Investigating the feasibility of EPI-X4 analogs for targeting CXCR4-expressing tumors *in vivo*

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The overexpression of the C-X-C motif chemokine receptor 4 (CXCR4) in more than 23 types of human cancer, followed by its role in tumor growth, angiogenesis, and metastasis designates it as an attractive target in oncology. The identification of an *endogenous peptide antagonist of CXCR4, termed EPI-X4* [1] and its optimized derivative EPI-X4 JM#21 [2], opens the space for the development of radiotracers for non-invasive molecular imaging and treatment (theranostics) of CXCR4-expressing cancers using the EPI-X4 as platform. One drawback of EPI-X4 and EPI-X4 JM#21, however, is their low stability in human plasma with $t_{1/2}$ of 17 min and 6 min, respectively. Since the N-terminus of these peptides is metabolically vulnerable in plasma, different modification were introduced at the N-terminus of EPI-X4 leding to derivatives with significantly increased stability ($t_{1/2}$ > 8 h). In addition, amidation at the C-terminus enabled further truncations of EPI-X4. A series of these derivatives was selected for conjugation to the DOTA chelator and labeling with Lutetium-177 (^{177}Lu) in order to assess for first time the CXCR4 targeting ability of EPI-X4-based radiotracers *in vitro* and *in vivo*.

In vitro characterization

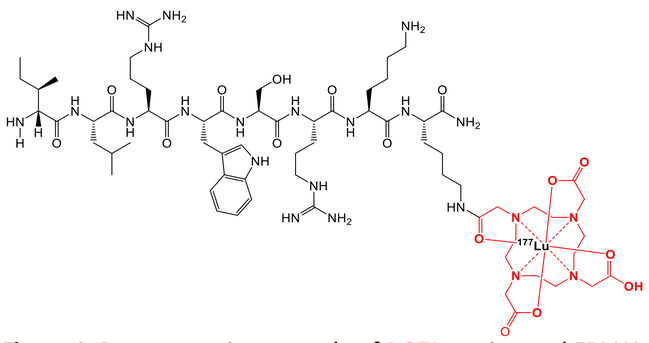


Figure 1. Representative example of DOTA-conjugated EPI-X4 (JM173) derivative labeled with ^{177}Lu.

Table 1. Peptide sequences and IC_{50} values of ^{nat}Lu-complexed DOTA-conjugated EPI-X4 derivatives.

Ligand	Sequence	Ghost-CXCR4	Jurkat
		IC_{50} value (nM ± SEM)	
AMD3100	-	1186 ± 307	489 ± 88
EPI-X4	LVRYTKKVPQVSTPTL	4544 ± 1355	1779 ± 338
JM#21	ILRWSRKLPCVS	183 ± 50	136±49
JM206	ILRWSRKLPCVSK(DOTA)	278 ± 103	208 ± 63
JM207	ILRWSRKLPSVSK(DOTA)	122 ± 45	49 ± 25
JM118	ILRWSRK(DOTA)-NH_2	197 ± 62	113 ± 39
JM29	(d-L)LRWSRKLPCVSK(DOTA)	1013 ± 233	465 ± 102
JM173	(d-I)LRWSRKK(DOTA)-NH_2	481 ± 106	435 ± 130
JM169*	IVRWSKK(Pal)VPCSK(DOTA)	30 ± 19	18 ± 5
JM235*	(d-L)LRWSRK(E-Pal)K(DOTA)-NH_2	6 ± 4	4 ± 1

*Pal = palmitic acid

EPI-X4-based radiotracers: The DOTA-conjugated EPI-X4 based peptides were incubated with [^{177}Lu]$LuCl_3$ (40-150 MBq) in ammonium acetate buffer (0.4 M, pH 5.0) for 20-30 min at 95°C. Quality control was performed by radio-HPLC. Radiolabeling yield was ≥97% and radiochemical purity ≥95% at apparent molar activities of 30-80 MBq/nmol.

Affinity: All the DOTA-conjugated peptides were complexed with natural Lutetium (^{nat}Lu) and the CXCR4 receptor affinity studies were performed using antibody competiton assay in CXCR4-expressing cells (Ghost-CXCR4 and Jurkat cells).
The affinity data revealed JM169 and JM235 (both bearing palmitic acid) to be the most affine EPI-X4 derivatives, followed by JM118 and JM207.

Log *D*: The octanol-water particion coefficient at pH 7.4 (log *D*) was determined by shake-flask method. The radiotracers displayed a broad spectrum of hydrophilicities, ranging from ^{177}Lu-JM207 being the most hydrophilic with a log *D* of -3.23 ± 0.23 to ^{177}Lu-JM235 being far more lipophilic with a log *D* of 0.29 ± 0.10.

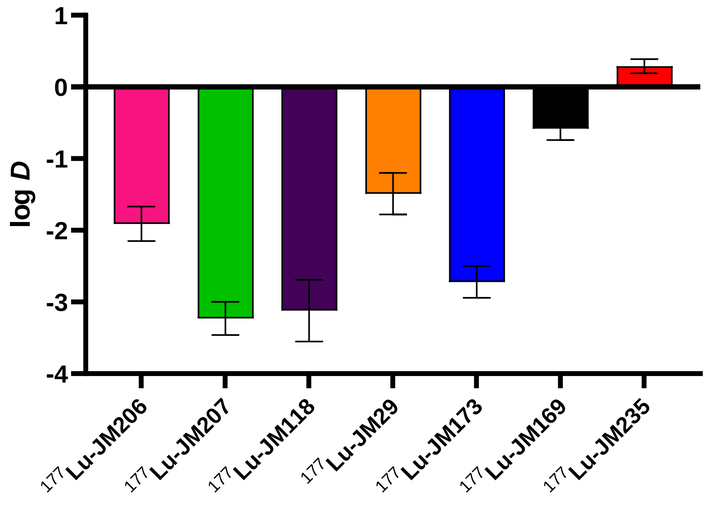


Figure 2. Lipohilicities of the ^{177}Lu-labeled tracers expressed by their log *D*.

Cellular Uptake: All the radiotracers were tested comparatively in Ghost-CXCR4 cells. A time dependent cellular uptake was observed for most of them.
Two of the radiotracers, ^{177}Lu-JM173 and ^{177}Lu-JM235, displayed a distinct higher total cellular uptake (7.90 ± 1.48 % and 3.25 ± 0.06 % of the applied activity (standard), respectively, after 60 min, Figure 2A), compared to all others radiotracers that exhibited a cellular uptake of around 1 %.
The cellular distribution (Figure 2B) showed that the two distinguished radiotracers are distributed differently; ^{177}Lu-JM235 is almost entirely internalized (filled bars, suggesting agonism), while ^{177}Lu-JM173 remaining mostly on the cell surface (dotted bars, suggesting antagonism).

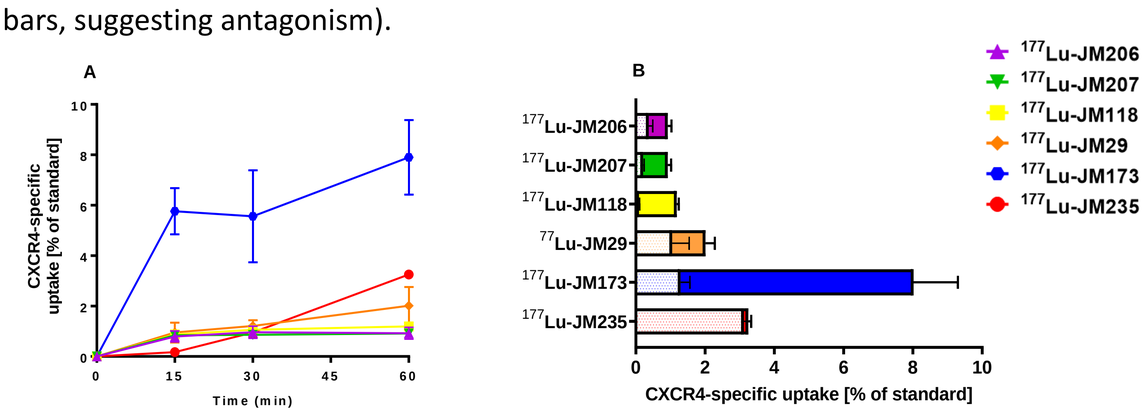
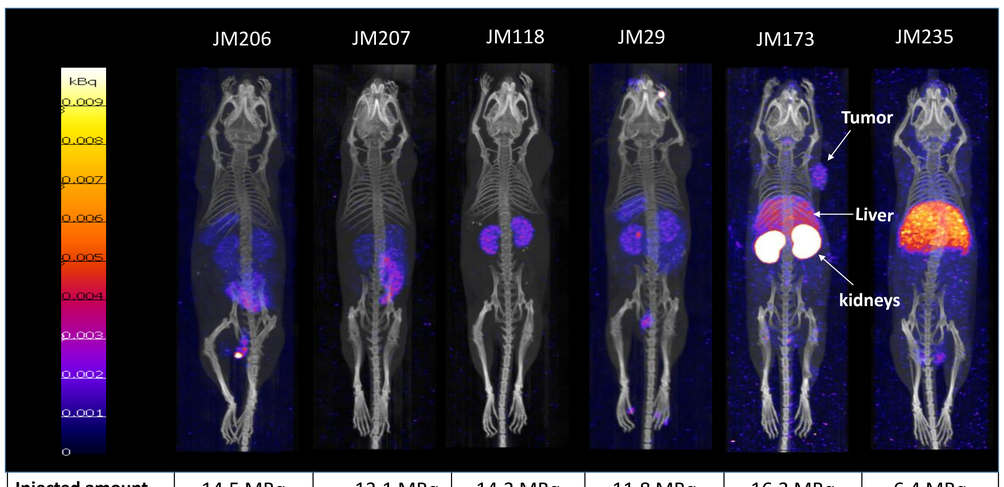


Figure 3. Cellular uptake (A) and distribution (B) of ^{177}Lu-labeled tracers; the cell surface bound fraction is indicated by the filled bars and the internalized fraction by the dotted bars.

In vivo studies

SPECT/CT imaging: Nude mice bearing Jurkat xenografts were euthanized 1 hour post injection (p.i.) of 200 pmol/~15 MBq of the ^{177}Lu-labeled tracers (Figure 4).
^{177}Lu-JM206, ^{177}Lu-JM207 and ^{177}Lu-JM29 were rapidly washed out, with only 5% of the total injected activity retained in the body at 1 h p.i. indicating metabolic instability of these radioligands.
^{177}Lu-JM173 and ^{177}Lu-JM235 remaining activity in the body was found to be around 55 and 66 %, respectively.
Among all the radioligands, only ^{177}Lu-JM173 visualized the CXCR4-expressing tumor and was mainly excreted via renal route along with limited hepatic uptake.



	JM206	JM207	JM118	JM29	JM173	JM235
Injected amount	14.5 MBq	13.1 MBq	14.3 MBq	11.8 MBq	16.3 MBq	6.4 MBq
Remaining amount	0.8 MBq **(6%)**	0.9 MBq **(7%)**	1 MBq **(7%)**	0.9 MBq **(8%)**	9 MBq **(55%)**	6 MBq **(94%)**

Figure 4. SPECT/CT images of ^{177}Lu-labeled tracers at 1 hour after injection and indicative whole-body washout values.

Biodistribution: Quantitative biodistribution was performed with ^{177}Lu-JM173 (200 pmol/0.8-1 MBq) in Jurkat xenograft tumor model at 1h and 4h p.i.. The tumor uptake was 2.25 ± 0.43 % injected activity per gram of tissue (% I.A./g) at 1 h and reduced to 0.90 ± 0.25 % at 4h. The radiotracer is predominately excreted via the urinary system as the high accumulation in the kidneys demonstrates.

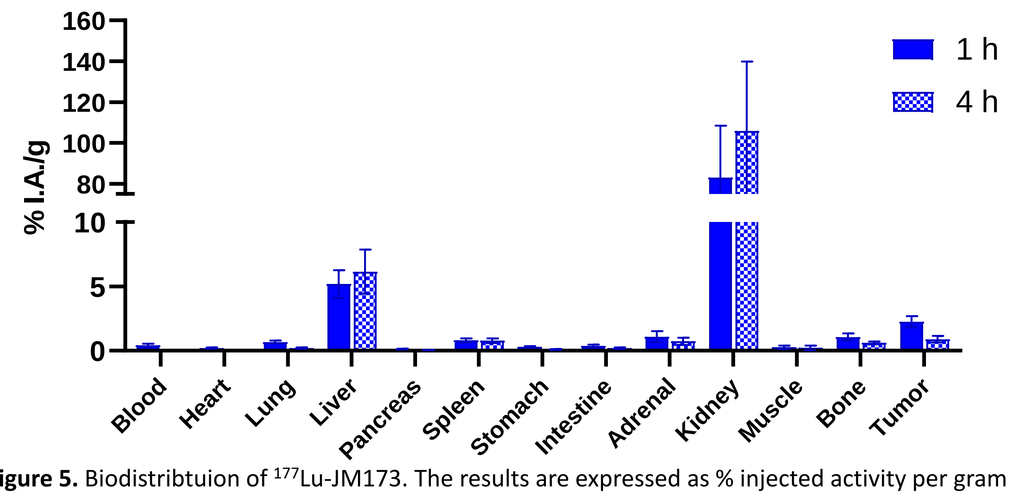


Figure 5. Biodistribtuion of ^{177}Lu-JM173. The results are expressed as % injected activity per gram of tissue.

Plasma protein binding: Mice were injected with 500 pmol/18 MBq of ^{177}Lu-JM173 and at 5, 15, 30, and 60 min blood was collected. Plasma was separated and centrifuged on the microcon-30kDa centrifugal filter unit tube. The amount of plasma-bound radiotracer was calculated based on the radioactivity measured in the filtrate (free fraction) relative to the corresponding loading solution.

Table 2. Plasma protein binding of ^{177}Lu-JM173 at different time points.

Radiotracer	% Plasma protein binding			
	5 min	15 min	30 min	60 min
^{177}Lu-JM173	37 ± 0.6	37 ± 0.8	37 ± 1	39 ± 0.9

Conclusion

- Our data confirm the feasibility of developing EPI-X4 derivatives as radiotracers for CXCR4 expressing malignancies.
- SPECT/CT imaging and *in vivo* data revealed the strengths and limitations of EPI-X4-based platform and designated the lead analog ^{177}Lu-JM173 for further optimization.

References

1. Ziraffi, O., et al., Cell Rep, 2015. **11**(5): p. 737-47.
2. Harms, M., et al., Acta Pharm Sin B, 2021. **11**(9): p. 2694-2708.