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One-week *in vivo* sustained release of a peptide formulated into *in situ* forming implants

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GRAPHICAL ABSTRACT

ABSTRACT

The LR12 peptide has been reported to reduce the size of infarct and improve both cardiac function and survival in myocardial infarction in murine models, after daily repeated intraperitoneal injections. In order to protect peptide from degrading and to prolong its release, *in situ* implants based on biocompatible biodegradable polymers were prepared and both *in vitro* and *in vivo* releases were evaluated after subcutaneous administration to Wistar rats. A progressive and complete release was obtained *in vitro* in 3 weeks. *In vivo*, a 7-day sustained release was demonstrated after administering the formulation once; bioavailability was improved by protecting the peptide against the degradation identified as a dimerization through disulfide bond formation. As a conclusion, *in situ* forming formulations are a suitable alternative for the therapeutic use of this peptide.

KEYWORDS

LR12

TREM-1 inhibitory peptide

Sustained delivery

In situ-forming depot

Bioavailability

Dimerization

TEXT

With 17.3 million deaths per year (a number expected to grow to more than 23.6 million by 2030), cardiovascular disease is the leading global cause of death. Related direct and indirect costs are estimated to be higher than \$320.1 billion, including health expenditures and loss of productivity (Mozaffarian et al. 2015). As the immune system and the inflammation are now recognized as key players in the establishment and exacerbation of cardiovascular diseases, new therapeutic strategies have been emerging to control them. In this context, the triggering receptor expressed on myeloid-cells-1 (TREM-1) has been identified as an interesting target. This immune-receptor is expressed by neutrophils, macrophages and mature monocytes, and acts as an amplifier of the innate immune response during both infectious and sterile inflammation (Bouchon et al. 2001, Gibot et al. 2009, Zhou et al. 2013). Recently, the modulation of TREM-1 signaling by an inhibitory dodecapeptide (LR12, **Figure 1A**) reduced the size of infarct and improved both cardiac function and survival in a murine model of myocardial infarction (Boufenzer et al. 2015). In that study, LR12 (5 mg/kg) was intraperitoneally (IP) administered once a day for 5 days. This therapeutic scheme should be advantageously replaced by a single injection of a sustained-release formulation. Moreover, LR12 oxidizes spontaneously in aqueous media with a short half-life ($t_{1/2}$) (approximately 14 hours in phosphate buffer saline (PBS) *in vitro*, and 0.5 hour in blood *ex vivo*): a disulfide bridge is formed between two peptides, generating a dimer (**Figure 1B**). While still to be confirmed by further investigation, preliminary experiments on cells suggested that the dimer is devoid of pharmacological activity. Consequently, the formulation should both sustain LR12 release for at least a 5-day period and, as much as possible, protect the drug from degrading by dimerization.

In this contribution, the *in vitro* and *in vivo* release of LR12 (both the monomer and its main product of degradation, *i.e.* the dimeric form) from *in situ* forming implants have been evaluated. *In situ*

58 forming implants are liquid formulations which, when injected into aqueous environments,
59 precipitate as solid polymeric matrices entrapping the drug (Parent et al. 2013a). Sustained releases
60 obtained with *in situ* forming implants have been described in the literature for a wide range of
61 drugs with various physicochemical properties. Herein, poly-lactide-co-glycolide (PLGA,
62 Resomer RG502H, 50:50 ratio LA:GA) and poly-lactide (PLA, Resomer R203S) were used as
63 biocompatible biodegradable polymers due to their frequent use in *in situ* forming formulations
64 and their lack of toxicity. Polymers were solubilized (18.7% w/w) in triacetin (TA, 74.8% w/w)
65 before adding the drug (6.5% w/w). The concentration of peptide used in the formulation allowed
66 to subcutaneously inject a dose of 80 mg LR12/kg to the animals. This can be compared to the total
67 dose of 25 mg/kg of free LR12 administered *via* several IP injections in the study, which
68 demonstrated the benefit of LR12 in myocardial infarction (Boufenzer et al. 2015). This dose is
69 compatible with a slow delivery of LR12 from the reservoir formulation during the time of
70 experiment, and it is likely without safety issues. Additionally, with this concentration, the
71 viscosity of the formulation remained suitable for an easy injection.

