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Phosphopeptides as a replacement of bisphosphonates for bone targeting

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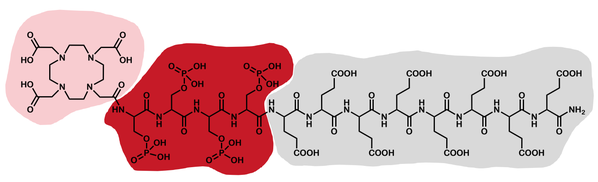
INTRODUCTION

The advancements in tumor biology have led to the development of highly specific therapies, notably in nuclear medicine, precisely targeting tumor-specific epitopes [1]. Bone-targeting agents utilize bisphosphonates, which bind farnesyl pyrophosphate synthase. Due to the enzyme's heightened activity this allows an effective targeting of metastatic bone disease [2,3]. Bisphosphonates are highly resistant to degradation. This results in long *in vivo* half-lives. While this is beneficial for inhibiting bone resorption, it is problematic for use in drug conjugates [4]. This study aimed to design novel bone-targeting peptides to increase their bone targeting affinity. NCPs (non-collagenous proteins) of the SIBLING (small integrin-binding ligand, N-linked glycoprotein) family are known for their high affinity to hydroxyapatite and their role in bone mineralization. Peptides derived from osteopontin (OPN) and matrix extracellular phosphoglycoprotein (MEPE, also known as OF45) with phosphorylated serine residues show inhibitory effects on mineralization and increased affinity for hydroxyapatite [5].

METHODS

Solid-Phase Peptide Synthesis

The peptides with unmodified serine or tyrosine residues were synthesized through solid-phase peptide synthesis (SPPS) employing the Fmoc strategy on a TentaGel resin. The protected chelator was coupled to the N-terminus using HATU as the activating agent.



A	DOTA RDDSSESSDSGS^PSS^PES^PDGD
B	DOTA DDSHQS^PDESHHS^PDES^PDEL
C	DOTA EEEEEEEE
D	DOTA $S^P S^P S^P S^P$
E	DOTA S^PEES^PEES^PEES^PEE
F	DOTA $S^P S^P S^P S^P$EEEEEEEE

The subsequent post-synthetic phosphorylation step was carried out using dibenzyl-N,N-diisopropylphosphoramidite. The deprotection, along with the cleavage of the peptide, was achieved under standard conditions. The phosphopeptides were purified by preparative HPLC utilizing a Gilson 321 pump HPLC system and lyophilized, resulting in white solids. The yields ranged from 20% to 25%.

In vivo Small Animal PET Imaging

Positron emission tomography (PET) measurements were performed with female Wistar rats aged 8-10 weeks, weighing approximately 250 g. The rats were anesthetized with 2% sevoflurane (Abbott, Wiesbaden, Germany) and oxygen at a flow rate of 0.5 L/min. They were scanned in a prone position using a Siemens Inveon micro-PET scanner (Siemens, Knoxville, TN, USA) at 1 hour and 2 hours after the injection of the respective ^{68}Ga-radiolabeled conjugate (100 to 150 MBq). Each scanning session included a 15-minute transmission scan followed by a 20-minute emission scan. PET images were reconstructed using the Ordered Subset Expectation Maximization (OSSEM) 3D Maximum A Posteriori (MAP) algorithm with the Inveon Research Workplace system software (Siemens, Knoxville, TN, USA).

SUMMARY

The newly developed phosphopeptides demonstrate pharmacokinetic characteristics comparable to those of bisphosphonates. Given their promising safety profile and low toxicity, these peptide compounds merit additional research as potential agents for targeting bone metastases in cancer therapy.

REFERENCES

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RESULTS

Effect of the Absence of Phosphorylation of MEPE-ASARM on Bone Hydroxyapatite-Binding *in vivo*

In Wistar rats, biodistribution studies were conducted using non-phosphorylated MEPE-ASARM peptide (^{177}Lu-DOTA-ASARM). The results showed that the radioactivity was primarily eliminated through the kidneys and bladder, with no significant bone accumulation observed one hour post-injection..

