Identifying small molecules for protecting chondrocyte function and matrix integrity after controlled compressive injury

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ABSTRACT

Objective: Articular cartilage injury is central for the development of post-traumatic osteoarthritis (PTOA). With few disease-modifying therapies successful at offsetting progressive osteoarthritis (OA), our goal is to use a high throughput screening platform of cartilage injury to identify novel chondroprotective compounds. Targeting articular cartilage damage immediately after injury remains a promising therapeutic strategy to overcome irreversible tissue damage.

Method: We constructed a single impact-cartilage screening method using a multi-platen system that simultaneously impacts 48 samples and makes use of engineered cartilage tissue analogs (known as CTAs). Drug libraries were screened and assessed for their ability to alter two crucial biological responses to impact injuries, namely matrix degradation and cell stress.

Results: Over 500 small molecules were screened for their ability to alter proteoglycan loss, matrix metalloproteinase activity, and cell stress or death. Fifty-five compounds passed through secondary screening and were from commercial libraries of natural and redox, stem cell related compounds, as well as protease, kinase and phosphatase inhibitors. Through secondary screening, 16 promising candidates exhibited activity on one or more critical function of chondrocytes. While many are mechanistically known compounds, their function in joint diseases is not known.

Conclusion: This platform was validated for screening drug activity against a tissue engineered model of PTOA. Multiple compounds identified in this manner have potential application as early protective therapy for treating PTOA, and require further study. We propose this screening platform can identify novel molecules that act on early chondrocyte responses to injury and provide an invaluable tool for therapeutic development.

1. Introduction

Osteoarthritis (OA) is a chronic joint degenerative disease that affects over 32 million adults in the United States alone. As a major source of pain and disability, OA is major burden to the health care system with direct costs estimated to be over 185 billion dollars annually. The pathology of OA involves progressive cartilage deterioration, osteoporosis, subchondral bone remodeling, and synovial inflammation [1,2]. However, at this time there is a limited number of effective disease-modifying drugs that can prevent or slow OA progression and restore joint function.

Current therapeutic strategies aim to minimize joint tissue structural changes, reduce inflammation, and minimize pain.

OA is considered an age-related disease and is highly prevalent in older populations, affecting over 80% of those beyond 55 years of age. However, those who engage in high-impact activities or have a history of joint injuries are at greater risk of developing OA and at an accelerated pace. This condition is referred to as post-traumatic OA (PTOA). PTOA, arising from excessive use or blunt force trauma, accounts for approximately 12% of all OA cases [3]. Our military services members are among those who are particularly at risk of developing OA due to the increased...
physical demands of military occupation. This is also the case for athletes who are at a higher predisposition to injuries and joint overuse, and as a result are almost twice as likely to develop knee and hip OA compared to non-athletes [4]. Individuals who sustain knee injuries, such as anterior cruciate ligament (ACL) or meniscus tears, are 4.2 times more likely to develop early onset OA [5,6]. Some reports show that half of those who experience knee injuries such as ACL or meniscus tears resulting in degenerative changes (i.e. chondral lesions and cartilage fissures), will develop PTOA 10–20 years after being diagnosed [7]. As many as 250,000 ACL injuries occur in the United States each year, most of which will affect individuals between the ages of 15 and 25 years old [8] and negative outcomes associated with PTOA can be expected to occur even as early as 3–10 years post-injury in this younger age group [9]. One of the challenges in treating PTOA is that disease progression does not correlate well with symptoms, which are highly variable. Patients are typically diagnosed only once they reach the symptomatic phase, at which point the condition is irreversible. The time course of onset varies from individual to individual. For some patients, symptoms may become apparent immediately after injury, while others remain asymptomatic for years before being clinically diagnosed [10]. Novel therapeutic strategies should focus on minimizing initial cartilage structural damage before reaching the symptomatic phase.