72 Formulations were prepared and *in vitro* release experiments were performed in physiological
73 buffer saline according to previously reported protocols (Parent et al. 2013b). An HPLC-UV
74 method was developed, then validated for selectivity, precision, accuracy and linearity according
75 to the FDA guidelines, and used to quantify LR12 (monomer and dimer) released in the aqueous
76 medium (**Figure 2**). The chromatographic system was the same as previously described (Parent et
77 al. 2016). The elution was isocratic (86/14 % v/v of water/acetonitrile + 0.1% trifluoroacetic acid)
78 with 20 μ L of injected sample. The detection was set at 220 nm. Linearity was verified for
79 monomer and dimer between 1.0 and 50.0 μ g/mL ($y = 14.30x - 17.55$ and $R^2 = 0.994$ for monomer,
80 $y = 14.15x - 11.87$ and $R^2 = 0.996$ for dimer). Peptides remaining in the implants were also

quantified with the same method after being extracted (dissolution of implants in ethyl acetate then liquid-liquid extraction with PBS added with 0.1% trifluoroacetic acid, recoveries of 102.5 ± 6.2 % for the monomer and 100.3 ± 4.2 % for the dimer, $n = 3$).

In vitro experiments demonstrated a 3-week sustained release of LR12 monomer from PLGA implants (**Figure 2A**), without any burst, as expected when using a solvent with low water solubility such as TA (Camargo et al. 2013). LR12 dimer also appeared progressively in the medium. The release profile from PLA implants was different (**Figure 2B**): the monomer release reached a plateau after 3 days, while the concentration of the dimer increased until the end of the experiment. Dimer appears, resulting from the degradation of monomer, which can occur either inside the implant or in the aqueous medium after its release. In the conditions of the *in vitro* release test, non-formulated LR12 monomer spontaneously oxidized within time to form the disulfide dimer, with a short half-life of 14 hours. Moreover, at the end of the release experiments (after 3 weeks), remaining LR12 (< 5.0 % of the initial load) was extracted and was shown to be mainly under the monomer form (95.0 ± 1.3 % for PLGA and 93.4 ± 1.1 % for PLA). The very low proportion of dimer inside the implants suggests that the degradation of LR12 likely occurs in the release medium rather than inside the formulations. To conclude, *in situ* implants offered a sustained *in vitro* release of LR12 monomer up to 3 weeks, while efficiently protecting the drug from dimerization.

In a previous study, blood concentrations of LR12 (monomer and dimer) after intraperitoneal injection (5 mg/kg) to male Wistar rats were monitored (Parent et al. 2016). The same protocol and

method were applied to quantify LR12 in blood after one single subcutaneous injection of *in situ* implants (80 mg LR12/kg) to male Wistar rats (280-350 g). Rats were subcutaneously injected with *in situ* forming formulation prepared with PLGA or PLA (final dose of 80 mg LR12/kg, 21 G needle used for injection). Under isoflurane anesthesia, blood was collected 1h, 1, 3, 7 or 14 days after the treatment, before sacrificing the animal and retrieving the implants for residual LR12 quantitation. Three animals were used for each time point for each polymer (total = 30 rats). Blood samples were treated and analyzed with the HPLC-fluorescence method as previously reported (Parent et al. 2016). Remaining LR12 was also extracted from the retrieved implants and quantified as described above for the *in vitro* study. At autopsy, no sign of irritation or inflammation of the tissues surrounding the implants was observed, whatever the polymer used (Representative photograph of extraction is supplied in Supplementary data S1).