Bone Hydroxyapatite-Binding of Phosphorylated OPN-ASARM Peptide *in vivo*

Phosphorylated OPN-ASARM peptides inhibit crystal growth and matrix mineralization in osteoblast cultures. The phosphorylated OPN-ASARM peptide shares 60% homology with MEPE-ASARM and both contain three phosphoserine residues. While both peptides showed maximal inhibition at 20 µM, their pharmacokinetics differed, with fast blood clearance for both conjugates. Phosphorylation increased bone accumulation of ^{68}Ga-DOTA-OPN-ASARM compared to the non-phosphorylated version.

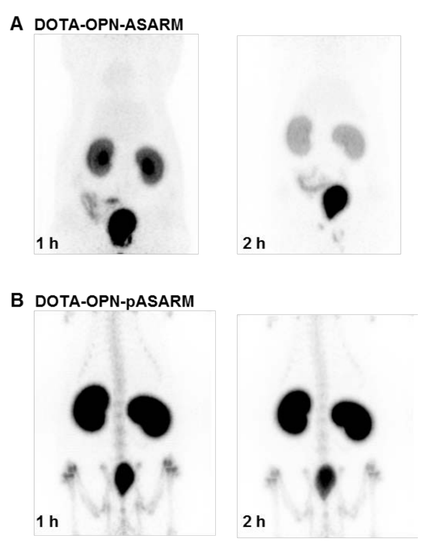

FIGURE 2.

PET-images of the ^{68}Ga-labeled non-phosphorylated (A) and phosphorylated (B) OPN-derived-DOTA-ASARM conjugate at 1h and 2 h post injection into female Wistar rats.

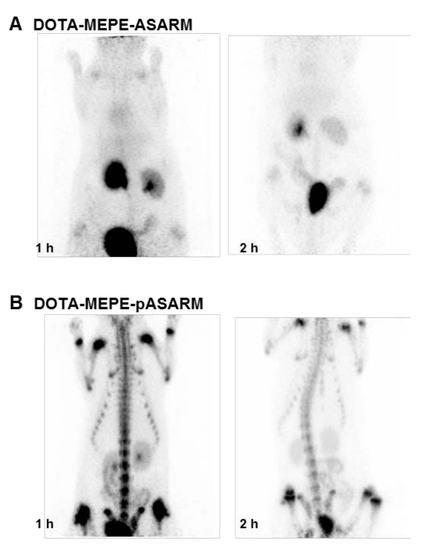

FIGURE 1.

PET-images of the ^{68}Ga-labeled non-phosphorylated MEPE-derived DOTA-ASARM conjugate (A) and its phosphorylated derivative (B) at 1h and 2 h post injection into female Wistar rats.

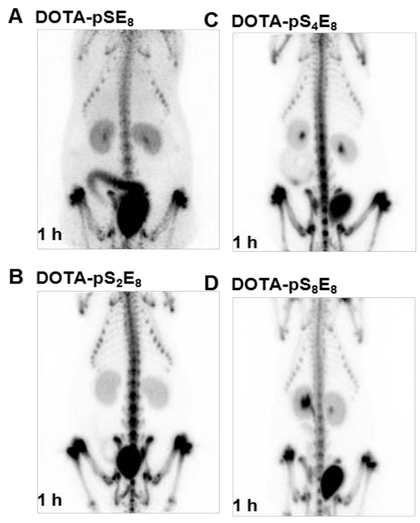

FIGURE 3.

PET-images of the ^{68}Ga-labeled phosphorylated conjugates with different sequence length DOTA-pS_nE_8 n=1 (A), 2 (B), 4 (C), 8 (D) at 1 h post injection into female Wistar rats.

Phosphopeptides of Aspartic Acid, Glutamic Acid, or DSS Repeats

Synthesized phosphorylated model peptides (DOTA-pS_4D_8, DOTA-pS_2E_8, DOTA-pS_4E_8, and DOTA-$pS_2(DSS)_4$) were tested in Wistar rats. Results indicated that phosphate groups enhance hydroxyapatite affinity, with ^{177}Lu-DOTA-pS_4D_8 and ^{177}Lu-DOTA-$pS_2(DSS)_4$ showing similar bone-binding potency to ^{177}Lu-DOTA-MEPE-pASARM. Replacing DSS repeats with glutamic acid significantly increased binding affinity, while aspartic acid did not alter it, confirming the specific binding of glutamic acid repeats to hydroxyapatite.

