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Research Paper

In Vitro–In Vivo Correlation on Delivery of Drug Candidates to Articular Cartilage

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Purpose. In the treatment of osteoarthritis (OA), some of the therapeutic approaches require delivery of drug(s) to the diseased cartilage. Presence of adequate drug levels in the cartilage is one of the important criteria in selection and ranking of lead compounds. The purpose of this study was to investigate the correlation in cartilage compound levels between *in vitro* experiments and *in vivo* animal studies.

Materials and Methods. Bovine cartilage samples were incubated with test compounds of various concentrations in a culture medium, in the absence or presence of 25 mg/ml of serum albumin which served as an artificial synovial fluid (SF). The test compounds were also dosed to rabbits, the animal model used for efficacy studies, over a six-week treatment period. Test article concentrations in plasma, SF, and cartilage were determined by LC/MS/MS analysis.

Results and Conclusions. A correlation in cartilage drug concentration was observed between *in vitro* and *in vivo* studies. Plasma protein binding and the test article's affinity to cartilage were the major determining factors for drug delivery to cartilage *in vivo*.

KEY WORDS: cartilage; drug delivery; *in vitro – in vivo* correlation; synovial.

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ABBREVIATIONS: $AUC_{\text{cartilage}}$, area under the compound concentration-time curve in cartilage; $AUC^{\text{unbound}}_{\text{diseased\_SF}}$ or $AUC^{\text{total}}_{\text{diseased\_SF}}$, area under the unbound or total compound concentration-time curve in diseased SF; $AUC^{\text{unbound}}_{\text{plasma}}$ or $AUC^{\text{unbound}}_{\text{serum}}$, area under the unbound compound concentration-time curve in plasma or serum; $AUC^{\text{unbound}}_{\text{SF}}$ or $AUC^{\text{total}}_{\text{SF}}$, area under the unbound or total compound concentration-time curve in SF; $AUC^{\text{total}}_{\text{plasma}}$ or $AUC^{\text{total}}_{\text{serum}}$, area under the total compound concentration-time curve in plasma or serum; $C_{\text{cartilage}}$, compound concentration in cartilage; C_{plasma} or $C^{\text{total}}_{\text{plasma}}$ or ${}^{drug}C^{\text{total}}_{\text{plasma}}$ or C_{serum} or $C^{\text{total}}_{\text{serum}}$, total compound concentration in plasma or serum; $C^{\text{protein}}_{\text{plasma}}$ or $C^{\text{protein}}_{\text{serum}}$, total protein concentration in plasma or serum; C_{SF} or $C^{\text{total}}_{\text{SF}}$ or ${}^{\text{drug}}C^{\text{total}}_{\text{SF}}$ or $C^{\text{total}}_{\text{healthy\_SF}}$, total compound concentration in SF; $C^{\text{unbound}}_{\text{diseased\_SF}}$ or $C^{\text{total}}_{\text{diseased\_SF}}$, unbound or total compound concentration in diseased SF; $C^{\text{unbound}}_{\text{plasma}}$ or $[C_{\text{plasma}} \times \%_{\text{free-fraction}}]$ or $C^{\text{unbound}}_{\text{serum}}$, unbound compound concentration in plasma or serum; $C^{\text{unbound}}_{\text{SF}}$ or ${}^{\text{drug}}C^{\text{unbound}}_{\text{SF}}$ or $C^{\text{unbound}}_{\text{healthy\_SF}}$, unbound compound concentration in synovial fluid (SF); ${}^{\text{drug}}C^{\text{bound\_to\_proteins}}_{\text{SF}}$ or ${}^{\text{drug}}C^{\text{bound\_to\_proteins}}_{\text{plasma}}$, SF or plasma compound concentrations that associated with SF or plasma proteins; $\%_{\text{free-fraction}}$ percentage of unbound compound in plasma; SF, synovial fluid.

INTRODUCTION

Apart from chronic pain, one of the most evident features of osteoarthritis (OA) is the progressive erosion of articular cartilage. Many proteases, notably, the matrix metalloproteinases (MMPs) and the aggrecanases (ADAMTs), play a role in this degenerative process (1–4). Inhibitors that modulate the functions of these collagen associated proteases have been extensively studied with the goal to slow down or even reverse the erosion of articular cartilage (5,6). Given the site of action, achieving adequate cartilage levels becomes one of the important sought after criteria for screening and ranking of drug candidates.

Articular cartilage, which serves as a cushion between bones, has limited blood supply. Joint fluid (synovial fluid, SF) plays a crucial role in lubricating, nurturing, and reducing wear and tear of cartilage (7,8). Presumably, SF is also the vehicle for delivering drugs to the diseased cartilage (9). Due to small sample sizes, analyzing drug candidate levels in SF and/or joint cartilage from *in vivo* studies requires a large number of animals, and consequently, consumes a substantial amount of test materials. As plasma and SF drug levels fluctuate, studying the kinetics of drug delivery to cartilage imposes an even more challenging task for early phases of drug discovery due to resource constraints. Therefore, a robust *in vitro – in vivo* correlation is useful in ranking lead candidates, projecting efficacious test article's cartilage levels *in vivo* and, more importantly, the relationship of drug levels in cartilage and plasma. This will ensure proper dosing regimen designs in long-term pharmacological studies.

In this report, we have studied the *in vitro – in vivo* correlation of delivering several MMP-13 inhibitors to knee cartilage and, in particular, the relationship of cartilage compound levels with plasma and SF drug concentrations.

MATERIALS AND METHODS

Test Articles and Materials

The test articles are biphenyl sulfonamide carboxylic acid derivatives prepared in house at Wyeth Research laboratory with purity no less than 97% by HPLC assay (10,11). Protein binding for the test articles was determined by equilibrium dialysis methods. For all test articles, the free fractions in plasma remained essentially unchanged throughout a concentration range of 0.1 to 10 μg/ml. Results from BIAcore assays indicated that the binding was nonspecific to albumin associated with fast on and off rates (data not shown). All chemicals, reagents and rabbit serum albumin (lyophilized powder) were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated in the text.

Determination of Test Article's Cartilage Accumulation *In Vitro*

Freshly harvested bovine knee cartilage from a local slaughterhouse was used as the primary matrix for *in vitro* screening. In general, wet cartilage discs of approximately 25~50 mg each were incubated in one mL of culture medium, in the absence or presence of 25 mg/ml rabbit serum albumin (served as artificial SF), containing test articles at various concentrations ranging from 1 ng/ml to 20 μg/ml. Rabbit serum albumin was used because rabbit was the animal model in the *in vivo* studies. The culture medium consisted of Dulbecco's Modified Eagle's medium (JRH Biosciences, Lenexa, KS), 50 μg/ml ascorbic acid (Wako, Osaka, Japan), 10 m*M* HEPES, pH 7.0 (Mediatech, Herndon, VA), 2 m*M* L-glutamine (Mediatech, Hollyhill, FL) and 100 U/ml antibiotic-antimycotic solution (Mediatech, Hollyhill, FL). The culture medium was maintained as sterile and filtered using a 0.20 μm filter prior to use.

The concentrations of total proteins in SF are approximately one third that found in plasma and consist primarily of albumin (12–14). Thus, a culture medium containing 25 mg/ml of rabbit serum albumin was used to mimic joint fluid (artificial SF). This amount was based on the total protein concentration of 20~30 mg/ml in SF and 57~80 mg/ml in plasma in animals and humans (12–15).

After incubation at 37°C for various lengths of time, the wet cartilage samples were milled and test article levels in the cartilage as well as in culture medium were determined by LC/MS/MS analysis. Individual test article's cartilage accumulation factors were computed as the concentration ratio between the cartilage and the culture medium.

Determination of Test Article's Cartilage Concentrations *In Vivo*

Male adult New Zealand White rabbits weighing between 2.5 and 5 kg (Charles River Laboratories, Wilmington, MA) were used in the study. The studies were performed at Wyeth Research Laboratory (Andover, MA) under the supervision of the Institutional Animal Care and Use Committee (IACUC). Test articles were administered either via chow food mixtures formulated in Oral Minitreat (Bio-Serv, Frenchtown, NJ) or by subcutaneous (SC) injection formulated in 0.5% methylcellulose (MC) and 2% Polysorbate 80 (Tween 80) aqueous suspension at 1 ml per injection or a combination of these two dosing schemes. For compound A, the three treatment groups comprised twice daily oral administration of 71 mg per rabbit in chow food (treatment group 1), twice daily oral administration of 71 mg per rabbit in chow food plus SC injection of 100 mg per rabbit (treatment group 2), and SC injection alone at 140 mg per rabbit every Monday, Wednesday and Friday (treatment group 3). The dose regimens for compound B and C were SC administration corrected by animal's individual body weight and were dosed to animals on every Monday, Wednesday, and Friday. The treatment groups were 5 and 25 mg/kg for compound B, 10 and 50 mg/kg for compound C, respectively. The SC doses were also formulated as aqueous suspensions in 0.5% MC and 2%Tween 80 and the dose volume was approximately 1 ml per rabbit. The total length of the treatment courses was 6 weeks for all the studies. The drug in chow food was given to animals around 8:00 A.M. and 5:00 P.M. The SC administrations were at approximately 8:00 A.M. On Week 2, Week 4 and on the last day of the 6-week treatment, blood samples of approximately 200 μl were collected from the marginal ear vein at pre selected time points for the plasma test article concentration measurement. At approximately 24~28 h post last dose administration of the 6-week treatment, animals were sacrificed. The plasma, SF as well as knee and shoulder cartilage were collected. The test article levels in plasma, SF and articular cartilages were determined by LC/MS/MS analysis.

The pharmacokinetic parameters and simulations were computed using WinNonlin (version 4.1, Pharsight, Mountain View, CA).

LC/MS/MS Assay for Test Article Levels in Plasma, SF, and Articular Cartilage

Calibration standards, study samples, and quality control samples were prepared using the same procedure on the day the assays were performed. Briefly, milled bovine cartilage samples of 20~25 mg were extracted by vortexing with 1.5 ml of acetonitrile containing the internal reference standard (IS), a structural analogue of the test article. This process was repeated twice for a complete extraction. The combined extracts were then dried under compressed nitrogen gas, reconstituted in 200 μl of acetonitrile and transferred to a 96 well plate for analysis. Supernatants (10 μl) were injected via an auto-sampler which was kept at 4°C for LC/MS/MS analysis. Plasma and SF samples of 50 μl each were precipitated by adding 100 μl of acetonitrile containing an appropriate IS. Supernatants (10 μl) were injected onto the LC/MS-MS system for quantitation.

The HPLC system was an Agilent 1200SL binary pumping system operated at a flow rate of 0.2 ml/min. The analytical HPLC column was a Waters Xterra C18 MS 2.1× 20 mm microbore maintained at 60°C. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). A gradient elution was started with an isocratic hold at 0% B for 0.5 min, followed by a linear gradient to 100% B over 3 min. The column was then re-equilibrated at 100% A, for 2 min prior to next sample injection. Mass spectrometry was performed on an API-4000 (Applied Biosciences, Framingham, MA)

Table I. Effect of Rabbit Serum Albumin (25 mg/ml) on Compound Accumulation in Bovine Cartilage *In Vitro*

		In absence of albumin			In presence of 25 mg/ml albumin		
Test Articles	Plasma Protein Binding	Medium Conc. (ng/ml)	Average Cartilage Conc (ng/gram)	Average Cartilage/ Medium Ratio	Medium Conc. (ng/ml)	Cartilage Conc. (ng/gram)	%Reduction in Cartilage by Albumin
A	~99.6%	10	113	10.1	10	BQL	NA
		100	1,081		100	34	96.8%
		1,000	8,194		1,000	501	93.9%
B	~99.8%	10	548	33.4	10	19	96.5%
		100	3,343		100	50	98.5%
		1,000	11,906		1,000	262	97.8%
C	~98.6%	1,000	5,053	5.1	1,000	1,148	77.3%

BQL Below quantitation level

tandem quadrupole mass analyzer in multiple reaction-monitoring (MRM) mode. Ionization was achieved by either electrospray ionization (ESI), or atmospheric pressure chemical ionization (APCI). The detection limits were approximately 1 ng/ml for plasma and SF, and 10 ng/g for milled cartilage, respectively. To increase the detection limit of the test article in cartilage, samples were pooled as needed.

RESULTS

In Vitro Cartilage Accumulation

In the absence of serum albumin, the cartilage compound accumulation, as defined by the ratio of the compound concentrations in cartilage (ng/g, assuming the density of cartilage is one) to the concentrations in culture medium (ng/ml), ranged from 5 fold to 33 fold for three Wyeth compounds tested (Table I). For the three compounds evaluated, there was a substantial reduction in cartilage compound levels in the presence of 25 mg/ml rabbit serum albumin. The ranking in reduction in cartilage accumulation, in the presence of serum albumin in the culture medium, was in agreement with the ranking of plasma protein binding for these compounds, *e.g.,* Compound B, which exhibited the highest plasma protein binding, manifested the largest reduction in cartilage levels, while Compound C, which had the highest free fraction in protein binding among the three, showed the least reduction in cartilage concentration.

Steady-state Test Articles Levels in Rabbit Plasma, Joint Fluid and Cartilage

Maintaining a relatively steady plasma drug concentration is essential for minimizing the fluctuation of compound levels in SF and cartilage. As shown in Fig. 1, the plasma levels for Compound A remained relatively consistent during the treatment cycle. All three compounds possessed low aqueous solubility (<10 μg/ml). Rabbits are known to be coprophagic. For oral administration of low solubility compound *via* chow food, along with coprophagy, the absorption of compound A was a slow but continuous process, which was close to zero-order kinetics. In the case of SC administration, in which the formulations were suspensions, the absorption was essentially determined by the dissolution rate at the injection site(s), which was also close to zero-order kinetics. In either case, the peak-to-trough fluctuation in plasma concentration-time profiles remained relatively small through-

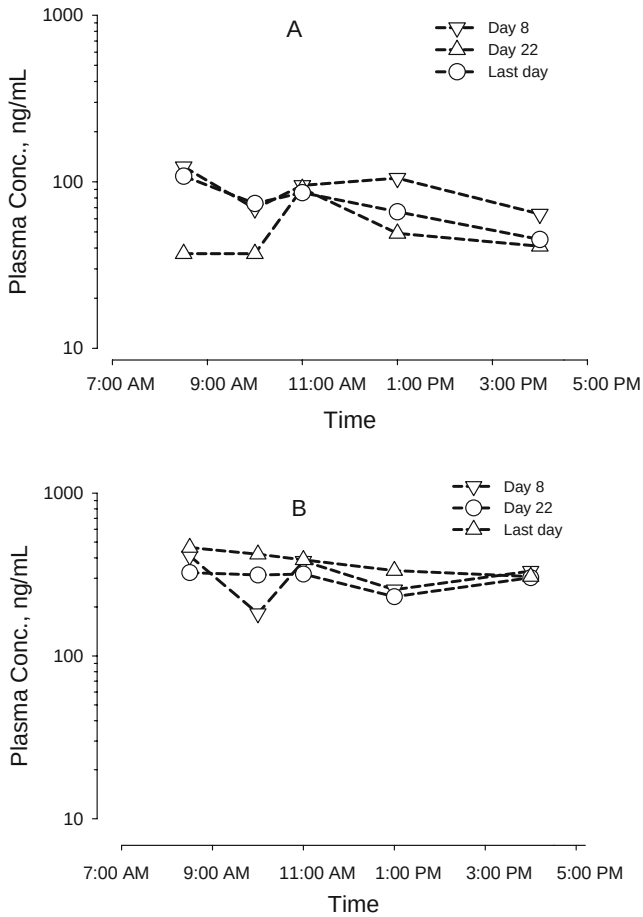

Fig. 1. Average plasma concentration of Compound A in rabbits after twice daily administration of 71 mg (per rabbit) (**A**) or combination of a twice daily oral dose of 71 mg plus SC administration of 100 mg (per rabbit) three times per week (**B**).

out the treatment cycle. The relatively consistent plasma exposures between Week 2, Week 4, and Week 6 also indicated the achievement of the steady-state early in the treatment cycle for compound A. Similar profiles were observed for Compounds B and C (data not shown). At the end of the 6-week treatment, the average SF drug levels, with respect to all test articles, were approximately 39% of that found in plasma (Table II). The cartilage test article levels are also summarized in the same table.

DISCUSSIONS AND CONCLUSIONS

Unlike the blood brain barrier, the blood synovium barrier appears quite leaky. Almost all proteins and solutes present in plasma can be found in SF. The SF to plasma concentration ratios for blood-derived macromolecules (e.g., proteins) are largely inversely related to the size of the compounds. Non-protein bound small molecular weight solutes, such as glucose, urea, and sodium, exhibit similar concentrations in SF and plasma (12–14). There are few reports on the presence of active transporters in or on the synovium membrane. Passive diffusion is generally the proposed mechanism for drug molecules distribution into SF (16,17).

By maintaining plasma compound levels relatively constant, one would be able to assess the relationship of the test article's distribution between SF and plasma. *In vivo*, the unbound and protein associated drug concentrations in SF and in plasma have following relationship,

$$C_{SF}^{proteins} + {}^{drug}C_{SF}^{unbound} \rightleftharpoons {}^{drug}C_{plasma}^{unbound} + C_{plasma}^{proteins}$$

$$\updownarrow \qquad\qquad\qquad \updownarrow$$

$${}^{drug}C_{SF}^{bound\_to\_proteins} \qquad {}^{drug}C_{plasma}^{bound\_to\_proteins}$$

At equilibrium, if a drug is not cleared at synovium or subjected to active transport, the unbound drug level should remain the same in SF and plasma, *i.e*, ${}^{drug}C_{SF}^{unbound} \approx {}^{drug}C_{plasma}^{unbound}$. For highly protein bound drugs, the majority of drug molecules are associated with SF or plasma proteins. Thus, ${}^{drug}C_{SF}^{total} \approx {}^{drug}C_{SF}^{bound\_to\_proteins}$ and ${}^{drug}C_{plasma}^{total} \approx {}^{drug}C_{plasma}^{bound\_to\_proteins}$. If the mechanism of protein binding is nonspecific and proportional to the SF and plasma protein concentrations, the observed drug concentration in SF should correlate with the total protein concentrations in SF and plasma, *i.e*.,

$$\begin{aligned} {}^{drug}C_{SF}^{total} \Big/ {}^{drug}C_{plasma}^{total} &\approx C_{SF}^{proteins} \Big/ C_{plasma}^{proteins} \\ &\approx [20 \sim 30 mg/ml] \Big/ [57 \sim 80 mg/ml] \approx 20 \sim 53\%. \end{aligned}$$

As shown in Table II, the average SF to plasma concentration ratios, ranging between 32% to 57% for all three compounds tested in the study, support this assumption, *i.e.,* total SF compound concentrations are about one third to one half of the correspondent total plasma compound concentrations.

In vitro, in the absence of serum albumin, test articles' cartilage accumulation factors were determined by the concentration ratio between wet cartilage and the culture medium. It is a measurement of the test article's inherent ability to accrue in the cartilage. When 25 mg/ml of serum albumin was incorporated into culture medium as "artificial SF", a reduction in cartilage compound levels was observed. *In vivo*, the cartilage accumulation was further reduced approximately by a factor of 3 in relation to the total plasma concentration (25 mg/ml serum albumin in a culture medium as "the artificial SF" *versus* 57~80 mg/ml total protein contents in plasma). The overall reduction in cartilage delivery was in agreement with test articles' protein binding determined by equilibrium dialysis. Also shown in Table II, the observed test articles' cartilage levels, with respect to their correspondent plasma concentrations, were in agreement with the projected values computed by the following proposed formula, $C_{cartilage} \approx \left[C_{plasma} \times \%_{free-fraction}\right] \times$ [cartilage accumulation factor]. This plasma to cartilage correlation in compound concentration validated the assumption that $C_{SF}^{unbound} \approx C_{plasma}^{unbound}$.

As plasma compound concentration fluctuates, the total and unbound compound concentration ratios between SF and plasma no longer remain constant. In general, solute

Table II. Observed and Predicted Test Article Levels in Rabbit Plasma, Synovial and Cartilage after a 6-week Treatment

Test Articles	Plasma Protein Binding	Average Cartilage/ Medium Ratio	Dose Regimens (mg/kg)	Plasma Conc. (ng/ml)	SF Conc. (ng/ml)	SF/Plasma Ratio	Observed Cartilage Conc. (ng/gram)	Projected Cartilage Conc.[a] (ng/gram)
A	~99.6%	10.1	P.O. 71 mg B.I.D	76±43	24±20	32%	4.1±0.4	4
			P.O. 71 mg B.I.D + 100 mg S.C. 3×/wk	383±138	219±97	57%	15.1±5.9	15
			140 mg S.C. 3×/wk	976±334	313±151	32%	32	39
B	~99.8%	33.4	5 mg/kg S.C. 3×/wk	729±248	291±11	40%	96±44	49
			25 mg/kg S.C. 3×/wk	1,791±693	688±176	38%	311±155	120
C	~98.6%	5.1	10 mg/kg S.C. 3×/wk	2,585±879	957±371	37%	306±179	185
			50 mg/kg S.C. 3×/wk	7,007±3,991	2,361±1,277	34%	821±464	500

[a] The projection was based on the following formula
$C_{Cartilage} = \left[C_{plasma} \times \%_{free-fraction}\right] \times$ [Cartilage accumulation factor]. Where $\%_{free\text{-}fraction}$=(1−%bound) and Cartilage accumulation factor= cartilage/medium ratio (in the absence of serum albumin).

exchange between plasma and SF compartments has been reported to be moderately fast. The mean transit times, defined as the average interval of time spent by a molecule from its entry into synovium to its exit, for compounds such as diclofenac, etodolac, ibuprofen, indomethacin, tenoxicam, and albumin, are between 2 and 7 h (17,18). The relatively short synovium transit time enables the establishment of equilibrium between SF and plasma drug concentrations soon after the systemic steady-state is achieved. However, the 2~7 h mean transit time may not be fast enough to ensure that the concentration ratio of the two compartments be synchronized with the fluctuation in blood drug concentrations. In fact, SF levels should lag behind blood levels slightly. This is simulated in Fig. 2. At the peak of plasma concentration, the direction of flux is from plasma to SF, and therefore, one would observe a low SF to plasma compound concentration ratio and likewise, a high SF to plasma ratio when the net flux reverses its direction at the trough of the plasma concentration. After many observations, it has been suggested that SF to serum concentration ratios of many NSAID are variable in respect to different sampling schedules, with the highest at trough of the dosing interval (19).

Although the ratios, both $C_{SF}^{\text{total}}/C_{\text{plasma}}^{\text{total}}$ and $C_{\text{SF}}^{\text{unbound}}/C_{\text{plasma}}^{\text{unbound}}$, may vary at different sampling time points as plasma drug concentration fluctuates, the overall SF drug exposures (expressed as AUC_{SF}) should correlate with plasma exposures ($\text{AUC}_{\text{plasma}}$) *i.e.*, $AUC_{\text{SF}}^{\text{unbound}} \approx AUC_{\text{plasma}}^{\text{unbound}}$ and, $AUC_{\text{SF}}^{\text{total}}/AUC_{\text{plasma}}^{\text{total}} \approx 20 \sim 53\%$ (*i.e.,* equal to the total protein concentration ratio of 20~53% between SF and plasma). Indeed, the $AUC_{\text{SF}}^{\text{total}}/AUC_{\text{serum}}^{\text{total}}$ ratios are 43% for Tenoxicam and 36% for Meloxicam and more importantly, the $AUC_{\text{SF}}^{\text{unbound}}/AUC_{\text{serum}}^{\text{unbound}}$ ratios are statistically no different at 90% for Tenoxicam and 108% for Meloxicam, respectively (20–22).

Articular cartilage comprises approximately 70% of water. The pores within the cartilage tissue are large enough to accommodate convective flux of water, yet small enough to exclude the penetration of macromolecules such as serum albumin (23–25). During routine exercise or activity, there is as much as 5~10% change in articular cartilage volume (26–28). This indicates that convective flow, *via* daily activity, plays a substantial role in delivering solutes into cartilage (25,26). Consequently, the time needed for achieving steady-state cartilage drug delivery is projected to be relatively rapid. Similar to the above discussion on SF to plasma ratios, the drug concentration ratios between cartilage and SF are also projected to vary with respect to different sampling time points (Fig. 2).

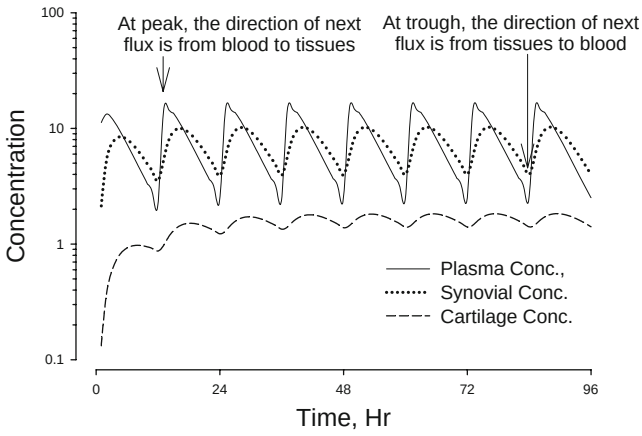

Fig. 2. The mean transit times in plasma, synovial and cartilage may vary for individual test articles. Therefore, the concentration ratios, among synovial fluid (*dotted line*), cartilage (*dashed line*) and plasma (*solid line*), are projected to be varied depending on sampling times and dosing regimen design.

In summary, the actual cartilage to SF and SF to plasma concentration ratios depend upon the test article's mean transit time in cartilage and SF as well as its plasma concentration-time profiles. Evidently, when the fluctuation of plasma drug levels becomes dramatic, the tissue (cartilage or SF) to plasma concentration ratios, with respect to the various sampling time points, can vary significantly.

In this study, we have observed a good *in vitro – in vivo* correlation of cartilage drug levels to the unbound plasma concentration. By maintaining plasma concentration relatively steady, the relationship of drug levels in cartilage and plasma can be described by $C_{\text{cartilage}} = C_{\text{plasma}}^{\text{unbound}} \times$ [cartilage accumulation factor]. In case the plasma compound concentration fluctuates, the following relationships should stand, $AUC_{\text{SF}}^{\text{unbound}} \approx AUC_{\text{plasma}}^{\text{unbound}}$ and, $AUC_{\text{cartilage}} \approx AUC_{\text{plasma}}^{\text{unbound}} \times$ [cartilage accumulation factor].

Directly linking compounds' cartilage levels with unbound drug concentration in plasma has several implications:

First, one can screen and rank leads for drug candidates based on *in vitro* assays. By integrating data from protein binding and cartilage accumulation measurements, together with test articles' *in vitro* potency data from cartilage explant assays and pharmacokinetics in animals, one will be able to project target plasma levels. This will help rationalizing dose regimen design for long-term animal studies, and eventually assess the *in vitro – in vivo* correlation for target and/or model validation.

Second, plasma drug levels can serve as an indicator for the cartilage concentrations. This reduces the need to sample SF or cartilage for drug levels. Frequent sampling of SF and articular cartilage requires a large number of animals in order to provide sufficient quantities of SF and cartilage for assay. Consequently, this approach consumes a substantial amount of test material. In the early phase of drug discovery, limited resources impose constraints on such practice. Moreover, as plasma drug levels fluctuate, one may observe variable results in tissue to plasma ratios that are caused by individual test article's unique mean transit times with cartilage and SF with respect to different plasma concentration–time profiles under various dosing regimens. Together with the challenges of collecting and handling the small sample size of animal SF and cartilage, a proper dosing regimen and the sampling schedule design have a crucial impact on the understanding of test article's kinetics among cartilage, SF and plasma.

The driving force for drug delivery to cartilage is the unbound drug concentration in SF, which correlates to the unbound drug concentration in the plasma. In some joint diseases such as rheumatoid arthritis (RA), the total protein concentration in an inflamed joint may be higher than that in normal joints due to increased leakage at synovium membrane site. For highly protein bound drugs, high protein infiltration into inflamed SF has been attrib-

uted to high total SF drug levels (19,20,29,30). However, the unbound drug levels are likely to remain unchanged in diseased and healthy SF. This has been supported by measuring total and unbound drug levels of ibuprofen in arthritis patients' synovial and serum samples, that $C^{total}_{disease\_SF} > C^{total}_{healthy\_SF}$, but $C^{unbound}_{serum} \approx C^{unbound}_{diseased\_SF} \approx C^{unbound}_{healthy\_SF}$ (30). Similarly for Naproxen in arthritis patients, Day *et al.* (31) has confirmed that there is no statistical difference between the unbound exposures in diseased SF and serum, *i.e.*, $AUC^{unbound}_{disease\_SF} \approx AUC^{unbound}_{serum}$. Hence, the high (total) SF drug levels presented in the diseased joints might not lead to a better delivery of drugs to diseased over normal cartilage.

In conclusion, we have observed good *in vitro – in vivo* correlations for cartilage delivery of drug candidates A, B and C. Test article's inherent affinity to cartilage and unbound drug concentration in plasma are the two essential determining factors for drug delivery to cartilage.

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