After one single administration of *in situ* forming implants, LR12 monomer was detected at therapeutic concentrations (between 100 and 400 ng/mL) for a week (**Figure 3**). The area under the concentration-time curve ($AUC_{0\rightarrow 7 \text{ days}}$) was calculated using the trapezoidal rule and was normalized by the received dose (**Table 1**). The highest observed plasma concentration ($C_{\max}$) and the time required to reach $C_{\max}$ ($T_{\max}$) were obtained from the concentration-time curves. Results indicated that *in situ* forming formulations significantly improved LR12 monomer bioavailability by a factor 50, without any difference between PLGA and PLA. This can be explained by the sustained-release properties of the *in situ* forming formulations, which increase the circulation time of LR12, and by the protection of monomer from degradation because it was encapsulated into the polymeric matrices. Although LR12 remaining inside the implants was mainly under the monomer form, the proportion of dimer inside the *in vivo* implants increased within time ($4.3 \pm 1.7 \%$ at day 1 to $27.3 \pm 2.4\%$ at day 7 for PLGA and $3.5 \pm 3.0\%$ at day 1 to $26.1 \pm 10.9\%$ at day 7 for PLA).

After one week, more than 95% of the initial load was released. Compared with these *in vivo* results, better LR12 protection (> 90% remaining as monomer after 3 weeks in the implants) and more sustained release (up to 3 weeks) with *in situ* forming implants were observed *in vitro*.

In the literature, only a few studies deal with *in vivo* peptide sustained delivery with *in situ* forming formulations. A 48 h – *in vivo* release was for example reported for enfuvirtide, an anti-HIV fusion inhibitor of 36 amino acids, when incorporated into implants made of PLGA and a mixture of DMSO and triacetin (Kapoor et al. 2012). A sustained effect of *S*-nitrosoglutathione, a nitric oxide donating tripeptide, was also observed over the same time length, using PLGA implants with *N*-methyl-2-pyrrolidone (NMP) as solvent (Parent et al. 2015). Regarding leuprolide acetate (9 amino acids), a 14-days *in vivo* release in rats was described from PLGA/NMP implants (Mashayekhi et al. 2013), but similar marketed formulations (Eligard®) allow therapeutic efficiency in humans from 1 to up to 6 months after a single administration. In this study, *in situ* forming formulations administered to healthy rats demonstrated their ability to deliver the dodecapeptide LR12 according to a smooth and sustained profile at therapeutic concentrations for 7 days. As a result, this single injection could be an interesting alternative to the current therapeutic scheme (free drug 5 mg/kg, daily injection for 5 days) proposed in myocardial infarction (Boufenzer et al. 2015). PLGA formulations should probably be preferred over PLA ones, because they will be degraded faster, while offering the same monomer bioavailability in this case, but with lower burst and less dimer blood exposure (dimer AUC/dose increased by 12 for PLGA implants compared to IP administration and by 18 for PLA ones, **Table 1**). Modifications of the peptide itself or of the formulation could also be envisaged to further increase the *in vivo* duration of release. For example, the PEGylation of a natural polysaccharide enhanced the *in vivo* mean retention time from 1.0 h to

2.8 days, and this result was drastically improved (up to 13 days) when the conjugate was formulated into PLGA *in situ* implants (Shi et al. 2014, 2015).

To conclude, this study demonstrates that *in situ* formulations are promising candidates for the therapeutic use of LR12, a TREM-1 inhibitory dodecapeptide useful in many conditions involving inflammation and exacerbated immune response. However, pharmacokinetics of LR12 could be modified in pathological situations, for example by the apparition of the soluble form of TREM-1 in the blood (Boufenzer et al. 2015). The benefit of *in situ* formulations for LR12 treatment should therefore be confirmed in animals suffering from myocardial infarction for example.

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REFERENCES

Bouchon, A., Facchetti, F., Weigand, M.A., Colonna, M., 2001. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* 410, 1103–1107.

170

171 Boufenzer, A., Lemarié, J., Simon, T., Derive, M., Bouazza, Y., Tran, N., Maskali, F., Groubatch,
 172 F., Bonnin, P., Bastien, C., Bruneval, P., Marie, P.Y., Cohen, R., Danchin, N., Silvestre, J.S., Ait-
 173 Oufella, H., Gibot, S., 2015. TREM-1 mediates inflammatory injury and cardiac remodeling
 174 following myocardial infarction. *Circ. Res.* 116, 1772-1782.

