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Review

Selective drug targeting to bone using acidic oligopeptides

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Introduction

Bone is susceptible to a range of diseases, like other tissues. However, it is generally difficult to deliver and retain drugs in the bone. Bone is composed unique materials such as the inorganic compound hydroxyapatite (HAP) and bone-matrix proteins, which are quite different from the components of other tissues. Among them, HAP may be a promising target for selective drug delivery to bone.

Now, two promising methods have been reported for selective drug delivery to the bone as a target HAP. One is a class of molecules known as bisphosphonates. Systemic administration of bisphosphonates commonly results in 20-50% deposition of the molecule at bone tissues, with minimal accumulation at other sites [1]. These compounds not only show anti-osteoporosis effect by themselves [2] but are useful as the drug delivery carrier to the bone [3-6]. Another is the oligopeptide having high affinity to the bone, described in this review.

The structures of several bone non-collagenous proteins that bind to HAP have a repeating sequence of acidic amino acids (Asp, Glu), which may serve as a HAP binding site. Osteopontin and bone sialoprotein, two major non-collagenous proteins in bone, have L-Asp and L-Glu repetitive sequences, respectively (Fig. 1), and rapidly bind to HAP after they are secreted in osteoblastic cell culture [7-9]. Thus, these acidic oligopeptides are candidate bone-targeting carriers. It was hypothesized that, after systemic administration, a drug tagged with such an oligopeptide would be selectively delivered and bind to bone, where the active drug would be released gradually during bone remodeling processes.

This review describes the properties of acidic oligopeptides and introduces the tagging of three model drugs, estradiol, quinolone antibiotics, and tissue-non-specific alkaline phosphatase (TNSALP), with an acidic oligopeptide to examine the clinical feasibility of the acidic oligopeptide strategy for selective drug delivery to bone.

Bioproperties of acidic oligopeptides

Homo-oligopeptides consisting of two to ten acidic amino acid residues, conjugated with 9-fluorenylmethylchloroformate (Fmoc) or fluorescein (FITC), were synthesized by routine solid-phase methods (Fig. 2). The dissociation constant (K_d) decreased with increasing numbers of acidic amino acid residues, and the binding rate (B_{max}) reached almost maximum at a length of six residues. The affinity for HAP was not related to the optical isomeric form (L or D) or the acidic amino acid species (Asp or Glu) [10]. When intravenously administered, fluorescein-labeled L-Asp hexapeptide (FITC-D_6) disappeared from the plasma with a biological half-life of 60 min, and 95% of the administered FITC was excreted within 24 h, but FITC-D_6 rapidly bound and retained on the bone and teeth (Fig. 3) and was detectable in bone tissues even at 14 d after administration, but it was undetectable in other tissues [11]. Among the isomeric forms of the amino acids, the retention time was much longer for D-amino acid oligopeptides than for L-peptides. However, the L-amino acid (Asp) hexapeptide was chosen, because the D-peptide may be insensitive to hydrolysis in bone thus would not release the tagged drug.

Oligopeptide-tagged estradiol for osteoporosis

Osteoporosis is a serious problem for postmenopausal and aged women, and estrogen deficiency plays a causative role in its development. Estrogen can act directly or indirectly on osteoblasts and osteoclasts through estrogen receptor-mediated mechanisms [12-16], resulting in an anabolic effect on bone formation in estrogen-deficient animal models [17, 18]. Estrogen replacement therapy is an effective treatment in postmenopausal women to prevent reduction of bone mineral density [19]. However, systemically administered estrogen is widely distributed to tissues other than bone, and prolonged therapy may increase the risks of endometritis, breast and endometrial cancer, and intrauterine hemorrhage [20, 21]. The development of osteoporosis therapies that are more efficient and selective for bone is desirable to avoid such adverse reactions.

