



Pharmaceutical therapeutics for articular regeneration and restoration: state-of-the-art technology for screening small molecular drugs

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Received: 24 May 2021 / Revised: 20 September 2021 / Accepted: 14 October 2021 / Published online: 16 November 2021

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Abstract

Articular cartilage damage caused by sports injury or osteoarthritis (OA) has gained increased attention as a worldwide health burden. Pharmaceutical treatments are considered cost-effective means of promoting cartilage regeneration, but are limited by their inability to generate sufficient functional chondrocytes and modify disease progression. Small molecular chemical compounds are an abundant source of new pharmaceutical therapeutics for cartilage regeneration, as they have advantages in design, fabrication, and application, and, when used in combination, act as powerful tools for manipulating cellular fate. In this review, we present current achievements in the development of small molecular drugs for cartilage regeneration, particularly in the fields of chondrocyte generation and reversion of chondrocyte degenerative phenotypes. Several clinically or preclinically available small molecules, which have been shown to facilitate chondrogenesis, chondrocyte dedifferentiation, and cellular reprogramming, and subsequently ameliorate cartilage degeneration by targeting inflammation, matrix degradation, metabolism, and epigenetics, are summarized. Notably, this review introduces essential parameters for high-throughput screening strategies, including models of different chondrogenic cell sources, phenotype readout methodologies, and transferable advanced systems from other fields. Overall, this review provides new insights into future pharmaceutical therapies for cartilage regeneration.

Keywords Cartilage regeneration · Osteoarthritis (OA) · Small molecular drugs · Disease-modifying OA drugs (DMOADs) · High-throughput screening

Introduction

Articular cartilage possesses a unique ability for weight-bearing and joint surface lubrication during an individual's lifetime. However, cartilage injury may lead to permanent

joint disability if not properly treated [1]. The incidence of knee cartilage surgery is 56 per 100,000 person years [2] and osteoarthritis, a worse condition caused by cartilage defects, currently affects approximately 250 million people worldwide [3]. Unlike other tissues, adult articular cartilage is neither innervated nor vascularized, and has extremely poor self-renewal capacity [4]. Articular

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cartilage consists of abundant extracellular matrix (ECM) but limited chondrocytes, which occupy only 1–3% of the total tissue volume [5]. Thus, defective tissue is rarely replaced by functional cartilage and additional therapeutic interventions are often required.

In recent years, the fields of cell-based therapy and tissue engineering have made remarkable improvements in the treatment of cartilage damage [6]. However, the clinical outcomes are unsatisfactory and challenges still exist. First, functional chondrocytes for articular cartilage recovery are lacking, hindering the application of chondrocyte implantation and necessitating the development of alternative cell sources. Second, chondrocytes with pathological phenotypes, representing either degradative or fibrotic cartilage ECM, cause functional impairment in cartilage diseases. This demands effective manipulation of pathological cellular phenotypes or processes.

Efforts have been made to address these problems. Among them, chemical compounds with molecular weights in the range of 0.1–1 kDa have gained increased attention as powerful tools for tissue regeneration and cell-fate control. Because of their small sizes, they easily cross the outer plasma membrane to target extracellular components such as cell surface receptors, protein domains attached to cell membranes, such as glycoproteins, and intracellular proteins such as kinases [7]. As a result, small molecular weight chemical cocktails have been demonstrated to drive direct reprogramming of fibroblasts into induced pluripotent stem cells (iPSCs), neural progenitor cells, cardiomyocytes, and other somatic cells [8–10]. Small molecular drugs have also been adopted for the treatment of various diseases, including cancers [11], fibrotic diseases [12], and asthma [13]. From a clinical perspective, small molecular drugs have considerable advantages: they are classified as low-immunogenic molecules (compared with biomacromolecules), and are easily synthesized, stored, transported, and standardized [14]. They offer rapid and temporal control over protein function, allowing their effects to be fine-tuned based on different concentrations and combinations [15]. In terms of drug discovery and design, small molecules typically mimic the interactions formed by peptides and place groups into hot-spot pockets with a smaller volume and tighter interaction in space [16]. Therefore, this review will highlight current achievements in the use of small molecules for cartilage regeneration, as well as rational high-throughput screening strategies used for the discovery of small molecules that facilitate chondrocyte generation or manipulation of chondrocyte degenerative phenotypes.

Generating chondrocytes using small molecules

Great advances have been made in the development of cartilage cell-based therapies and tissue engineering since the 1990s, providing new perspectives for achieving cartilage function recovery using alternative chondrocyte sources. Technically, chondrocytes can be expanded *in vitro*, differentiated from stem/progenitor cells, and reprogrammed from somatic cells. In other words, cell sources for cartilage regeneration include chondrocytes (autologous or allogeneic), stem cells (multipotent cells or pluripotent cells), and other trans-differentiated somatic cells [6]. Here, we discuss the routines to obtain functional chondrocytes, which can be effectively facilitated using small molecular compounds (Fig. 1).

Small molecules facilitating chondrogenesis of stem/progenitor cells

Stem cells with chondrogenic differentiation ability are of great interest in the field of cartilage regeneration. For example, mesenchymal stem cells (MSCs) have gained increasing popularity as an alternative cell source for the treatment of cartilage injuries in clinical trials [17]. However, many studies have reported inferior outcomes. There are still gaps in the precise regulation of MSC chondrogenesis because MSCs can differentiate into fibrochondrocytes, resulting in a mixture of fibrous cartilaginous tissue [4].

A 2012 study highlighted the exploration of a small molecule, kartogenin (KGN), which was dubbed a “game-changer in regenerative medicine” [18] for its ability to induce human MSC chondrogenesis *in vitro* by regulating CBF β -RUNX1. It was demonstrated to be an efficacious disease-modifying OA drug (DMOAD) in two OA mouse models as it did not enhance cartilage hypertrophy or calcification [19]. KGN is extensively used as a chondroprotective agent against cartilage degeneration and injury. To establish a controllable drug delivery system, Wang et al. combined KGN with PLGA microspheres for the superficial repair of articular cartilage in a rabbit model [20]. KGN can also be conjugated to Pluronic F127 thermoresponsive nanospheres with the anti-inflammatory drug diclofenac (DCF) [21], or to hyaluronic acid (HA)/PEG to suppress OA progression in rats [22]. Recently, other groups have conjugated KGN to polyurethane nanoparticles; their intra-articular injection increased type II collagen expression levels and decreased type I collagen expression, alleviating OA in a rat model [23]. In 2013, the compound TD-198946, discovered by Yano et al., showed remarkable promotion of chondrogenic differentiation of mouse stem cells and the cartilage progenitor cell line ATDC5 by activating *Runx1* to upregulate

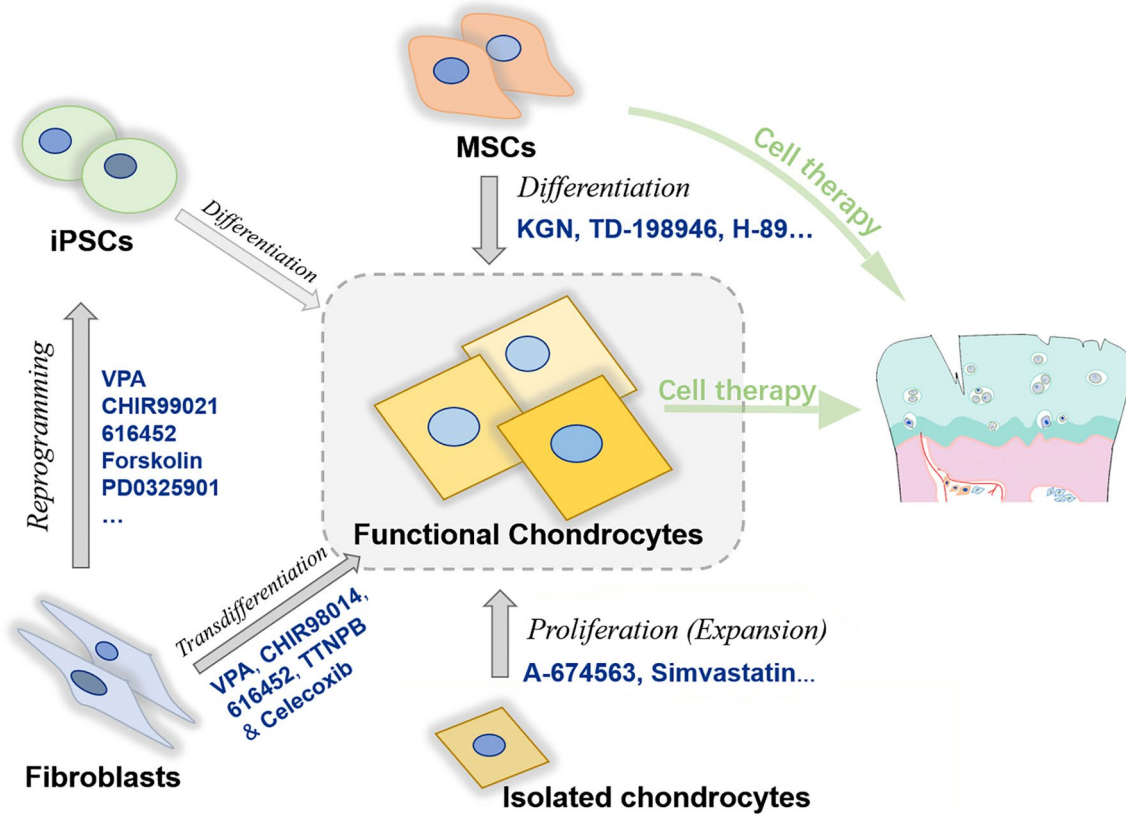


Fig. 1 Major applicable routines of small molecules for generating functional chondrocytes. *BMSCs* bone marrow mesenchymal stem cells, *iPSCs* induced pluripotent stem cells, *KGN* kartogenin, *VPA* valproic acid

Col2a1 and *Acan* gene expression without promoting chondrocyte hypertrophy [24, 25]. They also showed that, in combination with TGF- β 3, TD-198946 improved chondrogenesis of human synovium-derived stem cells [26].

In addition, through high-throughput screening, Hwang et al. discovered a protein kinase A inhibitor, H-89, which enhanced the chondrogenic differentiation ability of rat MSCs [27]. Using the structure of H-89, they synthesized a series of analogs and showed that, among them, compound 6 had the strongest ability to induce chondrogenesis of adipose-derived stem cells (ADSCs). Articular cartilage thickness was maintained with increased aggrecan levels by the injection of compound 6 or compound 6-treated-ADSCs in collagenase II-induced osteoarthritis rat models [28]. Heck et al. reported that GW0742, a PPAR- δ agonist, enhanced human MSC chondrogenesis and alleviated cartilage fibrosis/inflammation in an in vitro model by stimulating type II collagen/glycosaminoglycan secretion [29]. As Sirtuin-1 (SIRT1) is required for stem cell chondrogenesis, the SIRT1 activator resveratrol partially inhibits the inflammatory effect of IL-1 β on human MSC differentiation in vitro [30] and promotes human ADSC proliferation in a three-dimensional chondrogenic culture [31]. The small molecule

BIO (6-bromoindirubin-3-oxim) inhibits glycogen synthase kinase-3 (GSK-3) in the Wnt pathway and can upregulate cartilage-specific genes in mouse MSC in in vitro chondrogenesis [32]. Using a Col2GFP-ATDC5 system, Hojo et al. identified oxytetracycline, which acts as a chondrogenic inducer in a BMP-dependent manner and suppresses chondrocyte hypertrophy [33]. Similarly, the small molecule tetradecylthioacetic acid (TTA) was shown to improve ECM production in a fluorescently labeled collagen-binding probe system in ATDC5 cultures [34].