175

176 Camargo, J., Sapin, A., Nouvel, C., Daloz, D., Leonard, M., Bonneaux, F., Six, J.L., Maincent, P.,
 177 2013. Injectable PLA-based in situ forming implants for controlled release of ivermectin, a BCS
 178 class II drug: solvent selection based on physico-chemical characterization. *Drug Dev. Ind. Pharm.*
 179 39, 146-155.

180

181 Gibot, S., Massin, F., Alauzet, C., Derive, M., Montemont, C., Collin, S., Fremont, S., Levy, B.,
 182 2009. Effects of the TREM 1 pathway modulation during hemorrhagic shock in rats. *Shock* 32,
 183 633–637.

184

185 Kapoor, D.N., Katare, O.M., Dhawan, S, 2012. In situ forming implant for controlled delivery of
 186 an anti-HIV fusion inhibitor. *Int. J. Pharmaceut.* 426, 132-143.

187

188 Mashayekhi, R., Mobedi, H., Najafi, J., Enayati, M., 2013. In vitro/in vivo comparison of
 189 leuprolide acetate release from an in situ forming PLGA system. *DARU J. Pharm. Sci.* 21:57,
 190 doi:10.1186/2008-2231-21-57

191

192 Mozaffarian, D., Benjamin, E.J., Go, A.S., Arnett, D.K., Blaha, M.J., Cushman, M., de Ferranti,
 193 S., Despres, J-P., Fullerton, H.J., Howard, V.J., Huffman, M.D., Judd, S.E., Kissela, B.M.,

194 Lackland, D.T., Lichtman, J.H., Lisabeth, L.D., Liu, S., Mackey, R.H., Matchar, D.B., McGuire,
 195 D.K., Mohler, E.R. 3rd, Moy, C.S., Muntner, P., Mussolino, M.E., Nasir, K., Neumar, R.W.,
 196 Nichol, G., Palaniappan, L., Pandey, D.K., Reeves, M.J., Rodriguez, C.J., Sorlie, P.D., Stein, J.,
 197 Towfighi, A., Turan, T.N., Virani, S.S., Willey, J.Z., Woo, D., Yeh, R.W., Turner, M.B., on behalf
 198 of the American Heart Association, Statistics Committee and Stroke Statistics Subcommittee,
 199 2015. Heart disease and stroke statistics—2015 update: a report from the American Heart
 200 Association. *Circulation* 131, e29–e322.
 201
 202 Parent, M., Nouvel, C., Koerber, M., Sapin, A., Maincent, P., Boudier, A., 2013a. PLGA in situ
 203 implants formed by phase inversion: critical physicochemical parameters to modulate drug release.
 204 *J. Control. Release* 172, 292-304.
 205
 206 Parent, M., Boudier, A., Dupuis, F., Nouvel, C., Sapin, A., Lartaud, I., Six, J.L., Leroy, P.,
 207 Maincent, P., 2013b. Are in situ formulations the keys for the therapeutic future of S-nitrosothiols?
 208 *Eur. J. Pharm. Biopharm.* 85, 640-649.
 209
 210 Parent, M., Boudier, A., Maincent, P., Gibot, S., Ait-Oufella, H., Boufenzer, A., Jolly, L., Derive,
 211 M., Kouach, M., Goossens, J.F., Leroy, P., Clarot, I., 2016. LR12-peptide quantitation in whole
 212 blood by RP-HPLC and intrinsic fluorescence detection: validation and pharmacokinetic study.
 213 *Biomed. Chromatogr.*, doi: 10.1002/bmc.3877.
 214
 215 Parent, M., Boudier, A., Perrin, J., Vigneron, C., Maincent, P., Violle, N., Bisson, J.F., Lartaud, I.,
 216 Dupuis, F., 2015. In situ microparticles loaded with S-nitrosoglutathione protect from stroke. *PLoS*
 217 *One* 10, e0144659.

218

219 Shi, X., Lin, X., Zheng, X.W., Feng, Y., Shen, L., 2014. Injectable long-acting systems for Radix

220 Ophiopogonis polysaccharide based on mono-PEGylation and in situ formation of a PLGA depot.

221 Int. J. Nanomed. 9, 5555-5563.

222

223 Shi, X., Lin, X., Yao, C.X., Shen, L., Feng, Y., 2015. