Permeability of Articular Cartilage to Matrix Metalloprotease Inhibitors

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Purpose. To develop an *in vitro* cartilage permeation model for cartilage permeability study and to evaluate the effects of molecular hydrophilicity and cartilage location on the permeability of articular cartilage to matrix metalloprotease inhibitors.

Methods. An *in vitro* cartilage permeation model was developed and utilized to determine the permeability of articular cartilage to the matrix metalloprotease inhibitors of different hydrophilicity. Permeability coefficients were obtained by measuring the steady-state flux of the inhibitor compounds. HPLC methods were also developed and employed for the analysis of drug levels in assay media.

Results. The relationship between permeability and hydrophilicity of drug molecules was examined. Results indicated that the permeability coefficient increased with increasing hydrophilicity of the molecule. Additionally, the relationship between the permeability and the location of the cartilage section within the animal joint was investigated. Our results showed that the drug molecules penetrated faster in the surface layer cartilage than in the deep layer cartilage.

Conclusions. Increasing the hydrophilicity of a molecule would increase its permeability across articular cartilage. The *in vitro* cartilage permeation model developed could be used to rank order drug compounds according to their cartilage permeability profiles and to aid in drug selection and development.

KEY WORDS: cartilage permeability; matrix metalloprotease inhibitors; hydrophilicity; cartilage location; HPLC.

INTRODUCTION

To produce its therapeutic effects, a drug must be able to reach its pharmacological receptor at an adequate concentration. Therefore, how well a drug will be delivered to its site of action plays a critical role in drug discovery and development. In an effort to evaluate the drugs that treat the human diseases related to cartilage degradation, such as rheumatoid arthritis and osteoarthritis, an *in vitro* cartilage permeation model is often needed to assess the ability of the drugs to penetrate through cartilage. Such an *in vitro* model not only provides a means to rapidly determine the availability of drug molecules in cartilage, i.e., the target tissue, but also minimizes time consuming and expensive animal studies. In most cases, drugs are required to reach certain levels at the site of action at an appropriate time in order to yield desired pharmacological results, e.g., to inhibit the enzymes responsible for cartilage degradation in arthritis. A cartilage permeation model will allow the assessment of the extent and rate at which a drug permeates across cartilage tissue. Cartilage permeability to different types of molecules has been studied theoretically and experimentally over the years (1–5). There are several commercially available tissue diffusion devices for measuring the permeability of solute molecules through various tissues. But none of those is suitable for cartilage penetration experiments because of the fragility of cartilage tissues. In the present study, an *in vitro* cartilage permeation device has been designed and utilized to determine the permeability of several matrix metalloprotease (MMP) inhibitor compounds of different hydrophilicity (shown in Fig. 1). The MMPs are a unique family of enzymes that can degrade various components of extracellular matrix. The levels of MMPs are elevated in the cartilage in osteoarthritis (OA), causing cartilage degradation. The MMP inhibitors are designed to block the activity of MMPs and therefore slow or stop cartilage degradation in OA. It is apparent that the permeation of inhibitors through cartilage, the site of action, is critical for the effectiveness of the inhibitors. Our objectives of the study were first to design an *in vitro* cartilage permeation model capable of predicting how well a drug penetrates articular cartilage *in vivo*; second to evaluate the cartilage penetration abilities of selected MMP inhibitors of different hydrophilicity and correlate the permeability with hydrophilicity; and third to examine the relationship between the permeability of the drug and the location of the cartilage within the animal joint.

MATERIALS AND METHODS

Materials

Postmortem specimens of adult bovine articular cartilage from the knee joint were obtained. Each joint was cut into smaller sections with a bandsaw, quickly frozen, and stored in a freezer for later use. The cartilage sections of uniform thickness were obtained from a cryomicrotome. Research with animals adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). A phosphate-buffered saline (PBS) (pH 7.4) was obtained from Life Technologies (Gaithersburg, MD). MMP inhibitors, N-hydroxy-1,3-di-[4-methoxybenzenesulphonyl]-5-hydroxy-[1,3]cyclohexyldiazine-2-carboxamide (PGE-113288, compound I), N-hydroxy-1,3-di-[4-methoxybenzenesulphonyl]-[1,3]cyclopentyldiazine-2-carboxamide (PGE-9445565, compound II), N-hydroxy-1,3-di-[4-methoxybenzenesulphonyl]-[1,3]cyclohexyldiazine-2-carboxamide (PGE-9818266, compound III), and N-hydroxy-1,3-di-[4-methoxybenzenesulphonyl]-5,5-dimethyl-[1,3]cyclohexyldiazine-2-carboxamide (PGE-4410186, compound IV) were made by Procter & Gamble Pharmaceuticals (Mason, OH). Radioactive tracers [^{14}C]polyethylene glycol 4000 and [^{3}H]H_2O were obtained from Amersham (Arlington Heights, IL).

Sample Preparation

All MMP compounds were dissolved in PBS buffer in a concentration range of 50–1000 μg/ml depending on the solubility of the compound. Cartilage sections were immersed in the PBS buffer for about 2 hours prior to permeability measurement. The radioactive tracers, [^{14}C]PEG-4000 and [^{3}H] H_2O were dissolved in the PBS buffer at a concentration of 6 μCi/ml.

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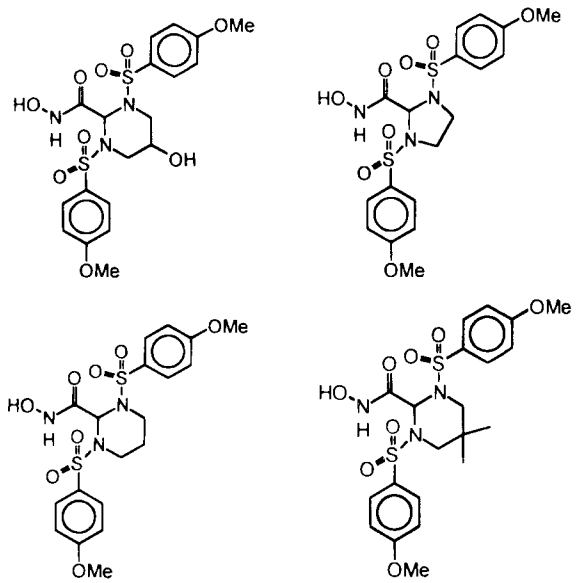

Fig. 1. Molecular structures of MMP inhibitors. Compound I (upper left), compound II (upper right), compound III (lower left), compound IV (lower right).

Equipment, Experimental Procedures and Chromatographic Conditions

The cartilage permeability device consisted of two chambers, donor chamber and receptor chamber, separated by a cartilage section covering a 5-mm diameter opening from each of the two chambers (as shown in Fig. 2). Teflon O-rings were used to ensure a good seal between the cartilage section and the edges of two chamber openings. Both the receptor- and donor-side solutions were thermostated at 37°C and circulated by gas lift (95%O_2/5%CO_2) at a flow rate of 4 ml/min throughout the experiment using gassing ports in the chambers. The circulation system reduced the thickness of the unstirred water layer at the surface of the cartilage tissue and enabled a rapid mixing of solutions in both chambers. A full-depth bovine articular cartilage was sectioned to several 300 μm thick × 9 mm dia slices using a cryomicrotome. These cartilage sections were then used for our cartilage permeability study.

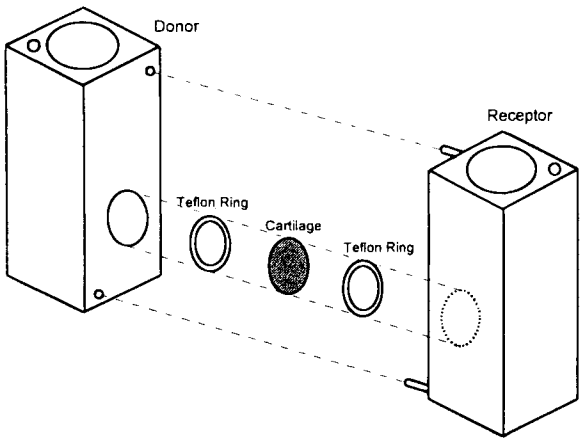

Fig. 2. Schematic diagram of an *in vitro* cartilage permeation device.

[^{3}H] H_2O and [^{14}C]PEG-4000 were used as controls to insure the integrity of the cartilage section and the permeation device. [^{3}H] H_2O was expected to penetrate faster in the cartilage while [^{14}C]PEG-4000 was expected to permeate much slower through the cartilage because of its large size. The concentrations of radioactive controls were determined using a scintillation counter. The permeability of these two controls were determined before and after each permeation run of the sample.

During the experiment, an aliquot of 200 μL of solution in the receptor chamber was removed at desired time intervals and replaced with fresh PBS buffer solution to maintain a constant sample volume, ionic composition, and hydrostatic pressure on the receptor side. In addition, the solution in the donor chamber was sampled at the beginning and end of the experiment for concentration determination by HPLC or scintillation counting.

Reversed phase HPLC methods were developed and utilized to measure the drug concentrations in both the donor- and receptor-side samples. The HPLC system consisted of a Waters (Milford, MA, USA) 600S controller, a 616 pump, an in-line degasser, a 717 plus thermostatic autosampler, and a 996 photodiode array detector. The HPLC system was controlled by the Waters Millennium 2020 data system. A Waters (Milford, MA, USA) analytical column (Symmetry C18, 150 × 3.9 mm I.D., 5 μm) was utilized. The mobile phase consisted of 5% acetonitrile, 95% water, 0.1% formic acid (mobile phase A) and 80% acetonitrile, 20% water, 0.1% formic acid (mobile phase B). A linear gradient elution with appropriate initial and final mobile phase compositions was applied. The flow rate was 1 ml/min with UV detection at 245 nm.

RESULTS AND DISCUSSION

The permeability coefficient Kp for the diffusion of the solute across the cartilage tissue was determined by the following equation (6):

$$Kp = \frac{1}{SCd}\left(\frac{dM}{dt}\right) = \frac{V}{SCd}\left(\frac{dCr}{dt}\right) \quad (1)$$

where Cd is the concentration of the solute in the donor-side chamber; dM/dt the amount of the solute penetrated in unit time t; V the volume of the receptor chamber; S the exposed tissue surface area; and Cr the concentration of the solute in the receptor-side chamber.

In our permeability experiments, the sample solutions in the receptor chamber removed at various times were measured by HPLC. The sample concentrations were determined based on three sample injections and reported as their mean values. The specificity of our HPLC methods were demonstrated in Fig. 3 for compound III. The analyte peaks were clearly separated from any other component peaks. For other three compounds, no peak interferences between the parent drug peak and other peaks were found. The calibration curves of the four compounds were linear from 0.1 to 100 μg/ml with the correlation coefficients of greater than 0.999. The method precision and accuracy were also investigated. The precision expressed as the relative standard deviation (C.V.%) based on 6 repetitive injections was less than 3% (intra-day) and 5% (inter-day) for all four compounds. The accuracy was found to be greater than 97% for all four compounds in three drug levels

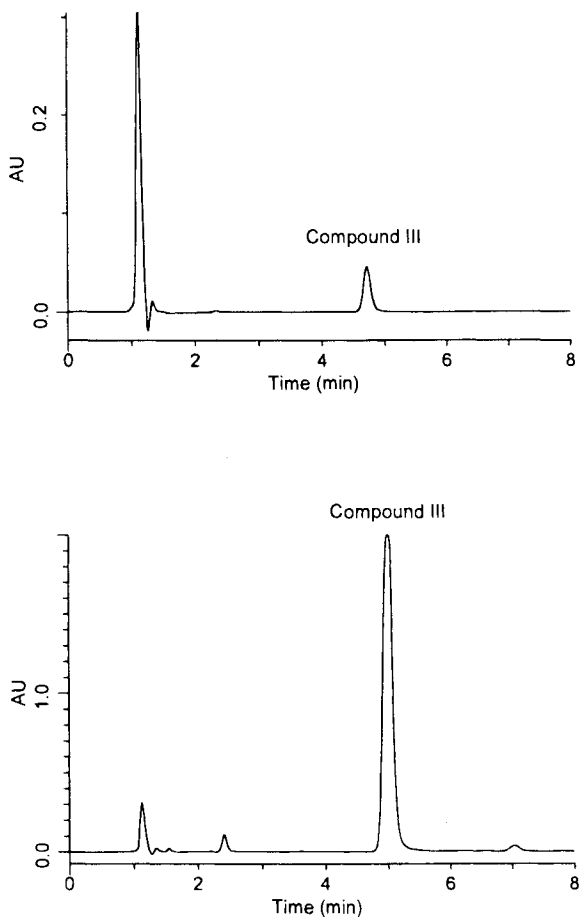

Fig. 3. Representative chromatograms of receptor- (upper) and donor-side (lower) samples of compound III at a permeation time of 6 hours.

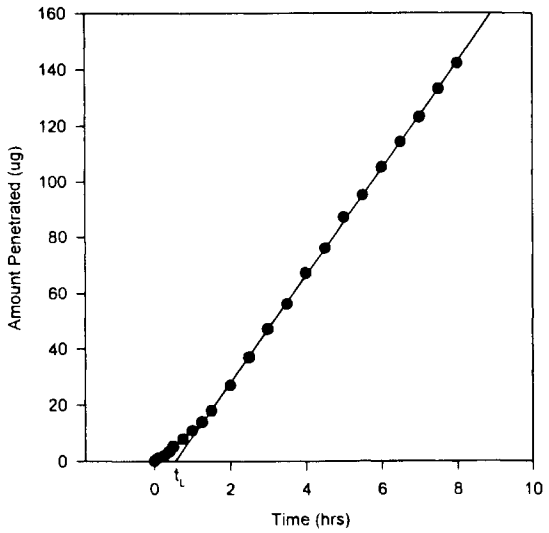

Fig. 4. Representative plot of penetrated amount versus permeation time for compound III using a surface layer cartilage section.

ranging from 0.5 to 100 μg/ml. The detection limit at a signal-to-noise ratio of 3 was 10 ng/ml.

For each compound, its permeability data were obtained as mean values based on the results from three separate experiments using three different cartilage sections. The results indicated that the drug concentration in the receptor chamber increased with permeation time. The drug concentrations of the sample solutions in the donor chamber were also measured at the beginning and end of the experiment. It was found that the drug concentration in the donor chamber was changed by less than 10% during the course of the entire experiment. This ensured the conditions for unidirectional flux used for the calculation of Kp in the above equation. The steady-state diffusion was reached when a uniform concentration gradient is established within the cartilage section. The permeability coefficients were calculated based on the steady-state condition. The time required to reach the steady-state condition, i.e., lag time t_L, was also determined from a curve of cumulative amount of solute (M) versus permeation time (t).

Figure 3 shows the representative HPLC chromatograms of donor- and receptor-side solutions taken at 6-hour time point. Figure 4 is a representative plot of the amount of drug penetrated versus permeation time. The calculated permeability coefficients and lag times are listed in Table 1.

To compare the effects of freezing and thawing of cartilage on the permeability characteristics of the cartilage, we conducted a comparison study using compounds I and IV on two pairs of fresh and freeze-thawed cartilage sections. No significant differences (within experimental error) in the permeability coefficient and lag time were found between fresh and freeze-thawed cartilage tissues. This insured the validity of the use of freeze-thawed cartilage tissues for our drug penetration studies.

Hydrophilicity Effects

Compounds of different hydrophilicity are chosen to understand the effects of hydrophilicity on the permeability of molecules. The hydrophilicity of a compound is traditionally expressed as Log P, defined as the logarithm of the octanol-water partition coefficient of the molecule. The use of Log P to understand the relationship between hydrophilicity and permeability of a molecule has been well documented (7,8). Here, the hydrophilicity of the drug molecules are represented by their calculated Log P values (Log P values were obtained using software developed by Advanced Chemistry Development Inc.). There is an inverse relationship between Log P and hydrophilicity, i.e., the greater the Log P, the less the hydrophilicity. As shown in Fig. 5, the permeability coefficient increases with increased hydrophilicity of the solute. An exception here is PEG-4000. Although it is quite hydrophilic, it penetrates cartilage much slower than do the drug molecules because of its much larger size. The proportional trend between hydrophilicity and penetration for solutes of similar sizes can be explained based on the cartilage tissue structure employed for the study. Articular cartilage is composed of mainly water (70–80% by weight) and a relatively low density of cells in a matrix of collagen, aggrecan, nutrients, and other matrix proteins (9). Solutes penetrate cartilage through two different routes, paracellular flux and transcellular flux. Because of the low density of cells in cartilage tissue, paracellular flux is apparently the main route for solutes to permeate through cartilage tissue. Therefore, the more hydrophilic the solute, the more easily the solute will diffuse into the tissue. This experimental finding represents, for the first time, a correlation between hydrophilicity and permeability of drug molecules in cartilage

Table 1. Permeability Profiles in the Surface Layer (n = 3) and Deep Layer (n= 3) of Bovine Articular Cartilage

Cartilage Location	Compound	Log P	h (μm)	Kp (10^{-4}cm/s) (mean ± S.D.)	t_L (h) (mean ± S.D.)
Surface Layer	[^{14}C]PEG-4000	−0.63	320	0.00882 ± 0.00044	2.1 ± 0.2
	[^{3}H]-H_2O	−1.38	320	14.0 ± 0.7	0.0
	Compound I	2.98	320	7.50 ± 0.48	0.2 ± 0.1
	Compound II	3.20	320	4.80 ± 0.43	0.3 ± 0.1
	Compound III	3.48	320	3.40 ± 0.31	0.5 ± 0.1
	Compound IV	4.51	320	1.30 ± 0.27	0.6 ± 0.1
Deep Layer	[^{14}C]PEG-4000	−0.63	300	0.00634 ± 0.00032	2.5 ± 0.2
	[^{3}H]-H_2O	−1.38	300	2.20 ± 0.11	0.0
	Compound I	2.98	300	0.834 ± 0.045	0.3 ± 0.1
	Compound II	3.20	300	0.518 ± 0.047	0.3 ± 0.1
	Compound III	3.48	300	0.451 ± 0.041	0.6 ± 0.1
	Compound IV	4.51	300	0.205 ± 0.038	0.7 ± 0.1

tissue. Our results are consistent with the correlation between the electrical charge of the charged molecule and permeability published in the literature (10). In addition, the lag time appears to increase with decreasing hydrophilicity as seen in Table 1, suggesting a major role for paracellular flux.

Cartilage Location Effects

Cartilage sections sampled from different locations within the joint were used to investigate the effects of cartilage location on the permeability of the solute. Cartilage is traditionally classified into four zones: superficial zone, middle zone, deep zone, and calcified zone (9). Here, we sectioned a full thickness cartilage into two layers, "surface" and "deep" layers. The surface layer is composed of the superficial and middle zones while the deep layer corresponds to the deep zone. As shown in Table 1, compounds were found to penetrate more rapidly in the surface layer than in the deep layer. This may be related to the content of water in those cartilage layers, as water content progressively decreases with increasing depth in articular cartilage (9). Our results were consistent with increasing rate of permeation with increasing hydration of cartilage. However, there was little correlation between the location of cartilage and permeation lag time as shown in Table 1. The lack of correlation may be due to the fact that penetration pathways across two cartilage tissues of different locations are similar, mainly through paracellular route, leading to the similar lag times as seen in the experiments.

The above results showed for the first time that for molecules of similar sizes, the more hydrophilic the compound, the greater the permeability. In addition, our results indicated that the drug molecules penetrated faster in the surface layer cartilage than in the deep layer cartilage. Once the permeability profiles of drug compounds are obtained from this *in vitro* cartilage permeation model, one could rank order compounds according to their permeability properties to accelerate drug development process.

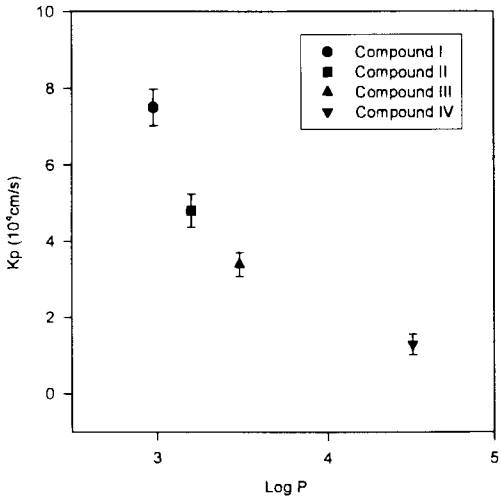

Fig. 5. The permeability coefficient as a function of Log P using a surface layer cartilage section.

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