With the rapid development in stem cell-based regenerative medicine, small molecules are promising tools for regulating stem cell behavior. However, challenges still exist in the precise regulation of chondrogenesis, particularly the functional phenotype of stem cell-derived chondrocytes. There is a need to uncover relevant critical targets for optimizing chondrogenesis efficiency, to harvest hyaline chondrocytes, rather than fibrotic or hypertrophic chondrocytes.

Small molecules facilitating chondrocyte expansion

To obtain sufficient cell numbers for chondrocyte implantation, in vitro expansion is necessary. However, chondrocyte

dedifferentiation, representing a loss-of-function phenotype of expanded chondrocytes, results in fibrocartilage formation and has severely hindered the clinical application of chondrocyte implantation [35]. This limitation motivated Kobayashi et al. to select A-674563 from 5822 compounds based on mouse chondrocyte dedifferentiated phenotypes. Based on the RNA sequencing data, A-674563 could inhibit the degradation of SOX9 protein by activating the gene that induces protein ubiquitination [36]. Simvastatin, a lipid-lowering agent used to treat hypercholesterolemia [37], has the ability to maintain the phenotype of passaged human chondrocytes by stimulating the expression of SOX9 [38]. Clodronate, a bisphosphonate, [39] exerts an anabolic effect on articular chondrocytes [40]. It can upregulate the expression of SOX9 in an in vitro pathological human MSC model, reducing osteoarticular pain and improving mental and physical performance in human patients 6 months after treatment [41].

Although the regulation of SOX9 is critical to the chondrocyte phenotype, the maintenance of cell viability and ECM homeostasis are also necessary for large-scale chondrocyte expansion. Paradoxically, a higher expression of degradative enzymes such as matrix metalloproteinase 3 (MMP3) [42], improves the outcome of chondrocyte expansion. The mechanism is potentially associated with MMP-mediated remodeling of the ECM [43]. In contrast, the expression of MMPs is tightly linked with chondrocyte functional phenotype loss, contributing to dedifferentiation during in vitro culturing [44].

Due to the lack of basic knowledge on chondrocyte dedifferentiation, the ideal approach for effectively maintaining chondrocyte phenotype has not been determined. More in-depth investigations are required before practical application of small molecular compounds. Developing chemical compounds that facilitate chondrocyte function is important, as these potential drugs are likely to have a direct effect on chondrocytes, enabling their adoption in cell-free cartilage repair at joint surfaces in situ.

Small molecules facilitating reprogramming to generate chondrocytes

It has been shown that induced pluripotent stem cells (iPSCs) reprogrammed from patient somatic cells such as fibroblasts can form scaffoldless hyaline cartilaginous tissue in vivo, which can then be integrated into native cartilage [45]. This offers new options for regenerating cartilage tissue without ethical issues. Initially, the generation of iPSCs required virus-mediated expression of defined transcription factors (TFs) [46]. However, accumulating studies are reporting that chemical cocktails can improve reprogramming efficiency and even replace some of the defined factors [47]. Hou et al. used a cocktail of seven small molecule compounds (valproic acid (VPA), tranilcyproline, CHIR99021,

616,452, forskolin, 3-deazaneplanocin, and PD0325901) to achieve virus-free reprogramming of mouse fibroblasts into iPSCs in a step-wise manner [8]. Because the induction efficacy and the long period required for chemical reprogramming were still problematic for practical application [48], Zhao et al. significantly improved the efficiency of chemical reprogramming from somatic cells to chemically induced pluripotent stem cells (CiPSCs) and identified unique intermediate cells (XEN-like state) [49]. They induced mouse embryonic fibroblasts (MEFs) using an optimized protocol and chemical combinations were adopted as follows: VPA, CHIR99021, 616,452, tranilcyproline, forskolin, AM580, and EPZ004777 in stage 1; VPA, CHIR99021, 616,452, tranilcyproline, forskolin, AM580, DZNep, 5-aza-dC, and SGC0946 in stage 2, and CHIR99021 and PD0325901 in stage 3. In 2019, they enhanced the reprogramming process by fine-tuning the chemical treatment and generated 500 CiPSC colonies from 100,000 re-plated cells from stage 1 [14], demonstrating that chemical reprogramming is a unique and promising strategy for generating functional iPSCs.

Somatic cells can be directly reprogrammed into other types of mature cells by bypassing pluripotency, meaning that chondrocytes can be generated through transdifferentiation. Chen et al. conducted a proof-of-concept study using a combination of five small molecular drugs (VPA, CHIR98014, 616,452, TTNPB, and Celecoxib) and induced MEFs into three-dimensional fibrocartilaginous tissues with in vivo mechanical function [50]. This approach avoids the pluripotent state, poses a lower risk of tumor development, and shortens the induction period, and may present a new opportunity for future pharmaceutically engineered cartilage. However, achieving cell-fate conversion of human somatic cells into functional hyaline chondrocytes via chemical reprogramming remains uncertain. Evidence suggests that more stringent chemical conditions are required for the reprogramming of human adult cells, but this will require a comprehensive understanding of human cell plasticity. More importantly, the low efficiency and long-term reprogramming period are technical concerns that still need to be addressed before any clinical application.

Several studies have demonstrated the convenience of using small molecular compounds for in vitro generation of functional chondrocytes. However, large gaps remain between the establishment of a well-recognized standard and the development of an in-house approach. First, most of the screening described above were conducted with reference to phenotypic measurements, not target-based drug design, and finding a hit was likely coincidental, although the targets could be identified using a deconvolution process [51]. In our opinion, the targets of chondrogenesis induction and reprogramming are highly relevant to major developmental signaling pathways. For example, the CBF β -RUNX1 complex activates important genes involved in the differentiation

of MSCs into cartilage. KGN can competitively bind to human cytosolic filamin A, a key binding partner of CBF β , to displace CBF β and accelerate its dimerization with transcription factor RUNX1 in the nucleus [52]. Similarly, TD-198946 strongly induces chondrogenic differentiation by regulating RUNX1 [24]. Cellular programs resembling cartilage development, including the activation of *Bmp6*, *Tgfb2*, and *Wnt4*, were demonstrated in a direct reprogramming study [50]. These compounds exert their effects by stimulating differentiation. SOX9 appears to be a common target in the modulation of the dedifferentiated phenotype [36, 38, 41], functioning as a master transcription factor determining chondrocyte differentiation and phenotype [53]. However, the main molecular events involved in chondrocyte expansion and dedifferentiation are still unclear, making it difficult to efficiently regulate the chondrocyte phenotype in long-term passaging.

Thus, boosting cartilage development may benefit chondrocyte generation. Nevertheless, there is a big caveat in developing a quality-control system for the cell source. Given that chondrocytes are not identical in different regions [54], a precise identification of the differentiation state or subtype is also urgently needed. Therefore, single-cell transcriptomic datasets are a powerful tool for dissecting differentiation trajectories and chondrocyte sub-identity [55–57]. The emergence of new techniques has made it more feasible to precisely evaluate cell quality and investigate the chemical condition of the medium used for differentiating cells into correct orientations.

Small molecules for manipulating chondrocyte pathological phenotype

Cell implantation is effective in the treatment of early focal cartilage defects [58]. However, OA is a more complex condition. Pharmaceutical interventions are usually administered to patients with OA during the painful process before disease end-stage as they contribute to symptom control, making them increasingly popular, with more than 1300 clinical trials registered worldwide in the last 10 years (<https://clinicaltrials.gov>). DMOADs are designed to slow OA deterioration and inhibit structural disease progression [59], but are still at the conceptualization stage.

Small molecular compounds may play a vital role in the development of DMOADs capable of controlling early OA in a more precise and defined manner. Here, we summarize a list of over 170 representative small molecular OA drugs previously or currently under clinical investigation (Table 1), according to the US National Institutes of Health database. Most of them are analgesics or anti-inflammatory agents. Understanding pharmacological activities that specifically

block pathological processes and inhibit certain OA pathways is important for the manipulation of chondrocyte pathological phenotypes. In this section, we discuss potential OA targets and the corresponding small molecular drugs (Fig. 2).

Small molecules inhibiting inflammation

OA is characterized by low-grade inflammation and chronic joint pain. Various inflammatory cytokines, including interleukin 1 (IL-1) and tumor necrosis factor (TNF) α , may play a vital role in OA progression, and a direct inhibition of inflammation controls pain and reduces cartilage matrix degradation [60]. Here, several representative anti-inflammatory candidates are introduced based on inflammation-induced models or OA inflammatory pathways.

Diacerein is a symptomatic, slow-acting drug with anti-inflammatory, anti-catabolic, and pro-anabolic effects on cartilage in rat and dog models [61, 62]. The European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) confirmed that the effect of diacerein in clinical trials was similar to that of non-steroidal anti-inflammatory drugs (NSAIDs), but with fewer side effects [63]. The mechanism by which diacerein inhibits inflammation and prevents cartilage ECM degradation is potentially associated with the inhibition of IL-1 β converting enzyme, the reduction of IL-1 β receptors on chondrocytes, and the increase of IL-1 receptor antagonists. Diacerein was also shown to affect matrix biosynthesis and subchondral bone remodeling in an arthritis sheep model [63, 64].

Necrostatin-1 (Nec-1) is an inhibitor of receptor-interacting protein kinase 1 (RIPK1) and specifically inhibits the phosphorylation of RIPK1, which regulates inflammation and cell death. Liang et al. found that Nec-1 dramatically suppressed the catabolic effect induced by IL-1 β in mouse chondrocytes, including the downregulation of MMP and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5). Nec-1 also significantly reduced the destruction of OA cartilage in a destabilized medial meniscus (DMM) mouse model [65].