The Effect of Sulfation on Binding to Hydroxyapatite

The phosphorylated model peptides (DOTA-pS_4D_8, DOTA-pS_2E_8, DOTA-pS_4E_8, and DOTA-$pS_2(DSS)_4$) were studied in Wistar rats. Results indicated that phosphate groups strongly enhance bone affinity, with ^{177}Lu-DOTA-pS_4D_8 and ^{177}Lu-DOTA-$pS_2(DSS)_4$ showing similar bone-binding potency to ^{177}Lu-DOTA-MEPE-pASARM. Replacement of the DSS repeats with glutamic acid significantly increased binding affinity, while aspartic acid did not alter it, confirming the specific binding of glutamic acid repeats to hydroxyapatite.

The Effect of Phosphopeptide Length on its Pharmacokinetic Profile

We studied phosphorylated glutamic acid repeat peptides and found varying bone uptake levels, ranging from 0.85 ± 0.10 %ID/g for ^{177}Lu-DOTA-D_8 to 1.94 ± 0.17 %ID/g for ^{177}Lu-DOTA-pS_2D_8. Notably, kidney uptake increased proportionally with phosphopeptide length. Unexpectedly, ^{177}Lu-DOTA-pS_8D_8, despite having more phosphorylated serine residues, showed lower bone accumulation, potentially due to enhanced renal excretion. PET imaging of ^{68}Ga-labeled phosphorylated conjugates with varying sequence lengths (DOTA-$pSnE_8$) corroborated these findings.

Phosphoserine Position within the Peptide Sequence of Glutamic Acid Repeats

We compared two phosphorylated peptides: DOTA-$(pSE_2)_4$, with alternating phosphoserine and glutamic acid residues, and DOTA-pS_4D_8, differing only in phosphoserine positions. In vivo PET imaging in Wistar rats showed that DOTA-$(pSE_2)_4$ had higher kidney and bladder signals but lower bone uptake (SUVmax 10.1) compared to DOTA-pS_4D_8 (SUVmax 13.7).

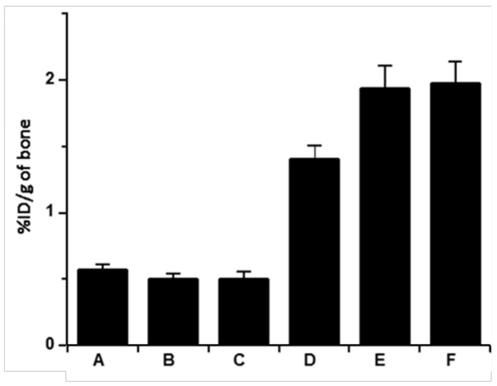

FIGURE 4.

The percentage of accumulation (%ID/g) of different ^{177}Lu-labeled phosphorylated peptides of DOTA-MEPE-pASARM (A), DOTA-$pS_2(DSS)_4$ (B), DOTA-pS_4D_8 (C), DOTA-pS_2E_8 (D), DOTA-pS_4E_8 (E) and ^{99m}Tc-MBP as reference (F) in the bone at 1 h post injection into female Wistar rats.

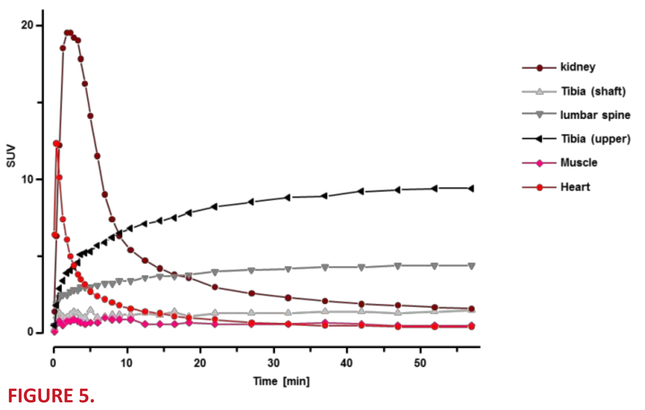

FIGURE 5.

Dynamic PET study of the ^{68}Ga-labeled phosphorylated conjugate DOTA-pS_4E_8.

Our Findings suggest:

- phosphorylated serine residues in the MEPE-ASARM peptide sequence are essential for bone accumulation
- serine phosphorylation is more effective for bone-binding than tyrosine phosphorylation
- binding potency depends on more than just net negative charge
- sulfation does not substantially improve peptide affinity to hydroxyapatite
- the bone targeting is dependent on both peptide sequence and net charge