The pathogenesis of PTOA is a multifactorial process that can be categorized into three phases: (1) the immediate phase relating to mechanical disruption of cartilage and other joint structures; (2) the acute phase characterized by chondrocyte cell death (apoptosis) and inflammation; and (3) the chronic phase when anabolic/catabolic activity has prevailed and there is an emergence of joint pain and dysfunction. There is a critical need to develop therapeutic strategies that modulate early post-injury events, both in the immediate and acute phase, in an effort to prevent or delay the onset of disease. The initial pathology depends on the severity of the joint trauma itself. Blunt force to the joint can cause irreversible cartilage damage to the articular surface and even create fragmentation that penetrates to the underlying subchondral bone. Articular damage ruptures the collagen matrix and initiates rapid release of glycosaminoglycans (GAG), and tissue swelling ensues. Chondrocyte cellularity diminishes as cells undergo necrosis, further impairing cartilage tissue regeneration capacity. The acute phase occurs within a period of several days post-injury, and is characterized by increased cell death/apoptosis, inflammation, and increased catabolic activity PTOA associated cell apoptosis is thought to occur primarily through the caspase pathway [11], which can be activated by the pro-inflammatory tumor necrosis factor alpha (TNFα). However, clinical studies using therapeutics to inhibit TNFα have not fared well [12]. Inflammatory chemokines such as the interleukin (IL)-1, IL-6, and IL-8 are major players in OA progression, orchestrating key catabolic events. Targeting IL-1β, an inducer of MMPs, seems to be the most effective strategy yet. In preclinical animal models of PTOA, inhibiting IL-1 signaling, via IL-1 receptor antagonist (IL-1ra), successfully reduced cartilage degradation and synovial inflammation [12].

Small molecule compounds remain a promising source of therapeutic and preventative drugs, and as such are routinely investigated in high throughput drug screens. Compounds that modulate early cellular responses are of particular interest. Several positive compounds have emerged for their ability to reduce cell loss and death, including caspase inhibitors, antioxidants or free radical scavengers (ROS), and amphiphilic surfactants [13,14]. It has been shown that pan-caspase inhibitors as well ROS and cysteine Rotenone are effective in increasing cell viability after injury [13,15]. Alternatively, amphiphilic surfactants such as P188 have been applied to ‘re-seal’ disrupted cell membranes damaged by impact, thereby preventing activation of signalling pathways involving IL-6, p38, etc. [14,16]. In a recent study, the small molecule BNTA was selected for its ability to stimulate cartilage matrix production in vitro models of cultured chondrocytes and cartilage explants. These positive results carried over into animals, where intra-articular injection of BNTA delayed PTOA disease progression in an injured induced rat model [17]. Another promising candidate is the small-molecule Wnt pathway inhibitor, SM04690 [18], which has had success reducing joint destruction in a rat OA model, by way of promoting chondrogenesis and inhibiting catabolic activity [19]. SM04690 has been ruled safe and well-tolerated in human patients in a phase 1 clinical study and is currently being investigated in a phase 2 development program (clinicaltrials.gov identifiers NCT02536833 and NCT03122860) [20]. The small-molecule compound flavopiridol, which inhibits the transcription regulating cyclin-dependent kinase 9 (Cdk9), has been shown to modulate expression of early responsive genes following traumatic joint injury [21–23]. In patients with symptomatic radiograph knee OA, the recombinant human fibroblast growth factor 18 (rhFGF18), also known as sprifermin, improved femorotibial joint cartilage thickness [24]. MIV-711 is another small molecule inhibitor with potential as a disease-modifying osteoarthritis drug and is currently being evaluated in phase 2 clinical trial for OA of the knee [25]. MIV-711 is a potent inhibitor of cathepsin K, a cysteine protease involved in bone resorption and cartilage degradation through the breakdown of collagen (clinicaltrials.gov identifier NCT02705625).

Despite surgical intervention, physiotherapy, and use of anti-inflammatory or pain-related agents, treatment strategies have failed to prevent progression of OA, especially to alter the post-traumatic sequence of events. There is a critical need to develop therapeutics that target joint destructive pathways within the acute phase post-injury, in an effort to prevent or delay disease onset. Our hypothesis is that by using our phenotypically correct, engineered cartilage analog system (CTA) and a customized device designed device to deliver a compressive injury to multiple samples, bioactive molecules can be identified that interfere with injury biomarkers, cell stress and matrix degradation. Using this our developed high throughput system and to screening hundreds of compounds in a single experiment, we hypothesize that we will more quickly identify potentially therapeutic agents for PTOA scenarios. Our strategies focus on targeting early molecular events associated with chondrocyte cell death, inflammation, and matrix degradation, as these events preclude the development of a fully diseased joint. Here, we present a systematic approach to predict novel therapeutics for acute traumatic injury as occurs in PTOA. In total, 511 small molecules from 6 chemical libraries were selected for high throughput screening. The libraries consisted of natural compounds, redox compounds, and a variety of inhibitors critical for cell function, including protease, kinase and phosphatase inhibitors (Fig. 1A). These libraries contain a diverse set of natural compounds and synthetic compounds that target a variety of relevant signalling pathways involved in matrix degradation, apoptosis, and inflammation.

Investigating cartilage-targeting therapies require a non-conventional drug screening process as cartilage must be artificially injured to initiate a sequence of events, either chemically or mechanically, to better understand a drug’s response to their pathological state. Previously, mechanical injuries were carried out using drop-towers - a weight-based method would be dropped on a cartilage explant. Drop-tower methods screen one to ten drugs at a time approximately, making them an extensively time-consuming method [26]. We have constructed an Instron controlled cartilage impact screening method to impact 48 samples at one time. We make use of uniformly engineered cartilage units, also known as cartilage tissue analogs (CTAs) that can be fabricated easily in a cost-efficient manner [27,28]. These reproducible cartilage surrogates are all tested simultaneously in a multiplate format, reducing problems with natural cartilage tissue heterogeneity and confounding variables between donors that may impact the overall study conclusion. Hence, this novel screening strategy mimics a single impact of cartilage with clinical relevance, to rapidly identify potential therapeutic compounds to accelerate the drug discovery process. There have been other attempts at establishing high throughput screening platforms to investigate chondroprotective therapeutics however with soluble injury or inflammatory mediators. The challenge that these studies face is the inefficient cartilage surrogates, cartilage explants or constructs that are often heterogeneous in nature. Willard et al. used induced pluripotent stem cells (iPSCs) to formulate an
in vitro cartilage model which was then treated with IL-1 to simulate osteoarthritic conditions in a 96-well format [29]. Similarly, another study attempted a cartilage construct model to screen for anti-rheumatic agents using a platform that acted as a transition from 2-D in vitro models to in vivo animal models [30]. Prior studies have also attempted to screen through thousands of drugs using virtual platforms and computer-aided drug design (CADD) by using molecular dynamic simulation and by looking at the biochemical structures of therapeutics that target aggrecanase-1 and MMP-13 [31,32]. All previous attempts have presented the clinical need and interest for such a platform and have provided informative outcomes, however, none use compression as a source of injury in a high throughput manner. Here we utilize a novel platform that mimics compressive injury and addresses the PTOA as a clinical target [28].

2. Results

2.1. Screening existing drugs for novel inhibitory properties against cartilage degradation

As proof of concept, an initial screen was conducted on well-known biologically active compounds that have been used to pharmacologically target stem cell associated signaling pathways, selecting for an inhibitory effect on cartilage matrix degradation (GAG assay) (Fig. 2A). From the stem cell signaling library, five of the six positive hits identified are selective potent inhibitors and have FDA approved trials for inflammatory diseases such as rheumatoid arthritis. These compounds affect OA-relevant pathways involved in inflammation and matrix synthesis.
Fig. 3. Novel chondroprotective agents identified through high-throughput screening. Compounds from 6 libraries (stem cell signalling, protease inhibitors, kinase inhibitors, natural compounds, phosphatase and redox compounds) were assessed based on their ability to prevent glycosaminoglycan (GAG) and lactate dehydrogenase (LDH) release following a compressive injury. Dual-flashlight plots for strictly standardized mean difference (SSMD) versus average fold change were used to score and rank (A) GAG and (B) LDH responses. The red shaded regions correspond to a log2 fold change less than minus 0.5 and SSMD score less than minus one. Data points within these regions satisfy gating parameters and are considered positive hits. Each black circle represents a single data point, n = 1.

Table 1
GAG positive hits identified in primary phase.

| Library                | Total # of Compounds | # of GAG Hits | # of LDH Hits |
|------------------------|----------------------|---------------|---------------|
| Stem Cell Signaling    | 89                   | 6             | 4             |
| Protease Inhibitors    | 53                   | 7             | 0             |
| Kinase Inhibitors      | 80                   | 18            | 1             |
| Natural Compounds      | 173                  | 19            | 38            |
| Phosphatase Inhibitors | 33                   | 8             | 4             |
| REDOX Compounds        | 83                   | 1             | 8             |

Table 1

| Protease             | Natural compounds                      | Kinase                      |
|----------------------|----------------------------------------|-----------------------------|
| Dec-RVKR-CMK         | Magnolol*                               | Tyrophostin 9*              |
| Z-VAD-FMK*           | Azomycin*                               | Ro 31 8220 mesylate         |
| TLCK-HCL             | L-[-]-Rhamnose Monohydrate*             | H-7 2HCl                    |
| P32/98 hemifumarate salt | Puerarin                                | Piceatannol                |
| DL-Thiorphan*        | Enoxolone*                              | AG-c490                     |
| PPACK                | (-)-Epigallocatechin Gallate*           | Quercetin’2H2O             |
| PKSi-527             | Emodin*                                 | BML-265*                    |
|                      | Myricetin*                              | SU 4312                     |
|                      | Kinetin*                                | SU1498                      |
| Phosphatase          | Artemether                              | GF 109203X                  |
| BML-260              | Costunolide*                            | AG-126                      |
| RWJ-60475*           | Gynostemma Extract*                     | SB-202190*                  |
| Endothail            | Cinchonidine*                           | PKC-412                     |
| L-p-Bromotetramisole oxalate bi-5 | Piperine*                               | TYRPHOSTIN 25               |
| OBA*                 | Hyodeoxycholic Acid (HDCA)*             | N9-isopropyl-olomoucline    |
|                      | Esculin*                                | TYRPHOSTIN 1                |
|                      | Myrtgenol*                              | SB-203580                   |
|                      | Phloretin*                              | U-0126                      |
|                      | Gramine*                                |                             |
| Stem Cell            | Redox                                   |                             |
| Filgotinib (GLPG0634)| AA-861                                  |                             |
| Oclacetinib          |                                        |                             |
| Wnt agonist 1        |                                        |                             |
| Tofcitinib (CP-690550) Citrate |                            |                             |
| Galunisertib (LY2157299) |                                      |                             |
| Pacritinib (SB1518)  |                                        |                             |

*Denotes positivity in more than one assay
2.2. Systematic screening of chemical libraries

Moving forward, an additional 5 libraries were systematically screened, selecting for compounds with protective effects against matrix degradation and cell stress and/or death. Each library was screened and assessed independently, with each compound scored and ranked amongst the individual experimental group. Dual flashlight plots in panels A and B of Fig. 3 represent a summary of six independent screening experiments, where changes in GAG and LDH levels were used as outcome measures. The table in panel C reflects the number of the positive hits identified from each library and categorizes the hits based on their effect, either matrix degradation (GAG) or cell death (LDH). In total, 59 GAG and 55 LDH hits were identified. See Tables 1 and Table 2 for a complete list of positive hits. Compounds are grouped by library and shown in ranking order of greatest outcome effect.

2.2.1. Library screening

The top responding natural compounds were subjected to an additional screen to determine whether results were consistent among different donors. In total 55 compounds were subject to a second screening with at least 3 technical replicates. The compounds selected for this screen include the 17 positive hits (Table 1) and an additional 38 which tested positive but failed to pass our scoring criteria. Approximately 75% of the natural compounds had a positive response in the GAG release assay (Fig. 4A), while about 70% reported positive LDH activity for the second time (Fig. 4B).

2.4. Inhibition of IL-1β induced MMP activity

As a secondary follow-up screen, positive GAG hits from the initial primary screens were evaluated for their inhibitory effects on IL-1β, a well-known cytokine involved in pathogenesis of OA and inducer of MMP activity. IL-1β induced MMP activity was completely inhibited by the following four compounds: triptolide, (S)-10-hydroxyamptetoxine, SB-202190 and tyrphostine-9 (Fig. 5). Six other compounds also reduced MMP activity, but to a lesser degree (e.g. Schisandrin, ursolic, RWJ-60475, Z-VAD-FMK, DL-thiophran and BML-265).

2.5. Targeting OA relevant signaling pathways

We mined though the literature to identify those signaling pathways most targeted in our screening approach. The chart in Fig. 6A summarizes our results and categorizes compounds based on their respective molecular targets. As shown, we’ve grouped our compounds of interest into 8 categories, most of which fall into one of seven major signaling pathways. Those which have not been well-characterized are represented by the group N/A (not available), which accounts for 19% of the compounds. The class of JAK inhibitors make up the largest group at 25% of all the compounds we analyzed. With this information we can begin to assess the potential of these compounds as OA therapeutics. While conducting our literature analyses, we also identify those compounds which have been previously investigated in OA studies. Therefore, we can prioritize those that have yet to be explored. Fig. 6B lists 16 promising candidates which warrant further investigation into their therapeutic potential.

3. Discussion

Here, we provide a novel approach to joint-related drug discovery, one that addresses the unmet need for developing disease modifying osteoarthritis drugs. The period immediately following traumatic joint injury, before the onset of disease, represents an opportune time for therapeutic intervention. Thus, our drug discovery strategy focused on identifying small molecule inhibitors that minimize chondral damage, with the goal of preventing or delaying the progression of OA structural changes. Specifically, our screen filtered for compounds that prevented mechanical injury-related cell death or proteoglycan degradation, which represent significant hallmarks of OA. The compounds we identified represent a diverse collection of small molecules that display a broad range of biological activities, many of which target relevant signaling pathways important for cartilage health. Despite compounds in the libraries having other activities or modes of action in other systems, our findings suggest some compounds have potential for disease-modifying activity in musculoskeletal injuries of cartilage.

Not surprisingly, several compounds emerged from our screen that have been previously investigated for OA therapeutic properties. While such compounds are well-characterized, these results validate our screening platform and strengthen our approach of using our engineered CTA as a way to accelerate the drug discovery process. Among those we identified is the pan-caspase inhibitor Z-VAD-FMK, which has been shown in animal models to inhibit chondrocyte apoptosis following joint injury and thus reduce cartilage lesion severity [1,2]. In our study, we found Z-VAD-FMK provided protection against chondrocyte cell death and proteoglycan loss. These findings align well with previous studies, and further support the link between chondrocyte cell death and matrix regression.

### Table 2

| Natural compounds | Protease | Phosphatase | Stem Cell | Redox | Kinase |
|-------------------|----------|------------|-----------|-------|--------|
| Paenoptilin*      | No hits  | BML-267 Ester | Filgotinib (GLPG0634)* | D-α-Tacopherylquinone | AG-494 |
| Vanillin*         |          | OBA*       | WHI-P154 | Seratrodast |       |
| Ursolic Acid*     |          | 9,10-Phenanthenquinone | Hesperetin | Thymoquinone |     |
| 5-HTP*            |          | Cyclosporin A | 1- Azakenpaulone | Thio urea |     |
| Biochanin A*      |          |            |           | bis-demethoxycurcumin |     |
| L-Arginine HCl*   |          |            |           | Ibupropan |      |
| Sinomenine        |          |            |           | Ciclopirox ethanolamine |   |
| Salicin*          |          |            |           | Probuclol |     |
| Tangeretin*       |          |            |           |        |       |
| (+)-Buculline*    |          |            |           |        |       |
| D-Mannitol*       |          |            |           |        |       |
| Triptolide*       |          |            |           |        |       |
| Guanosine         |          |            |           |        |       |
| Gastrodin*        |          |            |           |        |       |
| Santacruzamate A* |          |            |           |        |       |
| Dihydromyricetin* |          |            |           |        |       |
| Tetrahydrodopaverine HCl | | | | | |
| Schisandrin B*    |          |            |           |        |       |
| Sesamin*          |          |            |           |        |       |
| Salidroside*      |          |            |           |        |       |
| Silbinin          |          |            |           |        |       |
| Isoliquiritigenin |          |            |           |        |       |
| Lappaconite HB |          |            |           |        |       |
| Rotundine         |          |            |           |        |       |
| Apocynin          |          |            |           |        |       |
| Scareolide        |          |            |           |        |       |
| Dicerein          |          |            |           |        |       |
| Forskolin         |          |            |           |        |       |
| Scopolamine HBr   |          |            |           |        |       |
| Idebenone         |          |            |           |        |       |
| Tanshinone I      |          |            |           |        |       |
| Genipin*          |          |            |           |        |       |
| Rutacearpine      |          |            |           |        |       |
| Astragaloside A   |          |            |           |        |       |
| Abscisic Acid     |          |            |           |        |       |
| (S)-10-Hydroxyamptothecin* | | | | | |
| Xanthone          |          |            |           |        |       |
| Hordenine         |          |            |           |        |       |
| Synefrine         |          |            |           |        |       |

*Denotes positivity in more than one assay
destruction in the acute phase post-injury.

Another identified therapeutic in the screening is the NF-κB inhibitor, triptolide, which is an immunosuppressant and anti-inflammatory agent that has been investigated as a therapeutic for rheumatoid arthritis (RA). Although triptolide has shown promising pharmacological anti-inflammatory effects it is extremely toxic and therefore failed to advance through clinical trials. Several other compounds identified in the library screening have been investigated for potential effects in other investigations. The compound (S)-10-hydroxycamptothecin is a camptothecin derivative that inhibits DNA topoisomerase, and has been studied for its anti-tumor effects in several cancers including colorectal and liver. Tyrophostin 9 is a multiple tyrosine kinase inhibitor (i.e EGFR and PDGFR). Tyrosine kinase inhibitors have been effective at treating RA in animals, but none performed well enough to advance to clinical trials [3,4]. Currently, there are no reported studies using tyrophostin 9 in osteoarthritis research. We identified another tyrosine kinase inhibitor, RWJ-60475, which had previously been identified in a drug screen selecting for compounds that affected bone mineralization [5]. In general, tyrosine kinase inhibitors are studied for anti-tumor properties. SB-202190 is a selective and potent inhibitor of p38 MAP kinase, a pathway that is thought to play an important role in arthritis [6]. Small-molecule p38 MAPK inhibitors have been widely used for their anti-inflammatory properties.

Several of positive hits identified among the stem cell library screen (Fig. 1) are FDA-approved drugs (e.g. filgotinib, oclacitinib, tofacitinib, galunisertib, pacritinib). Interestingly, all but galunisertib belong to a family of JAK inhibitors used to treat inflammation associated with rheumatoid arthritis (RA) [7–11]. However, it remains to be seen how these drugs function in the setting of OA. While JAK inhibitors are potent modulators of cytokine signaling critical for immune and inflammation responses, they may have added benefits as regulators of MMP activity [12]. Our work supports their ability to modulate cartilage matrix damage. Here we show all five compounds reduce proteoglycan loss resulting from mechanical injury. These results suggest the potential for repurposing existing drugs as OA therapeutic agents.

A major benefit of our top-down approach is that we can target a
candidates are listed in panel B, along with the results from our screen (discovered performance), known signaling roles, and supporting literature. It is important for OA treatment. (A) This study concluded that 25% of the all the compounds we identified were selected for MMP activity screening. CTAs were cultured in soluble interleukin-1β (IL-1β) to induce MMP activity and treated with one of the respective compounds. Treatment conditions are shown on the horizontal axis. CNTL (green dashed line) represents basal MMP activity for non-treated uninjured control samples, while IL-1 (red dashed line) represents the average of IL-1β injured samples only. All data is represented as a percentage relative to the average of IL-1β only treated samples, which is normalized to 100%. For this analysis, conditioned media was collected four days post injury/treatment and processed for active MMP levels. Errors bars represent the standard deviation of 3 technical triplicates.

Fig. 6. Promising drug candidates impact relevant signaling pathways associated with OA. A literature analysis was conducted to identify signaling pathways important for OA treatment. (A) This study concluded that 25% of the all the compounds we identified are involved in JAK-STAT signaling. The second most favored signaling pathway was EGF/EGFR at 19%. A larger number of compounds have yet to be characterized, also at 15% (N/A). (B) The chemical names of potential drug candidates are listed in panel B, along with the results from our screen (discovered performance), known signaling roles, and supporting literature. One of our goals is to test the delivery of these or other compounds identified from with in vivo models using a developed drug delivery platform, such as a new therapeutic approach using mechanically activated microcapsules (MAMCs). Limitations for our study include that we used bovine tissue as a model system and that follow-up will be needed using human tissue. Our study is one of the few high-throughput screening approaches and we restricted the injury to two widely accepted and relevant forms, namely a single impact and a soluble insult from IL-1. As a limit to the type of response, we plan to in future studies explore the effect of different injuries including a mixture of inflammatory cytokines. Our reproducible CTA injury platform, with its physiological relevance, has been validated here as an effective high throughput screening platform permits the opportunity to test new libraries in an accelerated timeframe. The experiments described here demonstrate a higher throughput model system than previously described for PTOA-related therapeutic discovery. In conclusion, our investigation presents a well-characterized and validated screening platform for discovering new therapeutics that are sorely needed in the field of osteoarthritis and in particular required to prevent the progressive and negative sequelae arising from joint trauma.

4. Methods

4.1. Cartilage tissue analog (CTA) fabrication

Primary chondrocytes were obtained by enzymatic digestion of minced fragments of femoral articular cartilage from juvenile bovine knee joints (2–6 months old, Research 87, MA), as previously described [27,28]. After overnight collagenase digestion (Type II 298 U/mL Worthington, NJ, USA) isolated chondrocytes were filtered (100-μm and 70-μm and nylon meshes), washed with PBS, and cultured immediately for cartilage tissue analog fabrication. Cells were seeded in ultra-low adhesion polyHEMA coated 96-well plates (Corning, NY, USA) at 10⁶ cells/well, allowing for tissue-like biomasses to form within 24 h of initial seeding. CTAs were maintained in complete media (high glucose DMEM) containing 10% FBS, 100U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL Fungizone [PSF, Life Technologies, NY, USA], 1% MEM Vitamin Solution [Gibco], 25 mM HEPES buffer, and 50 μg/mL ascorbic acid for at least 3 months (fresh media given twice a week) before being subject to compressive or chemical injury and treatment [28].

4.2. CTA compression-induced injury

To create a PTOA-like injury response we used our previously...
established HTMS platform, suitable for screening 48 samples simultaneously [28]. In this previous study we identified the threshold needed to induce a fracture by histology and that elicited a robust response in outcome measures, LDH and GAG levels. We determined there was a 2-fold increase in GAG loss at 75% compared to 50% strain accompanied by extensive cell death adjacent to fissures. This study established our model system and validated it using CTA as compared to cartilage explants [28]. Prior to each experiment the height was determined of 10 CTAs and the average was taken to determine 75% strain. The height of the CTAs was measured using callipers prior to testing and the average height was 4.0 mm. Further information regarding height variability can be found in reference 28 which represents this variability in Fig. 3B [28]. CTAs, placed in a standard 48-well plate (BD Falcon), were compressed via polytetrafluoroethylene indenters, with the load displacement controlled by an Instron (Model 5848, Instron, MA). Injurious strain and strain rate were calculated based on the average height of constructs. CTAs were compressed to a strain of 75% (rate: 0.5 s⁻¹) to initiate an injury response. As a positive injury control, CTAs were treated with IL-1β (10 ng/mL). Non-compressed CTA samples served as uninjured controls. Small molecule drug treatment began within minutes after applying compression, as described below. CTAs were cultured in 0.5 mL of complete media for 4 days post-injury with sample culture media harvested at days 2 and 4. At the 2-day collection time point, 250 μL of media was collected and replaced with fresh media containing no drug. On day 4 the final 500 μL of conditioned media was collected and CTAs were flash frozen for future analysis.

4.3. Small-molecule treatment

Immediately following injury, CTAs were treated with a single dosage of a small molecule compound (10 μM, N = 1/compound; treatment applied <15 min post-injury) targeting kinase, protease, and phosphatase inhibitor pathways and Redox compounds with prooxidant or antioxidant activity (Enzo Life Sciences, Farmingdale, NY, USA), natural compounds and stem cell compounds (SelleckChem, Houston, TX, USA). Several controls were also included to aid in the identification of putative therapeutics: un-injured/un-treated CTAs served as a growth control, injured/un-treated CTAs as a baseline for the injury response, Triton-X (0.1%) and IL-1β (10 ng/mL) served as known inducers of apoptosis and matrix degradation respectively, and DMSO (10 μM) served as the delivery vehicle control (N = 3/plate). Each compound remained in the culture medium for the first 48 h following injury, at which point half of the media was harvested. Fresh, complete media was added back to the samples, followed by an additional 48-h incubation. CTA culture media harvested at 2- and 4-days post-injury and was used for analysis.

4.4. Evaluation of drug treatment response; GAG and LDH assays

Drug compounds were evaluated based on their ability to minimize proteoglycan degradation and cellular death, two hallmarks of cartilage injury response, following impact. Collected media samples, harvested at respective time points, were analyzed via the dimethylmethylen blue (DMMB) assay to measure GAG levels. Additionally, cellular stress was assayed for lactate dehydrogenase concentration (LDH; CytoTox-ONE Homogenous Membrane Integrity Assay, Promega, WI), which is released from cells that suffered cell damage.

4.5. MMP activity assay

A secondary screen was employed to assess the ‘hit’ compounds’ abilities to suppress IL-1β-activated MMPs. CTAs (10–12 weeks old) were transferred to 48-well plates and subsequently treated with the GAG-related compounds (10 μM, N = 1/well) that were identified in our primary screens, with or without IL-1β (5.0 ng/mL). Similar to our primary screening procedure, half of the media from each sample was harvested at 48 h post-treatment, which was then replaced with fresh complete media. Both Day 2 and Day 4 media samples were assayed for MMP activity via the SensoLyte® 520 fluorometric MMP activity kit (AnaSpec, Inc, Fremont, CA, USA).

4.6. Statistical analysis

Dual flashlight plots, a common tool to measure differences between two groups in high-throughput experiments, was used to score and rank potential drug candidates. Plots were generated depicting the strictly standardized mean differences (SSMD) vs. log₂ fold change [36,37]. A gating threshold of −1 for SSMD and −0.5 log₂ fold change was used to filter for positive responsive compounds. The log₂ fold change is calculated based on taking the log2 of the GAG measured in the media for a given treatment condition (i.e. one of the chemical compounds in the screening library) to the GAG measured in the media for the un-treated, injured control condition (the compounds should modulate the biological response to injury, in this case measured by GAG loss, with injury serving as the baseline signal). All samples received the same media volume during the course of the experiment and were normalized to CTA/volume. The SSMD score was calculated as the ratio of two components: (1) the difference between the measured value for a compound and the median of the negative reference (un-treated, injured control) and (2) the median absolute deviation, adjusted for sample size (SSMD based on UMVUE). The median absolute deviation adjusted for sample size follows this formula: 1.4826 * (median [assay value-median (all assay values)]) / sqrt(2 * (number of samples - 1)/(number of samples – 2.48)).

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Authors contributions

Conception and design: RLM, GRD; Acquisition, analysis and interpretation of data: JRM, SAJ, JC, FY, GD, MT, ER, BM, RLM, GRD; Analysis and interpretation of data, technical support, statistical expertise: JRM, SAJ, JC, FY, GD, MT, ER; Drafting and revising the manuscript: JRM, SAJ, JC, GD, BT, BM, RLM, GRD; Obtaining of funding: RLM, GRD; Revision and final approval of manuscript: SAJ, GRD.

Dr. George R Dodge (george.dodge@pennmedicine.upenn.edu) is the responsible person who is ultimately responsible for the integrity of the work as a whole, from inception to the finished article.

Competing interests

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Authorship

All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted. By signing below each author also verifies that he (she) confirms that neither this manuscript, nor one with substantially similar content, has been submitted, accepted or published elsewhere (except as an abstract). Each manuscript must be accompanied by a declaration of contributions relating to sections (1), (2) and (3) above. This declaration should also name one or more authors who take responsibility for the integrity of the work as a whole, from inception to finished article. These declarations will be included in the published manuscript.
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