Estradiol (E_2) was tagged with the L-Asp hexapeptide at the 3-position (E_2-3D_6) or the 17β-position (E_2-17βD_6) via a succinate ester (Fig. 4), and the pharmacokinetics and anti-osteoporotic effects of these compounds were examined [10, 22]. First, the binding affinities of these compounds at human estrogen receptors, ERα and ERβ, were tested *in vitro*. The affinities of E_2-3D_6 and E_2-17βD_6 to both estrogen receptors were about 100-fold and 10,000-fold, respectively, less than those of estradiol. Moreover, it is unlikely that the tagged estradiols can permeate through the plasma membrane, because of their hydrophilicity and large molecular size, and it seems probable that hydrolysis of the ester bond between estradiol and the L-Asp hexapeptide portion would be required for the tagged estradiols to show biological activity. At 6 h after a single intravenous administration (3.7 μmol/kg), the apparent tissue-to-plasma concentration ratio of the tagged estradiols was almost 1.0 in most tissues and organs, indicating that these compound could not distribute or accumulate in tissues. Among the tissues examined, untagged estradiol was most highly distributed in the uterus, where it might cause adverse effects such as endometritis, whereas the accumulation of the tagged estradiols in the uterus was clearly lower. On the other hand, tagged estradiol was distributed to bone at 50-fold the level of untagged estradiol. Moreover, the level of tagged estradiol in bone decreased very slowly, falling to basal level (10.5 ± 3.3 pmol/g) by 7 days, whereas untagged estradiol declined to the basal range within 1 day after injection.

It is well known that estradiol with a mono- or di-ester at position 3 or/and 17 shows prolonged estrogenic effects in the body due to the biological stability of esterified estradiol [23, 24]. This is so because removal of the ester is necessary for further metabolism and excretion of estradiol, and the de-esterification step appears to be the rate-limiting process in the elimination [25]. Indeed, at 6 h after the injection of untagged estradiol, the plasma concentration of estradiol had decreased almost to the basal level (7.6 ± 1.9 pmol/mL), whereas the tagged estradiols remained in the circulation at 6 h (E_2-3D_6; 26.7 ± 9.0 pmol/mL, E_2-17βD_6; 22.0 ± 12.0 pmol/mL, respectively). The lower total body clearance (CL_{tot}) of the tagged estradiols, compared with that of untagged estradiol, may result from superior biological stability. This would be favorable for the long-term action of tagged estradiol in bone.

When tagged estradiol (0.11, 0.37, or 1.1 μmol/kg, every seventh day) or untagged estradiol (0.37 μmol/kg, every third day) was intravenously administered into ovariectomized

(OVX) mice for 28 days, the tagged estradiol dose-dependently reversed the decreased bone mineral density (BMD), and the estrogenic effects were selective to bone. The untagged estradiol increased not only the BMD but also the weights of the uterus and liver [10, 22]. Estradiol caused hypertrophy of uterine tissues and fatty degeneration in the liver. These results indicate that tagged estradiol reduced the risk of systemic adverse effects of estradiol, while retaining the osteogenic effect in bone.

The retention of E_2-3D_6 in the blood circulation and bone and its anti-osteoporotic effects were greater than those of E_2-17βD_6 [10,22]. The difference may be attributable to the greater stability of E_2-3D_6 to hydrolysis, compared with E_2-17βD_6, so that the retention in bone is longer for E_2-3D_6 than for E_2-17βD_6. It is likely that the tagged estradiols bound to bone are gradually hydrolyzed on the bone surface, possibly by peptidases, acid secreted by osteoclasts, and/or non-specific esterases, and that the released estradiol then acts via estrogen receptors.

Thus, these acidic oligopeptide-tagged estradiols, E_2-3D_6 and E_2-17βD_6, are promising candidates for the treatment of postmenopausal osteoporosis. The prolonged effect and selective delivery to bone should extend the medication interval and reduce the adverse effects of estradiol. However, when considering the clinical use of these oligopeptide-tagged estradiols, intravenous injection is undesirable because of the potential for poor compliance. The bioavailability of E_2-17βD_6 after oral administration was less than 10%. Therefore, Yokogawa et al. have developed an intranasal preparation to increase the bioavailability of the tagged molecules [26]. The bioavailability of E_2-17βD_6 after intranasal administration was increased to 75% by incorporating

2,6-di-*O*-methyl-β-cyclodextrin into the formulation, thereby creating an inclusion complex with E_2-17βD_6 that could achieve prolonged residence in the nasal cavity. The acidic oligopeptide-tagged estrogen provided increased medication compliance because of its long-acting and easy administration, and it increased anti-osteoporotic efficacy because of selective delivery to bone and reduced adverse effects of estradiol, resulting in improved quality of life for patients.

Oligopeptide-tagged quinolone antibiotics for osteomyelitis

Osteomyelitis is a progressive infectious process resulting in inflammatory destruction of bone, bone necrosis, and new bone formation. Chronic osteomyelitis is generally treated with surgical debridement, following the administration of parenteral antibiotics such as β-lactams or aminoglycosides. Without adequate debridement, most antibiotic regimens fail, regardless of the duration of therapy. Even when all necrotic tissue has been removed, the remaining bed of tissue must be considered as contaminated with the responsible pathogens. Thus, it is recommended to treat the patient with antibiotics for at least 4 weeks [27-29]. Moreover, the serum bactericidal titer, which is defined as the maximal dilution of the patient's serum that is able to kill the infecting organism *in vitro*, should be 1:4 or greater for a cure [30]. Thus, high doses and long-term administration of antibiotics are required for the treatment of osteomyelitis, because the delivery of antibiotics to bone is difficult. As the fluoroquinolones have a broad spectrum of activity *in vitro*, including activity against Gram-negative organisms, *Staphylococcus aureus*, and *S. epidermidis*

[31], improving their bone distribution should increase their therapeutic effectiveness.

Takahashi et al. have designed levofloxacin (LVFX) tagged with the L-Asp hexapeptide at position 3 in the pyridone carbonic acid moiety, an active center of the drug, via a glycolate ester, (LVFX-3D_6) and norfloxacin (NFLX) tagged with the L-Asp hexapeptide at position N7 in the piperazinyl group via a glycolate and succinate linker (NFLX-7D_6)(Fig. 5) [32]. The minimum inhibitory concentrations (MIC) for anti-microbial activity of LVFX-3D_6 and NFLX-7D_6 against *S. aureus in vitro* were 100- and 60-fold less, respectively, than those of the respective untagged drugs. One reason for this may be the increased hydrophilicity, arising from the acidic oligopeptide tag. Fluoroquinolones act by inhibiting bacterial topoisomerase II, which is responsible for the replication of double-stranded DNA, and thus must cross the bacterial membrane to be effective [33, 34]. The marked reduction of the antimicrobial activity of tagged quinolones indicates that the release of the parent drug by hydrolysis was required for biological activity, as in the case of tagged estrogen.

The pharmacokinetic parameters of LVFX-3D_6 and NFLX-7D_6 after a single intravenous injection are shown in Table 1. The AUC value of each tagged drug was two-fold that of the respective parent drug. In contrast, the Vdss value was significantly less for the tagged drugs than for the parent drugs, indicating a low distribution of the tagged compounds into tissues. The biological half-life was not changed by conjugation. Although these fluoroquinolones themselves have some affinity for calcium or bone, the level of tagged drugs in bone was more than 100-fold that of the parent drugs for at least 7 days after injection. After injection of LVFX-3D_6, the resulting LVFX was detected in bone marrow at about 1% of the concentration of LVFX-3D_6 in bone and was continuously released for 7 days. It is known that fluoroquinolones show extensive tissue penetration, and as a result, can cause adverse effects in various tissues, including the cardiovascular system, central nervous system, skin, liver, musculoskeletal system, and kidneys [35, 36]. The risk of adverse events may be decreased by conjugation with an oligopeptide because of lower distribution into tissues due to the increased hydrophilicity.

The therapeutic effect of tagged drugs on osteomyelitis was evaluated using a murine model of osteomyelitis, which was prepared by inoculation of *S. aureus* into the tibia. The amount of *S. aureus* remaining in the tibia after a single intravenous injection of the drug (27.7 mmol/kg for LVFX and LVFX-3D_6; 31.3 mmol/kg for NLFX and NFLX-7D_6) was determined, but only a slight reduction in the number of *S. aureus* was observed after the administration of LVFX, and no effect was seen for NLFX and NFLX-7D_6. The anti-microbial effect of LVFX was temporary, and the number of *S. aureus* had recovered to the level of the untreated control group at 6 days after administration. On the other hand, LVFX-3D_6 suppressed the growth of *S. aureus* for at least 6 days, showing a prolonged effect. Nevertheless, the effect of LVFX-3D_6 was not sufficient to kill *S. aureus*, presumably because the LVFX concentration generated from LVFX-3D_6 in the bone was insufficient to kill *S. aureus*. Fluoroquinolones show a concentration-dependent killing effect for susceptible organisms, with higher concentrations of fluoroquinolones resulting in more complete killing [37, 38]. Thus, the hydrolytic rate of LVFX-3D_6 appears to be an important determinant of the maximal anti-microbial effect. NFLX-7D_6 did not release NFLX and did not show any effect. These findings emphasize the importance of ensuring an appropriate susceptibility of the acidic oligopeptide-tagged drug to biological hydrolysis. To improve the effectiveness of the tagged drug, further modification is required, possibly devising a linkage susceptible to osteoclast-derived acid, peptidase, or non-specific esterase.

Oligopeptide-tagged enzyme for deficiency of tissue-non-specific alkaline phosphatase

Hypophosphatasia is an inherited metabolic disorder of defective bone mineralization and is caused by a deficiency in tissue-non-specific alkaline phosphatase (TNSALP). Despite the presence of TNSALP in bone, kidney, liver, and adrenal tissue in healthy individuals**,** clinical manifestations in patients with hypophosphatasia are limited to defective skeletal mineralization, manifesting as rickets in infants and children and osteomalacia in adults [39]. ALP functions as an inorganic pyrophosphatase in bone [40, 41]. Inorganic pyrophosphate (PP_i) itself impairs the growth of HAP crystals and acts as an inhibitor of mineralization [42-45]. With insufficient TNSALP activity, PP_i is not adequately hydrolyzed, and the resulting build-up of unhydrolyzed PP_i in the perivesicular matrix inhibits the proliferation of pre-formed HAP crystals beyond the protective confines of matrix vesicle (MV) membranes.

Hypophosphatasia is caused by the deficiency of a single enzyme, TNSALP, making the disorder potentially amenable to enzyme replacement therapy (ERT). However, the results of ERT with intravenous infusion of plasma ALP or purified liver ALP in patients with hypophosphatasia have been disappointing [46-50]. Recently, one report has suggested that continuous delivery of a high dose of TNSALP to bone would be needed to induce physiological bone mineralization [51]. These observations suggest that the ALP enzymes administered intravenously were mostly consumed in the visceral organs and thus were not actually delivered to the bone at the physiological levels necessary to rescue the lesions. Therefore, it was tried to develop TNSALP targeted to bone by acidic oligopeptide.

The cDNAs were designed for two enzymes: anchorless recombinant human TNSALP (rhTNSALP) and anchorless human TNSALP (about 80 kDa) tagged with a stretch of six L-Asp residues at the C terminus (CD_6-TNSALP) (Fig. 6) [52]. When these cDNAs were transfected into Chinese hamster ovary (CHO-K1) cells, the enzymes were secreted from the cells into the culture medium, because they do not have the glycosylphosphatidylinositol anchoring signal peptide sequence from the C-terminus of human TNSALP cDNA. There was no significant difference in specific activity, Michaelis constant, or k_{cat} between the two enzymes. Thus, CD_6-TNSALP can restore mineralization in hypophosphatasia patients. Indeed, in cultures of hypophosphatasia patient-derived bone marrow cells, the tagged enzyme markedly improved mineralization, similar to the untagged enzyme rhTNSALP. Although PP_i completely inhibited the mineralization, even in the presence of P_i, the addition of CD_6-TNSALP restored the mineralization level to that of the PP_i-free control culture, as did TNSALP. Consequently, it seems that both the tagged and untagged enzymes may be taken up without modification into osteoblastic cells, possibly by endocytosis, and that both show similar bioactivity in terms of mineralization, by degrading PP_i in the cells.

To evaluate the pharmacokinetic tissue distribution of the enzymes, fluorescently labeled enzymes were prepared with the Alexa dye. CD_6-TNSALP showed about a 2-4-fold increase in bone retention, compared with untagged TNSALP, for at least 7 days after intravenous injection. The distribution to other tissues was not significantly different between the two enzymes, although the half-lives in the circulation were slightly different (rhTNSALP, 19.1 h; CD_6-TNSALP, 14.4 h) after a single intravenous injection. The different half-lives in the circulation may have been due to the different carbohydrate structures, i.e., the tagged enzyme was less sialylated than the untagged enzyme, based on the result of lectin affinity chromatography [52]. It is known that higher sialic acid residue content at the terminus of the carbohydrate chain contributes to a longer half-life of ALP in the blood [53, 54].

These results suggest the possible clinical application of ERT for hypophosphatasia, although further studies are needed to confirm *in vivo* and clinical effectiveness of the tagged enzyme and may encourage us to develop drugs of other large molecule such as growth factors or hormones tagged with acidic oligopeptides for some bone diseases and growth disorder.

Bisphosphonates and acidic oligopeptides as bone targeting carrier

Fujisaki et al. [3,4] designed a hybrid compound of estradiol and bisphosphonate. This compound was rapidly taken up by bone and increased BMD of OVX rats. But this hybrid compound also increased the uterine weight to have intrinsic hormonal action on estrogen receptors, indicating missing in selectivity to the bone. Many investigators have tried to selectively deliver of several biological active proteins to bone, but none has been successful so far in targeting the molecules to the bone and showing that they have the desired biological effect on the bone. Uludag et al. [5,6] have designed aminobisphosphonate conjugated with bovine serum albumin and succeeded in delivery the protein to bone with several fold higher concentration and longer retention than unconjugated protein. However, because the cleavage and release the bioactive protein from the conjugate may be difficult, it has not been proved until now to exhibit the bioactivity on bone after administration of the conjugate of bisphosphonate and some biologically active protein or peptide.

Our small peptide consisting of an acidic amino acid may be more practical as a bone-targeting carrier of various drugs for treatment of bone diseases, because it is selectively distributed to the bone, where it gradually releases the conjugated drug, whereas it is rapidly degraded and removed from plasma [10,11]. On the other hand, a genetically prepared TNSALP tagged with six L-Asp oligopeptide at the C terminus accomplished higher accumulation and longer retention in the bone than untagged enzyme [52]. Furthermore the tagged enzyme improved mineralization of bone-marrow cells from patient of hypophosphatasia, similar to the untagged enzyme [52], suggesting the carrier peptide does not influence the enzyme activity, because the molecular size is too small compared with the parent enzyme.

Conclusion

The cumulative results of the studies on acidic oligopeptide conjugation indicate a promising method of selectively delivering drugs to bone. Owing to the apparently strong affinity of acidic oligopeptides for HAP, the targeting system may be widely applicable for both small molecule drugs and large molecules as proteins in several bone diseases.

The presence of an acidic oligopeptide tag altered the drug's pharmacokinetic and biological properties, including blood clearance, distribution to visceral organs, and biological activity. The increased hydrophilicity created by the tag is thought to be responsible for these alterations. In considering clinical use, it is unlikely that tagged molecules would be suitable for oral administration because of their high hydrophilicity and their susceptibility to peptidases and esterases in the gastrointestinal tract. Other routes of administration, such as the intranasal route, may increase the bioavailability of tagged molecules, and the greater convenience may improve compliance and patient quality of life.

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Fig. 1. Amino acid sequences of bone matrix proteins

Bone sialoprotein

MKTALILLSILGMACAFSMKNLHRRVKI**ED**S**EE**NGVFKYRPRYYLYKHAYFYPHLKRFPVQGSS**D**
SS**EE**NG**DD**SS**EEEEEEEE**TSN**E**G**E**NN**EE**SN**EDED**S**E**A**E**NTTLSATTLGYG**ED**ATPGTGYTGLAAI
QLPKKAG**D**ITNKATK**E**K**E**S**DEEEEEEEE**GN**E**N**EE**S**E**A**E**V**DE**N**E**QGINGTSTNST**E**A**E**NGNGSSG**E**
DNG**EE**G**EEE**SVTGANA**E**GTT**E**TGGQGKGTSKTTTSPNGGF**E**PTTPPQVYRTTSPPFGKTTTV**E**Y**E**
G**E**Y**E**YTGAN**D**Y**D**NGY**E**IY**E**S**E**NG**E**PRG**D**NYRAY**EDE**YSYFKGQGY**D**GY**D**GQNYYHHQ

Osteopontin

MRIAVICFCLLGITCAIPVKQA**D**SGSS**EE**KQLYNKYP**D**AVATWLNP**D**PSQKQNLLAPQTLPSKSN
ESH**D**HM**DD**M**DDEDDDD**HV**D**SQ**D**SI**D**SN**D**S**DD**V**DD**T**DD**SHQS**DE**SHHS**DE**S**DE**LVT**D**FPT**D**LPAT**E**
VFTPVVPTV**D**TY**D**GRG**D**SVVYGLRSKSKKFRRP**D**IQYP**D**AT**DED**ITSHM**E**S**EE**LNGAYKAIPVAQ
DLNAPS**D**W**D**SRGK**D**SY**E**TSQL**DD**QSA**E**THSHKQSRLYKRKAN**DE**SN**E**HS**D**VI**D**SQ**E**LSKVSR**E**FH
SH**E**FHSH**ED**MLVV**D**PKSK**EED**KHLKFRISH**E**L**D**SASS**E**VN

Acidic amino acids are emphasized.

Fig. 2. Structure of FITC-D_6

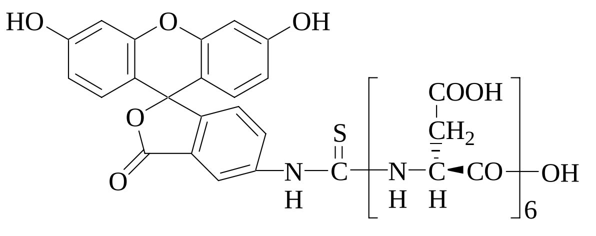
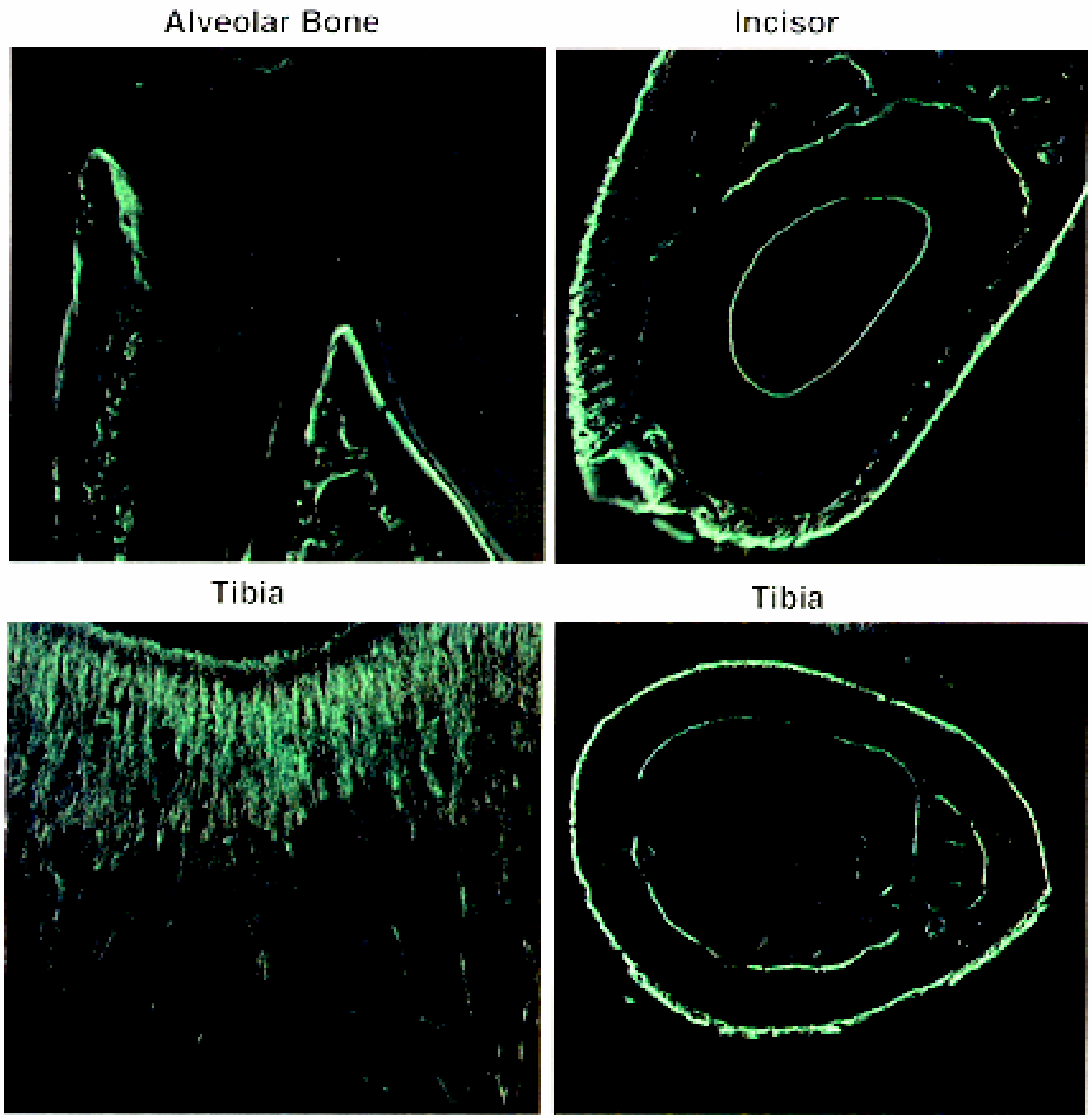
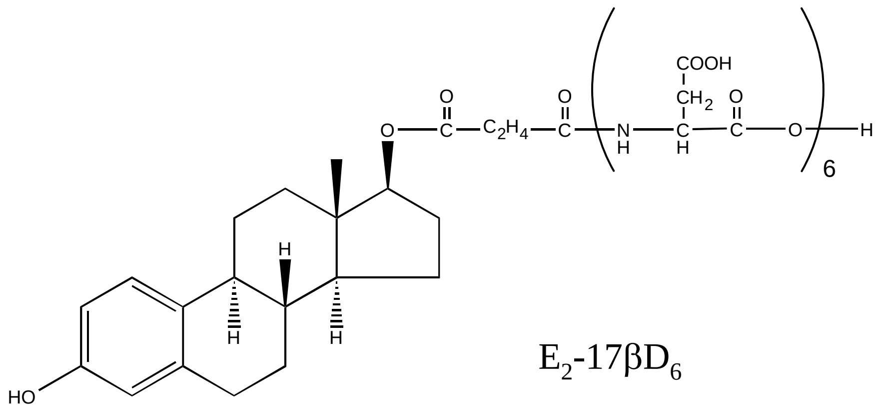


Fig. 3. Confocal laser scanning microscopic images of bones

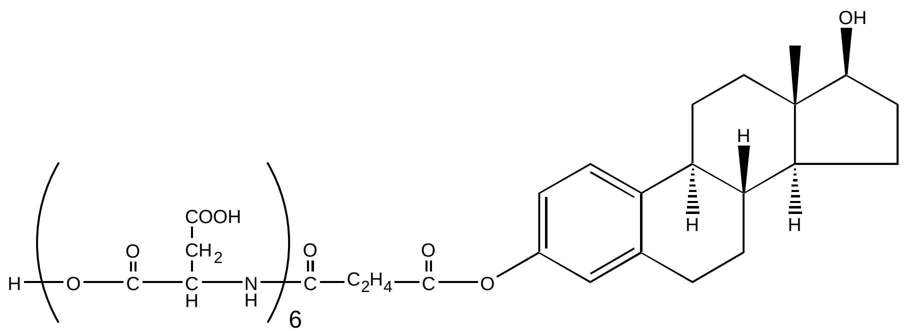


Ground sections of bones were prepared from rats 24 h after injection with FITC-D_6.

Fig. 4. Structures of E_2-3D_6 and E_2-17βD_6

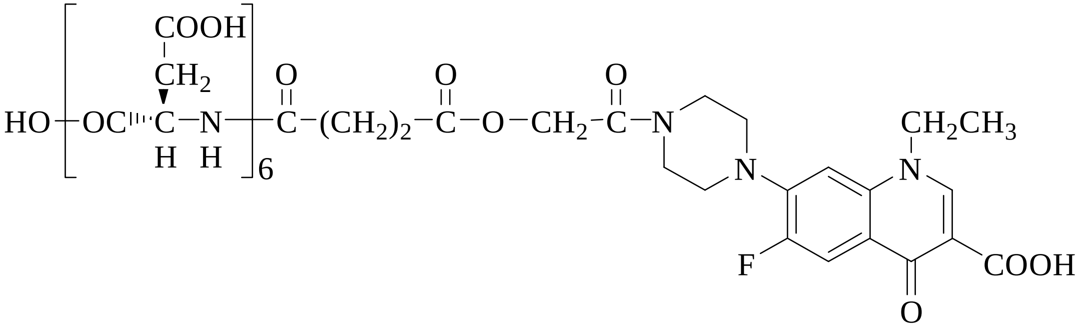


E_2-17βD_6

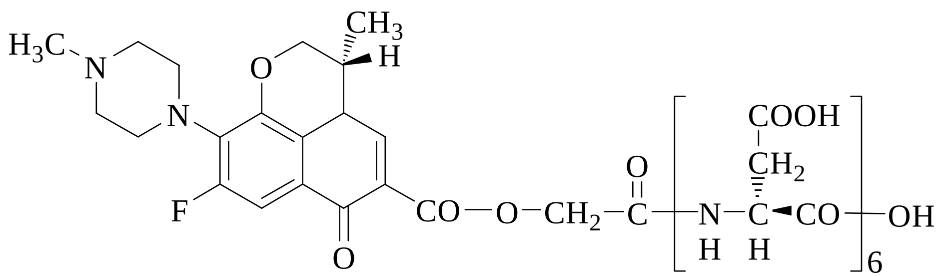


E_2-3D_6

Fig. 5. Structures of NFLX-7D_6 and LVFX-3D_6



NFLX-7D_6



LVFX-3D_6

Table 1. Pharmacokinetic parameters after an intravenous administration of fluoroquinolones and their Asp-oligopeptide tagged drugs

	LVFX (27.7 μmol/kg)	LVFX-3D_6 (27.7 μmol/kg)	NFLX (31.3 μmol/kg)	NFLX-7D_6 (31.3 μmol/kg)
$AUC_{0\text{-}2\,h}$ (nmol/mL・h)	8.77 ± 0.39	24.8 ± 0.8*	9.66 ± 1.70	20.7 ± 7.8*
Vd_{ss} (L/kg)	1.55 ± 0.04	0.27 ± 0.06*	1.86 ± 0.35	0.63 ± 0.14*
$t_{1/2}$ (h)	0.49 ± 0.09	0.38 ± 0.02	0.52 ± 0.09	0.62 ± 0.17

Each compound was injected into the jugular vein of mice at the indicated dose.
Each value represents the mean ± S.D. (n = 4).
*Significantly different from the parent drug at $p < 0.05$.

Fig. 6. DNA constract of CD_6-TNSALP