The transcription factor NF- κ B is a key molecule in classical inflammatory pathways [66]. Salubrinal, a specific inhibitor of eIF2 α phosphatase enzymes, attenuated cartilage degradation in mice with OA by inhibiting NF- κ B [67]. NF- κ B is also regulated by mechanical forces. Gremlin-1 can be elevated by mechanical stimulation in OA, which subsequently activates NF- κ B signaling and promotes matrix degradation by MMP13 and ADAMTS-5 [68]. NSC23766 and EHT1864 are inhibitors of Rac1 (an upstream regulator of Gremlin-1) and can downregulate the expression of Gremlin-1 [68]. Indeed, Rac1 was found to be highly expressed in human and mouse OA cartilage, while its intrinsic inhibitor, OCRL, was inhibited

Table 1 Representative small molecular OA drugs under clinical investigations

Name	Category	Identifier	Status	Update year and primary measurement
99Tc-MDP	Anti-osteoporosis/ Anti-RA	NCT02993029(NA)	Recruiting	2019 No results posted
ABT-652	Analgesic	NCT01444365(II)/NCT01207115(II)	Completed/ Completed	2013 No results posted/ 2013 No results posted
ADL5859	Analgesic	NCT00979953(II)	Completed	2015 Change in the Average Pain Score (NPRS)
AGG-523	Anti-matrix degradation	NCT00427687(I)/ NCT00380900(I)/ NCT00454298(I)	Completed/ Completed/ Completed	2008 No results posted/ 2007 No results posted/ 2009 No results posted
Alivium	Anti-inflammation/ Analgesic	NCT02409381(IV)	Completed	2016 No results posted
AMG 403	Analgesic	NCT02318407(I)	Completed	2014 No results posted
AMZ001	Anti-inflammation/ Analgesic	NCT03691844(II/III)	Completed	2020 Change in WOMAC pain subscore
Apremilast (Otezla)	Anti-inflammation	NCT01200472(II)	Completed	2013 No results posted
ARRY-371797	Anti-inflammation (p38 inhibitor)	NCT01366014(II)	Completed	2013 No results posted
ART-102	Anti-inflammation	NCT02727764(I)	Active, not recruiting	2018 No results posted
ASP7962	Analgesic	NCT01366014(II)	Completed	2019 No results posted
ATB-346	Anti-inflammation/ Analgesic	NCT03978208(II)	Recruiting	2019 No results posted
Atorvastatin	Other (statin)	NCT01645176(II)	Completed	2018 Change in synovitis by MRI after 16 weeks
AXS-02	Anti-osteoporosis/ Analgesic	NCT02746068(III)	Unknown Status	2019 No results posted
AZD1386	Analgesic	NCT00878501(II)	Terminated	2012 Terminated after the planned interim analysis
Balicatib (AAE581)	Anti-osteoporosis	NCT00371670(II)	Completed	2017 No results posted
Betamethasone	Steroid	NCT00685880(NA)/ NCT00542139(IV)/ NCT02612272(IV)	Terminated/ Completed/ Unknown Status	2012 Unable to recruit adequate numbers of patients/ 2008 No results posted/ 2016 No results posted
BI 1.026,706	Anti-inflammation	NCT02126826(I)	Completed	2019 Percentage of subjects with drug related adverse events
BI 113,823	Anti-inflammation	NCT01207973(I)	Terminated	2013 No results posted
Botulinum toxin type A	Analgesic	NCT01045694(IV)/NCT02230956(II)/ NCT01518257(II), etc.	Terminated/ Completed/ Completed	2017 Unable to acquire additional funding/ 2017 Change in the 7-day average daily pain score/ 2014 Change in the average daily worst pain intensity score at week 4

Table 1 (continued)

Name	Category	Identifier	Status	Update year and primary measurement
Bupivacaine (Exparel)	Analgesic	NCT02219087(NA)/ NCT02197273(NA)/ NCT02777749(IV), etc.	Completed/ Completed/ Completed	2018 Number of physical therapy sessions for discharge/ 2016 Length of stay in hospital/ 2019 Change in VAS pain scores from day 0 to 3
Buprenorphine	Analgesic	NCT00324038(IV)/ NCT02519387(IV)/ NCT01298765(III), etc.	Completed/ Completed/ Completed	2011 Average daily pain scores/ 2016 Change in Box Scale-11 pain score/ 2018 Change in NRS pain intensity
Calcitonin (SMC021)	Anti-osteoporosis/ Analgesic	NCT00486434(III)/ NCT00704847(III)/ NCT00486369(I), etc.	Completed/ Terminated/ Completed	2019 Joint space width (JSW) in the medial tibiofemoral knee joint by X-ray after 24 months: no statistical significant difference/ 2019 Male subjects were terminated due to an imbalance in prostate cancer events/ 2007 No results posted
Cannabidiol	Analgesic	NCT03693833(II)/NCT03825965(III), etc.	Recruiting/ Not yet recruiting	2021 No results posted/ 2021 No results posted
Capsaicin (Qutenza)	Analgesic	NCT03124407(IV)/ NCT03429049(III)/ NCT00995306(III), etc.	Completed/ Active, not recruiting/ Completed	2020 Percentage of subjects with 50% or greater pain score reduction/ 2020 No results posted/ 2011 No results posted
Cebranopadol (GRT6005)	Analgesic	NCT01709214(II)	Completed	2021 No results posted
Celebrex (Celecoxib)	Anti-inflammation/ Analgesic	NCT00346216(IV)/ NCT01768520(III)/ NCT00598234(IV), etc.	Completed/ Completed/ Completed	2021 The first occurrence of antiplatelet trialists collaboration (APTC) composite endpoint/ 2019 the change of total sum of K-WOMAC/ 2010 No results posted
CG100649	Anti-inflammation/ Analgesic	NCT01765296(III)/ NCT00530452(II)/ NCT01341405(II)	Unknown Status/ Unknown Status/ Completed	2013 No results posted/ 2008 No results posted/ 2012 No results posted
Chondroitin sulfate	Extracellular matrix	NCT01354145(III)/ NCT01425853(IV)/ NCT02823548(IV), etc.	Completed/ Completed/ Unknown Status	2016 Cartilage volume loss of the lateral compartment: significant superiority of chondroitin sulfate over celecoxib/ 2016 WOMAC pain subscale for 6 months/ 2016 No results posted
Cindunistat (SD-6010)	Anti-inflammation	NCT00565812(II)/ NCT01438918(III)	Completed/ Withdrawn	2016 Rate of progression of joint space narrowing up to 6 months: no difference vs placebo/ 2012 No results posted/ 2018 No results posted/ 2018 No results posted/ 2018 No results posted
Cingal	Extracellular matrix/Steroid	NCT03390036(III)/ NCT03191903(III)/ NCT03062787(NA), etc.	Completed/ Completed/ Completed	

Table 1 (continued)

Name	Category	Identifier	Status	Update year and primary measurement
Civamide	Analgesic	NCT00077935(III)/ NCT00995306(III)	Completed/ Completed	2011 No results posted/ 2011 No results posted
CL-108	Analgesic	NCT02462850(III)	Completed	2016 No results posted
Clodronate	ECM synthesis promoter	NCT03803839(early phase)	Completed	2019 No results posted
Colchicine	Anti-RA	NCT03913442(IV)/ NCT02176460(III)	Not yet recruiting/ Completed	2020 No results posted/ 2016 No results posted
Comarum palustre	Analgesic	NCT03530930(IV)	Completed	2019 No results posted
Cortisone	Steroid	NCT03580148(III)/ NCT01447160(III)	Active, not recruiting/ Unknown Status	2021 No results posted/ 2011 No results posted
COV155	Analgesic	NCT01722864(III)	Completed	2016 No results posted
COV795	Analgesic	NCT01451385(III)	Completed	2017 No results posted
CR845	Analgesic	NCT02524197(II)/ NCT02944448(II)	Completed/ Completed	2017 No results posted/ 2020 Change with respect to the weekly mean of the daily 24-h pain intensity
CRx-102	Steroid	NCT00521989(II)	Terminated	2014 CRx-102-006 study results, negative
Curcumin (Flexofy(tol))	Anti-inflammation/ Analgesic	NCT01909037(early phase)	Completed	2015 No results posted
Dapansutril (OLT1177)	Anti-inflammation/ Analgesic	NCT02104050(II)/ NCT01768975(II)	Completed/ Completed	2017 No results posted/ 2014 No results posted
Depomedrol	Steroid	NCT02176304(IV)	Completed	2017 No results posted
Devil Claw	Analgesic	NCT00295490(II)	Terminated	2011 Unexpected withdrawal of funding
Dexamethasone	Steroid	NCT02462148(IV)/ NCT02464176(IV)/ NCT01612702(IV), etc.	Completed/ Completed/ Completed	2018 Duration of sensory nerve block 2018 Duration of sensory nerve block 2013 Incidence of nausea and vomiting
Dextrose	Analgesic	NCT00685880(NA)/ NCT03238183(IV)/ NCT01210183(III), etc.	Terminated/ Unknown Status/ Completed	2012 Unable to recruit adequate numbers of patients/ 2017 No results posted/ 2014 No results posted
Diacerein	Anti-inflammation	NCT01120015(IV)/ NCT00685542(IV)/ NCT02688400(III), etc.	Completed/ Completed/ Active, not recruiting	2010 No results posted/ 2010 No results posted/ 2018 No results posted
Diclofenac	Anti-inflammation/ Analgesic	NCT01967550(III)/NCT02596451(III)/ NCT01383954(IV), etc.	Completed/ Completed/ Completed	2015 Pain on movement/ 2017 Absolute change in the total WOMAC/ 2017 Absolute change in the total WOMAC
Diractin	Anti-inflammation/ Analgesic	NCT00722852(III)/ NCT00716547(III)	Completed/ Completed	2009 No results posted/ 2009 No results posted
Donepezil	Anti-matrix degradation	NCT02927522(III)	Recruiting	2020 No results posted
Doxycycline	Anti-infective/ Anti-matrix degradation	NCT03115177(NA)/ NCT00000403(III)	Completed/ Completed	2018 Bacterial culture/ 2013 No results posted

Table 1 (continued)

Name	Category	Identifier	Status	Update year and primary measurement
Drogican	Extracellular matrix	NCT01425853(IV)/ NCT01893905(III)/ NCT02821468(IV)	Completed/ Completed/ Unknown Status	2016 WOMAC pain subscale/ 2017 Change in pain according to VAS/ 2016 No results posted
Duloxetine	Analgesic	NCT02335346(III)/ NCT01558700(IV)/ NCT02248480(IV), etc.	Completed/ Completed/ Completed	2019 Percentage of participants with drug related adverse events/ 2014 Change in brain gray matter volume/ 2019 Change on the brief pain inventory 24-h average pain score
EN3409	Analgesic	NCT01755546(III)	Completed	2018 Change in daily average NRS pain intensity score
Entelon Tab	Analgesic	NCT01768520(III)	Completed	2019 the Change of total sum of K-WOMAC
E-OA-07 (Lanconone)	Analgesic	NCT02417506(NA)	Completed	2015 Pain score as measured by visual analog scale
Epimorph	Analgesic	NCT01667445(IV)	Completed	2015 No results posted
Epinephrine	Steroid	NCT02658149(IV)/ NCT02462148(IV)/ NCT02464176(IV), etc.	Completed/ Completed/ Completed	2018 Pain score from 3–48 Hours/ 2018 Duration of sensory nerve block/ 2018 Duration of sensory blockade
Estrogen-basedoxifene	Anti-osteoporosis	NCT04036929(NA)	Active, not recruiting	2019 No results posted
Etoricoxib (MK0663, MK0966)	Anti-inflammation/ Analgesic	NCT00757627(IV)/ NCT01554163(III)/ NCT00140972(IV), etc.	Completed/ Completed/ Completed	2015 The percentage of participants achieving $\geq 30\%$ decrease in pain intensity as measured by WOMAC/ 2015 Time-weighted mean change in WOMAC pain subscale/ 2017 No results posted
Fasitibant	Anti-inflammation	NCT02205814(IV)	Completed	2015 Change in WOMAC
Fentanyl, Fentanyl citrate	Analgesic	NCT01774929(IV)/ NCT01742897(IV)/ NCT01795898(IV), etc.	Completed/ Completed/ Completed	2013 Pain intensity score at day 15/ 2013 Change in WOMAC score at day 30/ 2013 Change in BPI severity score at day 30
Flavocoxid	Anti-inflammation	NCT00294125(I)/ NCT00435292(NA)/ NCT00790985(NA)	Completed/ Completed/ Completed	2013 No results posted 2015 No results posted/ 2015 No results posted
Fulranumab	Analgesic	NCT02336698(III)/ NCT02289716(III)/ NCT02336685(III), etc.	Completed/ Completed/ Completed	2017 No results posted/ 2017 No results posted/ 2017 No results posted
FX005	Analgesic	NCT01291914(II)	Completed	2013 No results posted
Gabapentin	Analgesic	NCT01546857(IV)/ NCT03540030(IV)/ NCT03334903(IV)	Terminated/ Completed/ Completed	2016 No longer able to recruit subjects due to unavailability of orthopedic surgeon/ 2020 Post-operation pain/ 2021 Opioid consumption
Gefapixant	Analgesic	NCT01554579(II)	Completed	2019 The primary efficacy endpoint: weekly average daily NPRS (average pain)

