugs can further minimize potential nonspecific side effects due to small molecule drugs; thus, the dose–response relationship of small molecules on cell and tissue cultures should be evaluated carefully in order to determine the lowest possible effective concentration [136].

It should be noted that the osteogenic activities of many small molecules described here were studied in rodent models only. In the field of orthopedic research, the human MSC is the appropriate cell model to investigate the osteogenesis of a molecule or biomaterial because: they have demonstrated the ability to differentiate into the osteogenic lineages [101,137–139]; they offer an alternative cell source for bone tissue engineering procedures that involves a less invasive harvest technique [140]; and it is believed that the use of human MSCs for studying osteogenesis will bring basic science concepts closer to clinical studies [34]. Thus, further studies are needed to investigate the interspecies differences of small molecules' effects between rodents and humans in terms of osteoinductivity, dosages and pharmacokinetic properties. In addition, since a number of small molecules listed in this review were not tested for bone-forming activity in animals, further investigation using appropriate animal model is therefore needed to determine their *in vivo* bone-forming activity.

Many small molecules discussed in this review are primarily focused on osteoinductive potential. Further investigation is needed to determine whether the small molecules have the ability to promote neovascularization and to control bone remodeling, including osteoclastic resorption, because these two events are important to bone regeneration and bone graft repair success.

Small molecules are a useful tool for studying bone cell biology

The signaling mechanism underlying osteogenesis is still unclear in some cell types, including MSCs. Many small molecules described here could offer the unique opportunity to study osteogenic signaling pathways in cells and tissues because they can be utilized as molecular probes. For instance, cAMP analogs have been developed for decades and routinely used as biological tools for investigating various cAMP-mediated signal transduction pathways [23,33,79]. Therefore, the focus of the small molecule research here is not only on the development of new bone regenerative medicine, but also the development of an improved understanding of the mechanistic pathways that drive regeneration of cells and tissues [71].

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Executive summary

Bone growth factors

- Bone growth factors are usually polypeptide-based macromolecules such as BMP, FGF or IGF.
- Protein-based therapeutic agents usually carry a number of limitations including protein instability, high cost, contamination and immunogenicity.
- Small molecules with the ability to induce bone formation have recently gained a more prominent reputation as therapeutics because their inherent properties can minimize the limitations observed with protein growth factors.

Small molecules with bone regenerative potential

- In general, small molecules are low molecular weight organic compounds that can freely diffuse across the cell membrane and trigger a signal transduction system by activating a specific intracellular protein sensor.
- Identification of the small molecule that modulates the activity of the osteogenic signaling pathways (e.g., cAMP/PKA, BMP/Smad or Wnt) is the current drug discovery strategy for bone regenerative medicine.
- A large number of small molecules potentially with bone regeneration ability have been discovered in the last decade.

Future perspective

- There exists the potential for treatment of various musculoskeletal defects and disorders by combining osteoinductive small molecules with scaffold-based bone regenerative engineering.
- Future investigation will be needed to define the dosage, specificity, general toxicity and *in vivo* activity of small molecule drugs.
- It would be of great interest to explore whether the small molecules also have the ability to promote neovascularization and inhibit osteoclastogenesis.

Figure 1. Different forms of bone growth factors involved in osteoblast differentiation

(A) The protein ligand interacts with the extracellular domain of the membrane receptor, and in turn the intracellular domain of the receptor activates the target signal cascade. (B) The small molecules pass through the cell membrane and directly activate the intracellular target signaling cascade. Activation of the signaling cascade will lead to modification of the transcription factor. The modified transcription factor will then translocate from the cytoplasm to the nucleus and induce the expression of the osteoblast-associated gene.

Figure 2. 6-Bnz-cAMP at 100 μ M promoted matrix calcification at day 20 of human mesenchymal stem cells on a biodegradable polymeric scaffold of poly(lactic-co-glycolic) acid thin films

Briefly, for the qualitative determination of calcified matrix mineralization, at day 20, media were removed and cells were rinsed with calcium-free phosphate-buffered saline three-times. The calcium level was then measured with a calcium assay kit (Calcium LiquiColor[®], Stanbio Laboratory, TX, USA) according to the manufacturer's instructions [23]. Cells cultured in osteogenic medium served as a positive control, while cells cultured in control medium served as the baseline. To facilitate the comparison of different experimental settings, the 'control' values were set to one relative unit. *Statistical analysis was performed using the Student's *t*-test at a significance of $p < 0.05$.

Figure 3. Controlled release strategies (solid line) allow delivered drugs to stay within the 'therapeutic window' for a longer time in contrast to systemic, one-off delivery (dashed line) The extended period of time (t_{eff}) of controlled release strategies not only permits the drug to diffuse from the scaffold and be present in the tissue at a more sustained rate, but also avoids being present in high concentrations that could be toxic to the local environment. t_{eff} : Effective time. Adapted with permission from [123].

Table 1

Comparison of therapeutic proteins and small molecules.

Advantages		Disadvantages
Protein	Specific	Unstable Impurities High dose requirement High cost Immunogenic Given by injection
Small molecule	Low cost	Nonspecific
	Stable	Short half-life
	Nonimmunogenic	
	Can be given by oral route	

Adapted with permission from [24].

Table 2

Small molecules that promote osteoblast differentiation with known signaling targets.

Small molecule	<i>In vitro</i> effective dosage (μM)	Target signaling	<i>In vitro</i> activity (cell types)	<i>In vivo</i> activity	Ref.
6-Bnz-cAMP	100	PKA/CREB	MC3T3-E1; rat and human MSCs [†]	Not reported [‡]	[23]
XT-611	0.1	PKA/CREB	Rat and mouse bone marrow cells	Not reported	[80]
AICAR	1–2	PKA	Human amnion MSCs; rabbit MSCs	Not reported	[141]
AMG0892, AMG0309	0.01–0.2	CREB	MC3T3-E1	Not reported	[81]
Statins [§]	0.0001–5	BMP/Smad	MC3T3-E1; MG63; rat and human bone marrow stromal cells; mouse embryonic stem cells	Subcutaneous injection over the calvaria of mice; ovariectomized rats treated systemically	[49,53, 142–144]
Phenamil	10	BMP/Smad	Rat MSCs	Not reported	[71]
FK506 [§]	0.05–1	BMP/Smad	Rat bone marrow cells; rat MSCs; MC3T3-E1; ST-2; C2C12; C17	Subcutaneous implantation in rats	[63,65,145]
Rapamycin [§]	0.05	BMP/Smad	ROS 17/2.8; human embryonic stem cells	Subcutaneous implantation in mice	[69,70,77,146]
TH	1	BMP/Smad	MC3T3-E1; mouse primary osteoblast, mouse embryonic stem cells	Calvarial critical-sized defect in mouse	[60,72]
Icariin		BMP	MC3T3-E1; mouse primary osteoblasts	Ovariectomized mice treated systemically; calvarial defect model in mouse	[66,67,147]
Pumorphamine	1	Hh	C3H10T1/2 cells	Not reported	[82]
WAY-316606	0.03	Wnt	<i>Ex vivo</i> mouse calvarial organ culture	Not reported	[95]
TAK-778	10	ER dependent	Human bone marrow cells	Not reported	[89]
Oxysterols	2.5	Hh and Wnt	M2-10B4	Critical-sized rat calvarial defect model	[100,148]
Herbal medicines					
Puerarin	0.1	PI3K/Akt	Rat calvaria osteoblasts	Intramuscular implantation in rats; calvarial parietal bone defect model in rabbit	[149,150]
Genistin	1	ER dependent	Human bone marrow stromal cells	Calvarial parietal bone defect model in rabbit	[151,152]
Psoralen	100	BMP	Mouse calvarial osteoblasts	Calvarial parietal bone defect model in rabbit	[153,154]
Osthole	100	β-catenin/BMP	Mouse calvarial osteoblasts	Ovariectomized rats treated systemically	[155,156]
Machuoside A	80	BMP/MAPK	Rat MSCs	Not reported	[157]
Berberine	~150	MAPK	C3H10T1/2; MC3T3-E1	Not reported	[158]
Resveratrol	10	ER dependent	Human MSCs	Not reported	[159]

[†] Unpublished observations.

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[‡]Currently under investigation.

[§]Osteoinductive small molecules.

ER: Estrogen receptor; MSC: Mesenchymal stem cell.

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Table 3

Small molecules that promote osteoblast differentiation with unknown signaling targets.

Small molecule	<i>In vitro</i> effective dosage (μM)	<i>In vitro</i> activity (cell types)	<i>In vivo</i> activity	Ref.
Antibiotics or antivirals: cephalixin, doxycycline hydrochloride, gentamicin sulfate, minocycline hydrochloride, valacyclovir hydrochloride, chloroguanide hydrochloride Pain relievers: aspirin, acetanilide, antipyrine, nalbuphine hydrochloride Steroids: cholecalciferol, fludrocortisone acetate, fluorometholone, halcinonide, medrysone, triamcinolone diacetate, medroxyprogesterone acetate Psychotropic drugs: zolpidem, spiperone, olanzapine, inositol, isopropamide iodide, clopamide Miscellaneous: avobenzone, aminohippuric acid, tolaxamide, protoveratrine A, ropinirole tolaxamide, trimebutine maleate, ebselen, tuaminoheptane sulfate	0.1	Human MSCs	Not reported	[101]
Decalpenic acid	1	C3H10T1/2	Not reported	[160]
Glabrisoflavone	30	MC3T3-E1; mouse primary osteoblasts	Not reported	[102]
β-cryptoxanthin	10	MC3T3-E1	Not reported	[161]
Prostaglandins	0.01–10	Adipose tissue-derived MSCs	Systemic treatment of ovariectomized rats	[162,163]
OIC-A006	6.25	Bone marrow stromal cells	Skull defect model in rabbit	[103]
<i>Herbal medicines</i>				
Naringin	0.1	UMR 106; osteoblastic cells	Systemic treatment of ovariectomized mice	[164,165]
Quercetin	5	Human adipose stromal cells	Calvarial parietal bone defect model in rabbit	[166,167]

MSC: Mesenchymal stem cell.

Table 4

Osteogenic/osteoinductive small molecules that directly promote angiogenesis.

Small molecule	Study summary	Ref.
Statins	Both <i>in vitro</i> and <i>in vivo</i> angiogenesis were enhanced at low concentrations of statins (0.005–0.01 $\mu\text{mol/l}$). Pravastatin at 0.01 μM induced proangiogenic effects via the PI3K/Akt pathway	[168–170]
Icariin	Icariin stimulated <i>in vitro</i> angiogenesis by activating the MEK/ERK- and PI3K/Akt/eNOS-dependent signal pathways in human endothelial cells	[171]
Purmorphamine	Purmorphamine induced vascular lumen formation <i>in vitro</i> in the coculture of hMSCs and human umbilical vein endothelial cells	[172]
Puerarin	Puerarin (120 mg/kg) induced angiogenesis in the nonischemic and ischemic myocardium of rats	[173]
Prostaglandins	Prostaglandin induced angiogenesis in the myocardium of patients with ischemic heart disease	[174]
Olanzapine	Olanzapine treatment increased VEGF levels and angiogenesis in the rat hippocampus	[175]

hMSC: Human mesenchymal stem cell.

Table 5

Osteogenic/osteoinductive small molecules that inhibit osteoclast function.

Small molecule	Study summary	Ref.
Icariin	Icariin (10 μ M) inhibited the formation and bone resorption activity of osteoclasts in a mouse bone marrow culture	[176]
XT-611	XT-611 inhibited osteoclast formation in coculture of mouse bone marrow stromal cell line ST2 and adherent cell-depleted BMCs	[177]
Statins	Cerivastatin inhibited parathyroid hormone-stimulated bone resorption <i>in vitro</i> . Simvastatin inhibited osteoclastogenesis through suppression of ROS-mediated signaling pathways	[178,179]
FK506	FK506 induced osteoclast apoptosis in mouse BMCs	[180]
Rapamycin	Rapamycin inhibited osteoclast formation and bone resorption in giant cell tumors of bone	[181]
Osthole	Osthole inhibited bone resorption by decreasing the formation, differentiation and TRAP activity of osteoclasts derived from rat marrow cells	[182]
Berberine	Berberine inhibited osteoclast formation and survival through suppression of NF- κ B and Akt activation	[183]
Resveratrol	Resveratrol inhibited osteoclastogenesis through suppression of ROS-mediated signaling pathways	[184]
Prostaglandins	Prostaglandin E2 inhibited human osteoclast formation	[185]
Quercetin	Quercetin (0.1–10 μ M) decreased osteoclastogenesis via a mechanism involving NF- κ B and AP-1	[186]
Naringin	Naringin inhibited osteoclastogenesis and bone resorption via the inhibition of RANKL-induced NF- κ B and ERK activation	[187]
β -cryptoxanthin	β -cryptoxanthin induced osteoclast apoptosis and suppressed cell function in osteoclastic cells	[188]

BMC: Bone marrow cell; ROS: Reactive oxygen species.