Table 1 (continued)

Name	Category	Identifier	Status	Update year and primary measurement
GLPG1972	Anti-matrix degradation	NCT03595618(II)/ NCT02851485(I)/ NCT03311009(I)	Complete/ Completed/ Completed	2021 Change in cartilage thickness of the cMTFC as assessed by qMRI to week 52. no positive results/ 2016 No results posted/ 2017 No results posted
Glucosamine sulfate	Extracellular matrix	NCT01893905(III)/ NCT01906801(IV)/ NCT02604381(II), etc.	Completed/ Completed/ Completed	2017 Change in pain according to VAS / 2017 No results posted/ 2017 No results posted
GRT6005	Analgesic	NCT01357837(II)/ NCT01709214(II)	Completed/ Completed	2021 No results posted/ 2019 No results posted
GSK3858279	Analgesic	NCT03485365(I)	Recruiting	2021 No results posted
GW406381	Anti-inflammation/ Analgesic	NCT00120900(III)	Completed	2017 No results posted
GW842166	Analgesic	NCT00447486(II)/ NCT00479427(II)	Completed/ Completed	2009 No results posted/ 2017 No results posted
GZ389988	Analgesic	NCT02845271(II)/ NCT02424942(I)	Completed/ Completed	2017 No results posted/ 2016 No results posted
HLXL	Anti-inflammation/ Analgesic	NCT00755326(II)	Completed	2020 Pain normalized score
HOE140 (Icatibant)	Anti-inflammation	NCT00303056(II)	Completed	2012 No results posted
Hydrocodone	Analgesic	NCT01240863(III)/	Completed/	2017 Change in weekly average pain intensity (w/API)
(CEP-33237)	Anti-inflammation	NCT01223365(III)	Completed	2017 Participants with adverse experiences
Hydroxychloroquine	Anti-inflammation	NCT01148043(III)/ NCT01645176(II)	Completed/ Completed	2015 No results posted/ 2018 Change in synovitis
HZT-501	Anti-inflammation/ Analgesic	NCT00613106(III)/ NCT00450216(III)/ NCT00984815(III)/	Completed/ Completed/ Completed	2013 Number of participants with treatment- emergent adverse events/ 2013 Number of participants developing endo- scopically diagnosed gastric ulcers/ 2013 Number of participants with treatment- emergent adverse events
Ibuprofen	Anti-inflammation/ Analgesic	NCT00565084(I)/ NCT01496326(II)/ NCT01860833(IV), etc.	Completed/ Completed/ Completed	2015 Change in average pain intensities/ 2011 No results posted/ 2013 No results posted
IDEA-033	Anti-inflammation/ Analgesic	NCT00316784(III)/ NCT00265304(II)/ NCT00211549(II)	Completed/ Completed/ Completed	2009 No results posted/ 2009 No results posted/ 2009 No results posted
Imotun	Analgesic	NCT01756937(IV)/ NCT01743287(IV)	Completed/ Completed	2017 No results posted/ 2015 No results posted
Imrecoxib	Analgesic	NCT01985165(IV)/ NCT02426060(I)	Unknown Status/ Unknown Status	2013 No results posted/ 2015 No results posted

Table 1 (continued)

Name	Category	Identifier	Status	Update year and primary measurement
JNJ-39439335	Analgesic	NCT00933582(I)/ NCT01343303(I)	Completed/ Completed	2010 No results posted/ 2012 No results posted
JNJ-42160443	Analgesic	NCT01094262(II)	Terminated	2016 Logistic reasons associated with the FDA-imposed clinical hold
SKI306X (JOINS)	Anti-inflammation/ Analgesic	NCT01293955(IV)	Completed	2015 No results posted
JTA-004	Anti-inflammation/ Analgesic	NCT02740231(III)	Completed	2018 No results posted
Ketamine	Anti-inflammation/ Analgesic	NCT02220400(NA)	Unknown Status	2014 No results posted
Ketoprofen (HKT-500-US12)	Anti-inflammation/ Analgesic	NCT00722852(III)/ NCT00108810(II)/ NCT00647231(I), etc.	Completed/ Completed/ Completed	2009 No results posted/ 2012 No results posted/ 2015 No results posted
Ketorolac, Ketorolac Tromethamine	Anti-inflammation/ Analgesic	NCT03694821(IV)/ NCT02570503(IV)/ NCT02295189(NA)	Terminated/ Terminated/ Completed	2020 Terminated due to low enrollment/ 2021 Study's primary aims are no longer clinically impactful
Lacosamide	Analgesic	NCT00485472(II)/ NCT02342977(II)	Terminated/ Withdrawn	2014 No results posted 2017 Based on the outcome of the planned first interim analysis, it was decided not to continue the trial/ 2016 Unable to enroll any patients into the study
Lamaline	Analgesic	NCT00264225(III)	Completed	2008 No results posted
LBSA0103	Extracellular matrix	NCT02122601(III)/ NCT01510535(III)	Completed/ Completed	2015 No results posted/ 2013 No results posted
LEVI-04	Analgesic	NCT03227796(I)	Recruiting	2021 No results posted
Lidocaine(Lidoderm)	Analgesic	NCT00589979(II)/ NCT00904462(II)/ NCT00904605(IV)	Completed/ Completed/ Terminated	2017 Time-to-exit due to lack of efficacy/ 2010 No results posted/ 2010 Safety concerns with the COX-2 specific inhibitor class of drug
Lopain (MTX-071)	Analgesic	NCT02566564(I)	Completed	2021 No results posted
LT-NS001	Anti-inflammation/ Analgesic	NCT01063920(III)	Completed	2011 No results posted
Lumiracoxib	Anti-inflammation/ Analgesic	NCT00267176(IV)/ NCT00419796(IV)/ NCT00476034(III), etc.	Completed/ Completed/ Completed	2017 No results posted/ 2007 No results posted/ 2012 No results posted
LY2828360	Analgesic	NCT01319929(II)	Completed	2020 Change From Baseline to 4 Week Endpoint in Weekly Mean of Daily 24-Hour Average Pain Scores (APS)
LY2951742	Analgesic	NCT02192190(II)	Terminated	2019 Interim assessment: Lack of efficacy

Table 1 (continued)

Name	Category	Identifier	Status	Update year and primary measurement
LY545694	Analgesic	NCT00790790(II)	Completed	2012 Change From Baseline in Weekly Mean 24-h Average Pain Severity (APS) Score From Electronic Diary at 5 Weeks
M6495	Anti-matrix degradation	NCT03224702(I)/ NCT03583346(I)	Completed/ Completed	2019 No results posted/ 2020 No results posted
MEDI-578	Analgesic	NCT01072591(I)	Terminated	2013 Amended clinical development plan
Meloxicam	Anti-inflammation/ Analgesic	NCT01801735(III)/ NCT02183129(IV)/ NCT00635349(IV), etc.	Completed/ Completed/ Completed/	2015 Safety of Meloxicam 10 mg as Assessed by the Incidence of Adverse Events From Baseline to Week 52 or Early Termination/ 2014 No results posted/ 2013 Change in WOMAC total score
MEN16132	Anti-inflammation	NCT01091116(II)	Completed	2013 WOMAC VA 3.1 A score (total Pain)
Mepivacaine	Analgesic	NCT03838874(III)/ NCT03948386(IV)	Active, not recruiting/ Completed	2021 No results posted/ 2021 Percentage ambulating early after spinal anesthesia
Methotrexate	Anti-inflammation/ Analgesic	NCT01927484(II)/ NCT01654575(NA)/ NCT03815448(NA), etc.	Terminated/ Completed/ Recruiting	2020 Terminated due to COVID-19 pandemic/ 2012 No results posted/ 2021 No results posted
Methylsulfonylmethane	Analgesic	NCT01836757(I)	Unknown Status/	2013 No results posted/
Milnacipran	Analgesic	NCT01188213(NA) NCT01780389(IV)/ NCT01510457(IV)/ NCT01329406(IV)	Completed/ Completed/ Completed/ Unknown Status	2010 No results posted/ 2015 Change in pain visual analog scale (VAS)/ 2015 McGill pain questionnaire/ 2011 No results posted
MIV-711	Anti-matrix degradation	NCT03037489(II)/ NCT02705625(II)	Completed/ Completed	2019 Safety and tolerability / 2019 Numeric rating scale (NRS) average target knee pain Score
MK0686	Anti-inflammation	NCT00296569(I)	Completed	2014 No results posted
MK0822	Anti-osteoporosis	NCT00397683(II)	Terminated	2015 No results posted
Modafinil	Other (stimulant)	NCT02155257(IV)	Completed	2019 Resting pupil diameter
Morphine	Analgesic	NCT00901628(IV)/ NCT02658149(IV)/ NCT02292082(IV), etc.	Completed/ Completed/ Completed	2013 Pain (visual analog scale)/ 2018 Pain score at 3 Hours/ 2019 Time to meet physical therapy discharge criteria
Motore	Anti-inflammation	NCT02409381(IV)	Completed	2016 No results posted
MT-5547	Analgesic	NCT03245008(III)	Active, not recruiting	2019 No results posted
Multiprofen	Anti-inflammation/ Analgesic	NCT03199417(III)	Terminated	2019 Terminated prematurely due to feasibility issues with rate of recruitment
Naltrexone	Analgesic	NCT03008590(II)	Completed	2021 Brief pain inventory—pain interference

Table 1 (continued)

Name	Category	Identifier	Status	Update year and primary measurement
Naproxen	Anti-inflammation/ Anti-inflammation	NCT00541489(III)/	Completed/ Terminated	2009 No results posted/ 2013 No results posted
		NCT01207973(I)	Completed	2011 No results posted
		NCT00662896(I)	Completed/ Unknown Status/ Completed	2015 Change in time weighted average (TWA) pain intensity (PI)/ 2015 No results posted/ 2016 No results posted
NEO6860	Analgesic	NCT02712957(II)/ NCT02337543(I)	Completed/ Completed	2017 No results posted/ 2016 No results posted
Neridronate	Analgesic	NCT01803360(II)	Unknown Status	2013 No results posted
NISE (Nimesulide)	Anti-inflammation/ Analgesic	NCT02922712(IV)	Completed	2016 No results posted
NKTR-181	Analgesic	NCT01619839(II)	Completed	2014 No result posted
ONO-4474	Analgesic	NCT02997696(II)/ NCT02454387(I)	Terminated/ Completed	2018 Stopped for business reasons. No safety issues were reported/ 2016 No results posted
OROS hydromorphone	Analgesic	NCT00980798(III)/ NCT00399048(III)/ NCT00411164(III)	Completed/ Completed/ Completed	2014 Analgesic effect as assessed by brief pain inventory (BPI) item 5 score (pain on average)/ 2010 No results posted/ 2013 No results posted
Oxycodone (PF-00345439)	Analgesic	NCT00361504(III)/ NCT00784277(III)/ NCT00784810(IV), etc.	Completed/ Completed/ Completed	2014 Number of participants with treatment-emergent adverse events/ 2012 5-Day sum of pain intensity difference (SPID5)/ 2011 Average daily pain score Box Scale-11 (BS-11)
Paracetamol	Anti-inflammation/ Analgesic	NCT02311881(III)/ NCT01105936(IV)/ NCT03781544(IV), etc.	Completed/ Completed/ Recruiting	2017 Time-weighted mean change in WOMAC pain/ 2017 Blood oxygen level-dependent (BOLD) response in the tibiofemoral joint of knee osteoarthritis/ 2021 No results posted
Parecoxib	Anti-inflammation/ Analgesic	NCT02198924(IV)/ NCT01311804(III)	Completed/ Unknown Status	2019 No results posted/ 2011 No results posted
Pelubiprofen	Anti-inflammation/ Analgesic	NCT02682524(IV)	Completed	2016 No results posted
Pennsaid	Anti-inflammation/ Analgesic	NCT01511939(III)/ NCT01119898(II)	Completed/ Completed	2014 Change in laboratory results of prothrombin time/ 2019 Pain when walking on a flat surface
PF-04136309	Other	NCT00689273(II)	Completed	2012 Change in WOMAC pain domain score

Table 1 (continued)

Name	Category	Identifier	Status	Update year and primary measurement
PF-04457845	Analgescic	NCT00981357(II)	Completed	2017 No results posted
PF-05089771	Analgescic	NCT01529671(I)	Completed	2012 No results posted
PG201	Anti-inflammation	NCT01576419(III)/ NCT01122485(II)	Completed/ Completed	2012 No results posted/ 2019 No results posted
PG-530742	Anti-matrix degradation	NCT00041756(II)	Completed	2011 Change in minimum joint space width in the medial compartment of the tibiofemoral joint: no significant difference
PH-797804	Anti-inflammation	NCT01102660(II)	Completed	2014 No results posted
PLA-695	Anti-inflammation	NCT00396955(II)	Terminated	2007 Terminated due to imbalance of gastrointestinal and the lipase events
PN400	Anti-inflammation/ Analgescic	NCT00527904(III)/ NCT00664560(III)/ NCT00665431(III)	Completed/ Completed/ Completed	2010 Number of subjects monitored for long-term safety/ 2012 Change in WOMAC pain questionnaire subscore 2011 Change in WOMAC pain questionnaire subscore
Prednisolone, Methylprednisolone	Steroid	NCT00492973(NA)/ NCT00682357(NA)/ NCT03082092(IV), etc.	Completed/ Completed/ Completed	2013 Length of hospital stay 2016 Change in serum osteocalcin/ 2019 No results posted
Pregabalin	Analgescic	NCT00442546(III)	Completed	2021 Subject reported worst pain score in daily diaries
Resiniferatoxin	Analgescic	NCT04044742(III)/ NCT03542838(I)	Not yet recruiting/ Recruiting	2020 No results posted/ 2021 No results posted
Resveratrol	Anti-inflammation/ Anti-oxidation	NCT02905799(III)	Recruiting	2021 Mean change from baseline in knee pain
Rofecoxib	Anti-inflammation/ Analgescic	NCT00568295(III)/ NCT00092352(III)/ NCT00092365(III), etc.	Completed/ Completed/ Completed	2011 No results posted/ 2017 No results posted/ 2017 No results posted
Ropivacaine	Analgescic	NCT00901628(IV)/ NCT01042093(NA)/ NCT00562627(IV), etc.	Completed/ Completed/ Completed	2013 Pain (visual analog scale)/ 2013 Numerical rating scale (NRS) pain scores/ 2011 Pain at rest (VAS)
SAR113945	Anti-inflammation	NCT01598415(II)/ NCT01511549(I)/ NCT01463488(I)	Completed/ Completed/ Completed	2014 No results posted/ 2012 No results posted/ 2012 No results posted
SB-061	Anti-matrix degradation/ Analgescic	NCT03231280(II)/ NCT02802709(II)	Completed/ Completed	2018 No results posted/ 2018 No results posted
SI000413	Anti-inflammation/ Anti-matrix degradation	NCT01525758(II)	Unknown Status	2013 No results posted

Table 1 (continued)

Name	Category	Identifier	Status	Update year and primary measurement
SM04690, Lorecivivint	Anti-inflammation	NCT03928184(III)/ NCT03122860(II)/ NCT03727022(II), etc.	Active, not recruiting/ Completed/ Active, not recruiting	2021 No results posted/ 2021 Change osteoarthritis (OA) pain in the target knee/ 2021 No results posted
Spirolactone	Analgesic/Steroid	NCT02046668(IV)	Completed	2019 No results posted
Strontium chloride- hexahydrate	Analgesic	NCT00954629(III)	Unknown Status	2010 No results posted
Strontium ranelate	ECM synthesis promoter	NCT03937518(NA)	Completed	2020 No results posted
SUPARTZ	Extracellular matrix	NCT02110238(III)/ NCT00479687(III)	Completed/ Completed	2021 WOMAC VAS pain subscale score change/ 2021 Visual analog scale (VAS: 0–100) for pain on movement
Synera	Analgesic	NCT01654302(II)	Completed	2015 Index knee pain scores on a numeric rating scale (NRS)
Tapentadol Hydrochloride, JNS024ER	Analgesic	NCT00983073(III)/ NCT00486811(III)/ NCT00487435(III), etc.	Completed/ Completed/ Completed	2019 Change of the average pain intensity score/ 2019 Change of the average pain intensity/ 2014 Number of subjects with treatment-emergent adverse events
TDS-943	Anti-inflammation/ Analgesic	NCT00546507(III)/ NCT00546832(III)	Completed/ Completed	2010 No results posted/ 2010 No results posted
TLC599	Steroid	NCT03754049(II)/ NCT03005873(II)/ NCT02803307(II)	Recruiting/ Completed/ Completed	2021 No results posted/ 2018 No results posted/ 2016 No results posted
Tramadol, Tramadol HCl (JNS013)	Analgesic	NCT00833794(III)/ NCT00833911(III)/ NCT00635349(IV), etc.	Completed/ Completed/ Completed	2012 Pain intensity score/ 2012 Number of patients having experienced an adverse event/ 2013 Change in WOMAC total score
Triamcinolone (Zilretta)	Steroid	NCT03378076(II)/ NCT03895840(IV)/ NCT01230424(IV), etc.	Completed/ Active, not recruiting/ Completed	2019 Measure the concentration of triamcinolone acetamide in blood plasma/ 2021 30 s chair standing test 2017 Change in mean cartilage thickness in the index compartment: greater cartilage loss
TV-45070	Analgesic	NCT02068599(II)	Completed	2020 Change in the average evening pain intensity in the target knee
UBX0101	Anti-apoptosis	NCT03513016(I)	Completed	2020 No results posted
V120083	Analgesic	NCT03028870(II)	Completed	2019 Daily "average pain over the last 24 h" score
Valdecoxib	Anti-inflammation/ Analgesic	NCT00652808(III)/ NCT00648258(III)/ NCT00650624(II)	Completed/ Completed/ Completed	2008 No results posted/ 2018 No results posted/ 2018 No results posted
Venlafaxine	Analgesic	NCT00611676(IV)	Completed	2008 No results posted

Table 1 (continued)

Name	Category	Identifier	Status	Update year and primary measurement
Verapamil	Anti-matrix degradation	NCT01645709(II)	Terminated	2014 Sponsor decision to terminate study
Vitamin D	Other (vitamin)	NCT00599807(IV)/ NCT01176344(III)/ NCT00306774(II), etc.	Completed/ Completed/ Completed	2014 No results posted/ 2015 No results posted/ 2011 No results posted
VM902A	Analgesic	NCT02847702(II)	Terminated	2017 Company decision to terminate study
VX-150	Analgesic	NCT02660424(II)	Completed	2020 No results posted
WIN-34B	Anti-inflammation/ Anti-matrix degradation	NCT01873053(II)	Completed	2015 No results posted
YH23537	Analgesic/ Anti-degradation	NCT02759198(II)	Completed	2019 No results posted
YYC301	Anti-inflammation/ Analgesic	NCT03850587(II)	Not yet recruiting	2021 No results posted
Zoledronic acid	Chondroprotector	NCT01218035(IV)	Completed	2017 No results posted

Data were collected from www.clinicaltrials.gov

RA rheumatoid arthritis, NA not applicable

[69, 70]. NSC23766 successfully suppressed chondrocyte hypertrophy and ADAMTS-5 expression in an OA mouse model [69].

Prostaglandin E2 (PGE2) is an important mediator of inflammation [71]. Otsuka et al. investigated the effect of PEG2 on the repair of chondral and osteochondral defects in rabbits using a selective agonist of the PEG2 receptor EP2 (ONO-8815Ly). They demonstrated that microspheres loaded with ONO-8815Ly could promote the regeneration of COL2-positive tissues, particularly at 12 weeks post-operation [72]. Similarly, the oral administration of KAG-308, a selective agonist of the PEG2 receptor EP4, suppressed the development of OA in mice by reducing chondrocyte hypertrophy and TNF secretion [73]. In addition, NSC117079, which targets Phlpp protein phosphatases, attenuated mechanical allodynia and slowed articular cartilage degradation in a murine OA model. It also increased the production of cartilage ECM in human OA cartilage explants [74].

Bradykinin (BK) is an intrinsic nonapeptide for vasodilation and has been identified as an inflammatory mediator in OA [75, 76]. BK mediates chronic inflammatory responses, inflammatory factor release, and NF- κ B signaling pathway activation through the B2 receptor. Icatibant and fasitibant are two inhibitors of BK B2 receptors [75]. A clinical trial (NCT02205814) demonstrated that fasitibant had a moderate effect on OA.

The renin-angiotensin system (RAS) is associated with metabolic bone diseases and arthritis [77, 78]. RAS components were also expressed in hypertrophic or IL-1 β /TNF- α stimulated chondrocytes [79], and Aliskiren, a RAS inhibitor, suppressed IL-1, TNF- α , and ECM degradation and chondrocyte hypertrophy in an OA rat model [80].

Transcriptional factor c-Fos/activator protein-1 (AP-1) has been shown to regulate inflammation and matrix degradation [81, 82]. In 2008, Aikawa et al. designed a de novo small molecular inhibitor of c-Fos/AP-1, T-5224, using three-dimensional (3D) pharmacophore modeling. Notably, administration of this compound prevented or resolved arthritis by reducing inflammatory cytokines and MMPs in mice [83]. In 2018, the same research group demonstrated that T-5224 prevented articular cartilage destruction by inhibiting MMP13 expression in a C57Black6/J OA model and prevented OA osteophyte formation [84].

Since rheumatoid arthritis (RA) is also characterized by inflammatory symptoms, RA drugs can potentially function like DMOADs and inhibit inflammation and pain. Methotrexate (MTX), a drug targeting synovitis in RA, was found to relieve pain and improve joint function in patients with OA after a 6-month treatment course. However, the outcome after 12 months of treatment was not significantly different from that of the placebo group [85]. Ketamine is an anesthetic clinically used to treat chronic pain [86]. It was recently shown to alleviate RA by antagonizing the

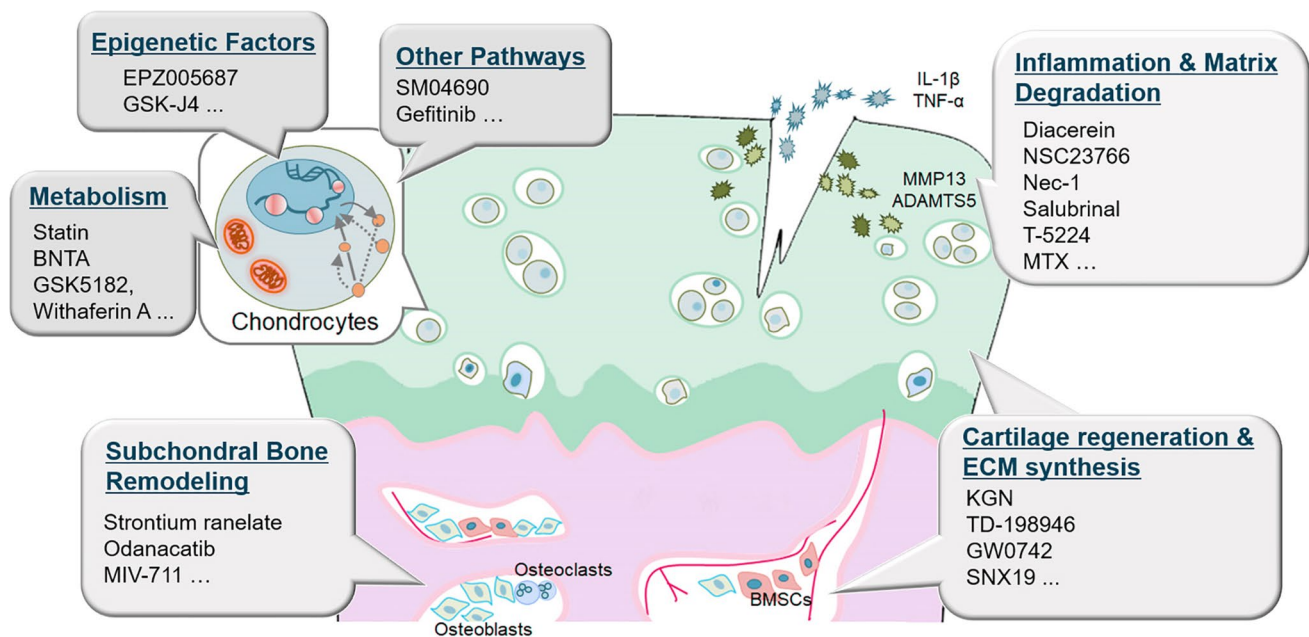


Fig. 2 Potential OA targets and representative developing small molecular drugs. *BMSCs* bone marrow mesenchymal stem cells, *ECM* extracellular matrix, *IL-1 β* Interleukin 1 β ; *TNF- α* tumor necro-

sis factor α , *MMP13* metalloproteinase 13, *ADAMTS-5* a disintegrin and metalloproteinase with thrombospondin motifs 5

N-methyl-D-aspartate (NMDA) receptor and inhibiting inflammatory and Th17 cellular responses [87–89]. In an OA rabbit model, ketamine protected articular cartilage from joint inflammation [88].

Although small molecules had great anti-inflammatory effects on OA, careful consideration must be given to the clinical heterogeneity of OA, as only parts of the patients are in the condition of acute high-level inflammation. Numerous studies have shown that simply blocking inflammation may not initiate the regenerative processes in cartilage [90]. Therefore, there is a need to increase our understanding of OA pathology and clarify the role of inflammation in tissue regeneration.

Small molecules for treating pain from OA

There is a strong association between inflammation and pain in patients with OA and several studies have suggested that pain from OA is driven by inflammation [91–93]. This theory has not been conclusively confirmed due to the complexity of measuring pain and OA pathogenesis [94]. However, as a major symptom of OA, pain is an important target and the primary outcome measure of OA disease management. There is an urgent need to develop analgesics with satisfactory clinical outcomes for many patients. Generally, both NSAIDs and glucocorticoids can target inflammation to treat OA pain. However, their efficacies are often short-lived and there are adverse effects associated with their long-term use [95–99]. Recent years have witnessed several

promising candidates for pain control, including antibodies against nerve growth factor and some chemical small molecule molecules. These are discussed below based on their molecular targets.

G-protein coupled receptors (GPCRs) are highly drug-gable targets and are expressed in almost all cells that constitute the components of pain signaling in humans [100]. They include opioid receptors. Morphine is a strong analgesic that binds to opioid receptors and blocks the transmission of nociceptive signals, but its use is restricted due to safety concerns. Miller et al. provided a good summary of the drugs in this class [101]. Among them, CR845 has been shown to benefit OA. CR845 is a second, peripherally restricted, selective agonist of kappa opioid receptors [102]. In a phase 2B clinical trial (NCT02944448), oral CR845 (Cara Therapeutics) was well tolerated, with a statistically significant effect on pain. Another new drug, cebranopadol, targets both nociceptin/orphanin FQ peptide receptor and m opioid receptor. It showed more promising outcomes and better safety profiles in clinical trials compared with traditional opioids [103, 104]. However, results from clinical trials on OA are yet to be reported.

Apart from GPCRs, ion channels are also well-studied components in the pain signaling pathway [105], including OA chronic pain [106]. In this class, CNTX-4975, a highly purified, synthetic trans-capsaicin targeting the transient receptor potential vanilloid 1, has been tested in clinical trials. Based on results from a 24-week, randomized, double-blind study [107], a single injection of CNTX-4975

had analgesic effects on pain associated with walking, knee stiffness, and physical function. Additionally, NEO6860, an oral transient receptor potential vanilloid 1 blocker, showed positive clinical outcomes in a proof-of-concept study [101].

In conclusion, analgesics are the most commonly used OA drugs (Table 1), as pain relief remains the most direct need, considering patients' perspectives during OA therapy. Unfortunately, even after decades of research in this field, drugs that can delay or prevent OA progression are still unavailable. One possible reason for this is that pain killers and disease-modifying drugs are often investigated separately in different fields of study [94, 108]. Importantly, we still lack evidence showing that analgesics can induce structural changes.

Small molecules regulating metabolism

Increasing evidence indicates that lipid metabolic disorders are relevant to the development of OA [109–111]. Statin drugs competitively bind to HMG-CoA reductase [112], thereby inhibiting cholesterol synthesis. In 2014, Yamashita et al. discovered that lovastatin treatment could rescue the FGFR3 skeletal dysplasia phenotype in a patient-derived iPSC chondrogenesis model [113]. Interestingly, recent studies have reported that statins have potential as chondroprotective reagents. Pathak et al. discovered that atorvastatin protects rat OA cartilage explants from IL-1 β -stimulated degeneration in vitro [114]. Yudoh reported that, in a mouse model which spontaneously develops OA, statins could reduce articular degeneration [115]. However, clinical outcomes of statin therapy for OA remain controversial, and only partially indicate that statin use is associated with a lower risk of knee OA progression and knee pain [116–118]. The pharmacological mechanisms underlying these effects may be associated with the serum lipid control and anti-inflammatory ability of statins [118].

Estrogen-related receptors (ERRs) are involved in the metabolic activities of organs such as the liver, heart, and skeletal muscles, and are also associated with type II diabetes and metabolic syndrome [119, 120]. One of the ERR isoforms, ERR γ , was shown to be highly expressed in the cartilage of human patients with OA and in mouse models, and promoted OA development by upregulating MMP3 and MMP13. In the presence of the small molecule inhibitor GSK5182, ERR γ activation was inhibited and the OA phenotype was markedly ameliorated in a temporomandibular joint OA rat model [121].

Mitochondrial dysfunction has been observed as a typical feature of OA chondrocytes and is a promising pharmacological target for the maintenance of cartilage homeostasis [122, 123]. Inflammation is thought to be responsible for mitochondrial impairment, resulting in the increased production of inducible nitric oxide (iNO) by chondrocytes.

Using an IL-1 β -treated OA chondrocyte model, Eitner et al. showed that activation of cAMP by forskolin could prevent both iNO release and mitochondrial dysfunction [124]. However, the compound's effect on the animal model was not confirmed.

Oxidative stress is a major factor that can cause mitochondrial dysfunction [125] and increase MMP, collagenase, and gelatinase levels in OA. Hence, antioxidation can be regarded as a therapeutic strategy for OA. Curcuminoids are strong, natural antioxidants. A randomized controlled trial showed that curcuminoids could increase serum superoxide dismutase (SOD) activity and decrease malondialdehyde (MDA) concentrations to relieve oxidative stress in OA, although its direct effect was not validated [126]. In 2019, Shi et al. discovered a potential DMOAD, BNTA, which stimulated ECM expression and suppressed inflammatory factors. In a trauma-induced rat OA model, intra-articular injection of BNTA delayed disease progression through superoxide dismutase 3 (SOD3) [127]. This study confirmed that SOD3 can act as a disease-modifying target. Withaferin A (WFA) is a plant extract that has been utilized as a drug in India. Choudhary et al. showed that treatment with WFA upregulated the expression of *SOX9* and inhibited reactive oxygen species (ROS) synthesis, mitochondrial depolarization, and chondrocyte apoptosis induced by IL-1 β or TNF- α . Daily oral administration of WFA for 28 days alleviated articular cartilage erosion and improved the subchondral bone structure in rats [128].

Mitochondria are widely distributed in cells and are in charge of complex processes required for cell survival, thus many endogenous protective pathways are involved in the regulation of mitochondrial function. Autophagy and mitophagy are, therefore, potential targets. Autophagy is a degradative process that breaks down subcellular content in response to various stimuli and is a protective behavior that prolongs cell survival [129]. Autophagy impairment has been associated with OA [130]. Notably, trehalose, a natural disaccharide, can exert cell protective effects under various stress conditions in many cells [131] by activating autophagy through the mammalian target of rapamycin (mTOR)-independent pathway. In a study of OA, trehalose promoted autophagic flux and attenuated mitochondrial dysfunction, and this was validated using a DMM mouse model [132]. Similarly, mitophagy is a quality-control process that governs basal autophagy by targeting and eliminating dysfunctional mitochondria [129]. Urolithin A is a natural dietary compound that induces mitophagy and thus prevents the accumulation of dysfunctional mitochondria [133]. Exploring its effect on a mechanical loading model using human chondrocytes in a hydrogel showed that Urolithin A protected chondrocytes from mechanical injury and supported cartilage homeostasis [134].

Despite the importance of mitochondria in tissue homeostasis, few mitochondria-targeting drugs are available in OA clinical trials [135]. This may be due to lack of non-invasive methods for measuring mitochondrial function in human cartilage *in vivo*. Interestingly, Harms et al. used the protoporphyrin IX triplet state lifetime technique to noninvasively measure mitochondrial oxygen tension (mitoPO₂) in a rat model [136], providing a potential tool for clinical diagnosis. In addition, extensive investigations are needed to identify OA metabolic subtypes.

Small molecules regulating epigenetic factors

Various physiological and pathological processes are epigenetically regulated, including skeletal development and OA occurrence [137–141], indicating that pharmaceutical interventions targeting epigenetics are promising as OA therapies. H3K27 methylation is generally gene repressive [142]. Enhancer of zeste homolog 2 (EZH2) is an H3K27 methylase that is highly expressed in some human patients with OA [143]. The small molecule EPZ005687 inhibited EZH2, downregulated ADAMTS-5 and MMP13 expression, and ameliorated chondrocyte hypertrophy in a murine model [139]. Consistently, the expression of H3K27 demethylase KDM6B was reduced in human patients with OA, and KDM6B knockout in mice accelerated OA progression [138]. The KDM6B (JMJD3) inhibitor, GSK-J4, prevented *ex vivo* cartilage destruction in humans and suppressed disease development in a DMM-induced OA mouse model [140, 144].

Thus, it is clear that epigenetic factors play an important role in OA, but knowledge on whether or how they exacerbate the disease is currently lacking. A continuous evolution of the technique in this field may provide high-resolution insights and a comprehensive understanding, enabling manipulation of specific targets at specific stages of OA.

Small molecules targeting other pathogenic pathways

Wnt signaling is one of the most essential pathways for chondrogenesis, osteogenesis, and OA progression [145–147]. Deshmukh et al. performed high-throughput screening and found that SM04690 significantly inhibited Wnt signaling, promoted chondrogenesis of human mesenchymal stem cells (MSCs), and suppressed the expression of members of the MMP family in chondrocytes [148]. A rodent model showed that this small molecule could improve cartilage regeneration, with increased cartilage thickness [103]. The underlying molecular mechanism was identified in 2019, and it suggested that SM04690 (lorecevivint) modulated the Wnt pathway by inhibiting CLK2 and DYRK1A [149]. The drug demonstrated general safety and tolerance without serious

systemic adverse effects in phase I and phase II clinical trials. The study also showed clinical improvements in pain and joint function following a single intra-articular administration of 0.07 mg lorecevivint [150, 151]. No results are currently available for multiple rounds of administration of the drug. However, if the ongoing phase III trials exhibit good clinical efficacy profiles and marginal side effects [152], lorecevivint will become a promising DMOAD approved for knee OA therapy compared with intra-articular corticosteroid and hyaluronate injections [153]. Lietman et al. also reported that inhibiting Wnt/ β -catenin signaling could ameliorate OA [154]. They successfully reduced cartilage degeneration and synovitis in a mouse model using a small molecular weight inhibitor, XAV-939 [154].

EGFR signaling is vital in cartilage development and OA [155–158]. EGFR was recently shown to be activated in a subpopulation of patients with OA. The specific inhibitor, Gefitinib, which has been used in the treatment of non-small cell lung cancer (NSCLC) for decades, inhibited EGFR activation and re-activated chondrocyte autophagy, restoring the balance between ECM synthesis and degradation. This was demonstrated and validated in a study of Col2a1-creER^{T2} conditional knockout mice [159]. However, another study illustrated that EGFR is important for articular superficial layer maintenance and inhibiting EGFR accelerated OA occurrence in mice lineages specifically overexpressing heparin-binding EGF-like growth factor (HBEGF) [160].

As these pathways are essential for cell survival and growth, simply inhibiting them may raise safety concerns for practical use. However, designing a delivery system to suppress a target in a specific chondrocyte subpopulation is doable. It also highlights the importance of single-cell analysis in OA pathology, which uncovers significant changes in cell cluster distribution.

Small molecules targeting bone remodeling

Subchondral bone undergoes structural remodeling as OA progresses [161] and is a potential drug target. Strontium ranelate (SrR) regulates the balance between bone formation and resorption. Recently, SrR was shown to be capable of ameliorating OA with clinical improvement in joint structure and symptom control [162–164]. However, a separate study found that SrR did not improve cartilage in an OA porcine model and instead led to osteophyte overgrowth [165]. Odanacatib (MK0822) is a cathepsin K (CatK) inhibitor used to treat osteoporosis. High *CatK* expression in OA leads to cartilage matrix degradation, making it a promising therapeutic target for DMOADs [166]. Unfortunately, a 2006 clinical trial studying the effect of treating OA using odanacatib was terminated [167]. Other clinical profiles indicated that the development of odanacatib was stopped due to undesirable adverse effects such as higher incidence

of cardio-cerebrovascular events [168]. Another cathepsin K inhibitor, MIV-711, was also tested in a clinical trial, but no significant effect was observed.

Increased attention has been paid on subchondral bone remodeling in OA programs. However, the complex interactions between cartilage and bone are still poorly understood. Future advancements in this field will rely mainly on the development of new methodologies for clinical diagnosis for identifying OA subtypes relevant to bone remodeling. The corresponding small molecules may work as potential DMOADs or be used in combination with agents targeting cartilage in OA pharmacological treatments.

Nevertheless, OA is not only caused by cartilage deterioration or bone remodeling, but also shows a complex and heterogeneous syndrome with multiple tissue involvement [169]. Therefore, it is difficult to design a “one fits all” molecule able to treat all tissues, including cartilage, bone, ligaments, synovium, and meniscus. Interestingly, a few such cases exist. For example, efforts have been made to explore the effect of KGN, a star molecule in cartilage regeneration, on other musculoskeletal tissues. As confirmed in various animal models, KGN serves as an efficacious therapy in the treatment of meniscus damage, intervertebral disc degeneration, and tendon-bone interface injury [170–172]. However, until meaningful clinical outcomes have been shown, designing a “one fits all” molecule suitable for the whole joint remains a considerable challenge.

Strategies for screening small molecules for cartilage regeneration

To efficiently develop new small molecular drugs, high-throughput and high-content screening have become important steps in the drug discovery workflow [173]. High-throughput systems allow automatic, rapid, sensitive, and specific screening of large-scale chemical libraries, taking advantage of advanced image equipment and algorithms. In this section, we briefly introduce the key elements in the primary screening of new drugs for cartilage regeneration (Fig. 3), including *in vitro* screening models, specific phenotype readout, and advanced methodologies that prospectively have an impact on future chemical cartilage regeneration.

In vitro screening models

Chondrogenesis models

Cells capable of chondrogenic differentiation may become an additional source for cell-based therapy in cartilage injury intervention. It is, therefore, reasonable to set up chondrogenesis models. Practically, easily obtained cells and simple models are widely used. For example, in the discovery of KGN [19], the authors used primary human bone marrow MSCs in their chondrogenesis model. In their image-based high-throughput screening, human bone marrow MSCs were seeded in 384-well plates to form chondrogenic nodules. Early cell condensation on day 3 was positively stained using rhodamine B. This fluorescent tag did not identify

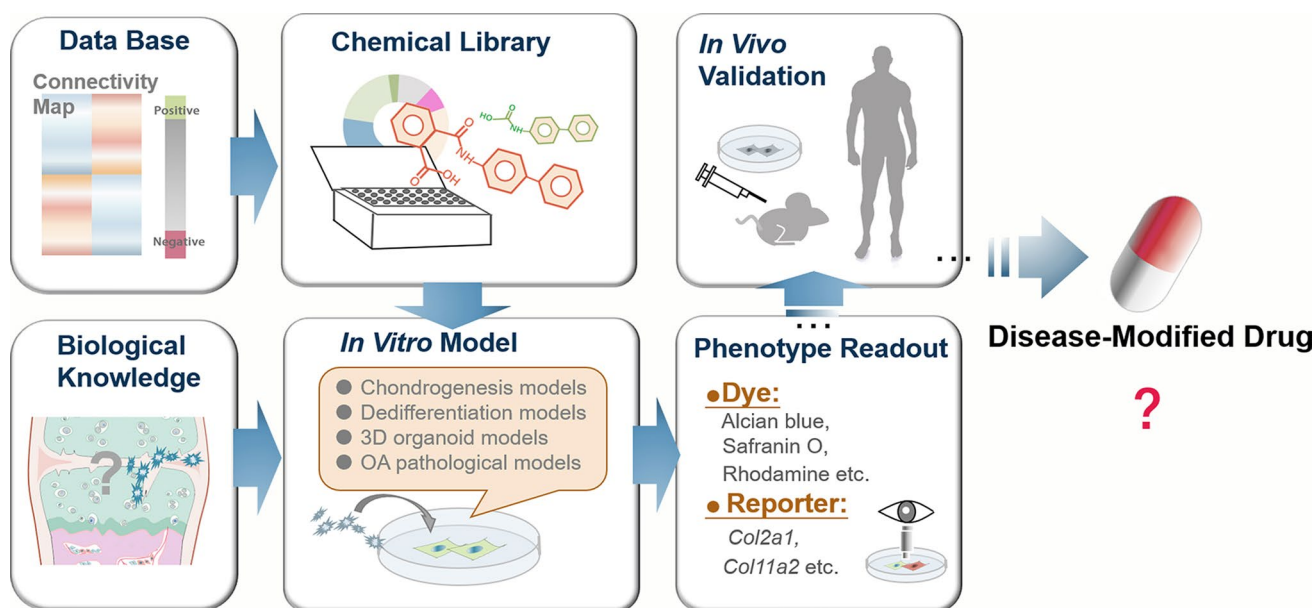


Fig. 3 Rational screening strategy of small molecular drug discovery for cartilage regeneration

chondrocyte ECM, but distinctly labeled cell condensation in the imaging system. In this way, KGN was identified as a hit among 22,000 structurally diverse molecules. In other studies, the chondrocyte progenitor cell line, ATDC5, is the most extensively adopted cell source because it is proliferative and stable under expansion. The ATDC5 cell line is derived from mouse teratocarcinoma and can form cartilage-nodule-like cell aggregates in the early stages of chondrogenesis induction [174]. Shi et al. treated ATDC5 cells with 2320 natural and synthetic small compounds for 7 days [127]. The cartilage-specific matrix component, proteoglycan, was assessed using Alcian blue. Additionally, the initial identification of TD-198946, SNX19, and oxytetracycline all relied on the ATDC5 screening system [24, 34, 175]. iPSCs are promising cell sources for drug screening because of their genetically defined chondrogenic differentiation potential [176]. Willard et al. used high-density micromass culture to chondrogenically differentiate mouse iPSCs and establish an iPSC-derived cartilage OA model [177]. Using a high-throughput screen, they found that the NF- κ B inhibitor, SC-514, effectively reduced cartilage damage in response to IL-1 α .

Dedifferentiation model

In addition to stem/progenitor cells, primary chondrocytes are useful tools for exploring the molecular features of cartilage degeneration and regeneration, although chondrocyte dedifferentiation remains unsolved [178]. Thus, primary isolated chondrocytes were less likely to be used in high-throughput screening. However, Kobayashi et al. used induced chondrogenic cells (iChons), which were trans-differentiated from mouse dermal fibroblasts by defined factors [179]. They exploited doxycycline-treated Col11a1 EGFP iChons as a dedifferentiation model in the screening assay and identified a novel compound which inhibited SOX9 degradation [36].

3D organoids

Significant progress has been made in organoids research. Specifically, the concept of organ-on-a-chip has spread in a range of fields, including cancer therapies, as well as studies of the liver, blood vessels, and lungs [180]. Organoids are believed to be more sensitive to individual-specific drug screening. Moreover, organoid models are estimated to be cost-effective, simulate *in vivo* tissues, and respond more accurately to candidate drugs. Cartilage is susceptible to mechanical microenvironmental stimuli. Nevertheless, current drug screenings are often performed in 2D monolayer cultures because 3D cultures require a larger number of cells and longer Z-stack imaging. Tuan et al. [181] introduced an adult MSC-based 3D microsystem of the osteochondral

complex for elucidating the pathogenesis of degenerative joint diseases and assessing the efficacy of potential therapeutics. The design of this 96-well microsystem involves a layered osteochondral tissue (bone, osteochondral interface, cartilage, or synovium) and mechanical loading modalities. The microtissue mimics tissue interactions and cellular responses to mechanical and inflammatory stimuli [181].

Cartilage explant models

Cartilage tissue explants are also an alternative model frequently used in cartilage regeneration [182–185]. Compared with isolated cells, they are estimated to simulate *in vivo* tissue and can serve as a “native cartilage” positive control [177]. When used in high-throughput screening, standardized *ex vivo* models provide a valuable platform. For instance, Schwab et al. established an *ex vivo* culture system for the long-term assessment of cartilage repair treatment strategies [186]. They isolated pig osteochondral biopsies and inserted them into an in-house developed six-well plate, where the cartilage and bone were cultured in separate compartments containing specific media. The viability, matrix content, and structural and mechanical properties of this model were maintained for at least 56 days, making the system a suitable tool for the characterization of cartilage regeneration treatment. Because the limited source of human joint tissue poses a challenge in large-scale experiments, Spinnen et al. developed a novel method that enables the preparation of 100 explant slices from a single human joint sample [187]. They used an orthopedic tissue punch to create an osteochondral cylinder punch (20 \times 10 mm) from a tibial plateau and inserted the cylinder into a custom 3D-printed microtome. The cylinder was then divided into eight disc-shaped cuts with a thickness of 500–800 μ m. The slices showed positive sensitivity to pharmacological testing after being cultured for three weeks. These findings indicate that *ex vivo* models can be applied in large-scale drug screening.

In vitro pathological models

OA inflammatory *in vitro* models are mostly based on the cellular responses of chondrocytes to inflammatory cytokines. When treated with IL-1 β or TNF- α , chondrocytes exhibit an OA-like phenotype, with higher levels of expression of MMPs and ADAMTS4/5, and reduced expression of ECM genes. Chen et al. also showed that IL-1 β substantially affected the mechanical properties of chondrocytes *in vitro* [188]. Alternatively, pathogenic inflammatory factors; IL-1 α , IL-6, and TGF- α , have also been adopted in OA inflammatory models [189, 190].

Nevertheless, current inflammation-related models do not fully reflect OA conditions. In these models, chondrocytes were treated with one or two specific cytokines, for

example IL-1 β or TNF- α . However, the concentrations of other cytokines such as IL-8, vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1), are all elevated in OA joints [191], but these cytokines are rarely used. Moreover, cytokine-based models often use concentrations higher than those found in vivo [192]. To simulate the complex conditions associated with inflammation in OA, Hoff et al. added synovial fluid from patients with OA to a cell culture [193]. Synovial membranes isolated from patients with OA can also be used in co-culture models [194]. Mechanical injury is a major cause of OA. Jin et al. applied a mechanical pressure model with a self-designed mechanical pressure container that generated 10 MPa pressure on normal human chondrocytes for 60 min. In their study [195], mechanical pressure induced early apoptosis of chondrocytes. In response to mechanical injury, chondrocytes were forced to upregulate their expression of *VEGF* and downregulate their expression of *Smad4*. In 2013, Kim et al. [196] found that the accumulation of advanced glycation end-products (AGEs) in aged cartilages was the major cause of ECM stiffening. Treatment with ribose (one of the AGEs) significantly increased MMP expression and reduced ECM molecule expression in chondrocytes embedded in collagen matrices, but not in those in 2D culture. Supplementation with β -aminopropionitrile (BAPN), a specific inhibitor of the LOX family, reversed these changes. This study validated the feasibility of establishing mechanical-associated in vitro OA models.

Phenotype readout

Dyes for cartilage ECM

Reliable phenotypic indicators for image recognition are essential. Dyes for cartilage histological characterizations, including Alcian blue, Safranin O, and Toluidine blue, are regularly adopted in screening as they can positively mark cartilage ECM composites. Le et al. used a fluorescently labeled collagen-binding probe, CAN 35-Alexsa 488, to quantify the total collagen content of ATDC5 cultures [34]. These phenotypic indicators provide clues for drug selection but may not be completely specific to the cartilage lineage.

Reporter systems

Reporter systems make it easier to detect molecular phenotypes using imaging systems. The most widely used cartilage reporter genes are *Col2a1* and *Col11a2*. Both are highly expressed in all cartilages and are essential for chondrocyte differentiation and skeletal morphogenesis [197].

A 1998 study indicated that *Col11a2* chondrocyte-specific enhancer elements share many similarities with the *Col2a1* 48-bp enhancer, and *Col11a2* and *Col2a1* might coordinately regulate chondrocyte differentiation [197]. Yano et al. and Hojo et al. used a Col2GFP-ATDC5 reporter system for high-throughput screening of potential DMOADs [24, 33]. Similarly, Kan et al. built an ATDC5-C2ER system to optimize the detection and analysis of fluorescence [175]. Horton et al. generated a new mouse strain harboring a Col2-pd2EGFP reporter transgene. Because pd2EGFP has a much shorter half-life than EGFP, it made a real-time reporter for *Col2a1* expression in vivo and in vitro. They also demonstrated that this reporter has illustrated the decrease in *Col2a1* in cultured articular chondrocytes [198]. Tsumaki et al. prepared chondrocytes from Col11a2-EGFP transgenic mice to provide a time-course of chondrocyte dedifferentiation [178]. They generated a Col11a2-EGFP human iPSC cell line using the piggyBac vector system and induced it to form hyaline chondrocytes [45]. They used the Col11a2 reporter cell line to screen 5822 compounds and found that A-674563 enhanced the expression of chondrocyte markers [36]. It is worth mentioning that the lineage-specificity of these biomarkers remains controversial and knowledge on hyaline chondrocyte biomarkers is still lacking. Generally, it is not advisable to identify small molecule drugs using a single-biomarker reporter in the primary screen. Therefore, efforts need to be made to identify more reliable cartilage biomarkers and establish multi-molecular reporter systems.

Mechanical property

As the mechanical microenvironment has been hypothesized to modulate chondrocyte function and fate [199], the biomechanical properties of cartilage explants or single chondrocytes are critical for cartilage regeneration tests. During the establishment of a post-traumatic OA model, Mohanraj et al. induced injury compression on engineered cartilage analogs and explants using a custom high-throughput mechanical screening device [200]. This device allowed for the compression of 48 samples in a standard 48-well plate and continuously recorded the load during injury. Although mechanical properties were not included as a phenotype readout, this case illustrated the possibility of applying mechanical stimulation and examination in high-throughput screening. In addition to cartilage tissue, chondrocyte mechanobiology has also been extensively studied [199, 201–203], and the atomic force microscope (AFM) has acted as a powerful tool for the characterization of single cells [201, 204]. The mechanical features of normal chondrocytes differed from those of OA chondrocytes, indicating that biomechanics could be a useful technique for investigating OA-related changes in human chondrocytes. However, as current measurements are time-consuming, a customer-friendly

instrument optimal for large-scale testing, which supports easy and fast detection during high-throughput screening, is urgently required.

Advanced methodologies in other fields

Optimal reporter cell line

Ideas and methods for improving screening efficiency can be borrowed from other fields. Here, we introduce an interesting study that used a genetically modified reporter cell line for lung cancer drug discovery. Kang et al. developed a method for systematically identifying optimal reporter cell lines for annotating compound libraries (ORCALs) [205]. They trans-genetically built a library of reporter cell lines tagged with different fluorescence and allowed the algorithm to choose which ORCALs optimally classify drugs in a large compound library. They obtained an A549 reporter cell line with fluorescence-labeled nucleus, cell membrane, and a specific protein, XRCC5, which could accurately identify a training set of known drugs. This proof-of-concept study presented a new strategy for improving the efficiency, scale, and accuracy of phenotypic screening by optimizing reporter cell lines.

Bioinformatic-based screening

Bioinformatics is generally used in genome and proteome dissection, image/structure organization, and network analysis. It has also been adopted in multiple drug discovery to help predict drug targets and drug-target interactions using large-scale datasets, which can greatly improve screening efficiency. The Connectivity Map system (www.broadinstitute.org/cmap/), created by Lamb et al. in 2006, aims to create a database that links clinical drug effects with human genomics to provide more valuable information [206]. In their study, they used ~5000 small molecule compounds and ~3000 gene regulatory agents to process a variety of cell lines. Through the development of the L1000 gene rapid detection side system, they collected genome sequencing data from these cell lines following drug treatment. The effects and cell states were then ranked. As a result, their connectivity Map indicated connections among small molecules with similar chemical mechanisms or physiological processes. In 2016, Kidd et al. examined the responses of 221 immune cell types to 1309 drugs [207]. This network can effectively predict 69,995 drug-immune cell interactions and potentially predict immune responses or side effects in future clinical applications. In 2018, Brum et al. identified molecular targets that could positively regulate osteoblast differentiation using a connectivity map [208], which is a good demonstration of the applicability of this system for drug development.

Chemistry synthetic toolbox

Despite multiple advances in drug discovery, transforming a screening hit into a commercial drug is still slow and expensive. The cost of each drug launch was estimated to be in excess of 2.6 billion dollars [209]. Thus, increasing attention is being paid to compounds that can be easily translated. After the primary identification of candidates, the second step is to optimize them through design-make-test-analysis cycles [210]. The key mission for experts in medicinal chemistry is to design and synthesize bioactive compounds that have the potential for use as drugs. They get access to chemical databases with reaction data (such as SciFinder and Reaxys) to search for an exact structure for precedence. If the exact compound is not found, a substructure search is conducted. There are many cases where biological discoveries are made by synthetic chemists exploring various chemical routes, such as the development of benzodiazepines, penicillin, tetracyclines, and taxanes [211, 212]. The role of medicinal chemistry has changed considerably, and expanding the synthetic platforms and design toolbox should enhance the impact of new methodologies in future drug discovery. Advances in chemistry are expected to greatly accelerate the development of DMOADs.

Challenges and outlooks

Increasing numbers of small molecular drugs for cartilage regeneration or OA modification have been tested in clinical trials, but so far, none has had a significant long-term effect. In our opinion, several issues and challenges warrant further investigation.

First, the induction efficacy and time required for phenotype control are major concerns. The fact that small molecules directly affect proteins, but not the genome, may become a double-edged sword for practical applications. It may cause induction failure, as chemical compounds are documented to only temporarily or reversibly alter cellular phenotypes. Chemical methods also require long-term step-wise induction. Second, individual patients can respond differently to the same chemical. Accumulating evidence supports the existence of OA subtypes [213–215]. Magnetic resonance imaging has been used to classify OA subtypes, but challenges still exist when it comes to diagnoses providing meaningful guidance for personalized therapeutic strategies. This necessitates a new diagnostic criterion allowing for early and individualized treatment, which not only relies on traditional imaging examinations, but also molecular detection. Third, a more in-depth understanding of cartilage biology and pathology is necessary to provide complete and informative data. This is important for the optimization of different chemical

combinations to manipulate cell fate more precisely in a defined manner. However, since cartilage injury and OA are complex conditions, there are still obstacles when it comes to selecting compounds that work against multiple targets without excessive side effects, thus further investigations need to be conducted. Last, cartilage-regenerative drug screening systems are not satisfactory compared with those in other fields. Previous cell models merely simulated responses to one or two factors and the biomarkers for chondrocyte phenotype readout are limited in number. A continuous evolution in techniques is required to build a 3D cultured screening system allowing for multiple-factor analysis.

In this review, we have summarized the latest research on novel small molecular compounds which target cartilage regeneration. Advanced drug discovery strategies derived from new concepts of regenerative medicine contribute to the identification of potential small molecular drugs for cartilage repair. Small molecule drugs are convenient and cost-effective to design, modify, and synthesize for personalized pharmaceutical management, and will make a strong push for the development of new pharmaceutical therapies for cartilage regeneration.

Funding This work was supported by the National Key R&D Program of China (2017YFA0104900), the Natural Sciences Foundation of China (81630065, 82002319), and the China Postdoctoral Science Foundation (2020M681892).

Availability of data and material The data and material that support this review are openly available.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Consent for publication was obtained from all participants.

Conflict of interest The authors declare that they have no conflicts of interest to this work.

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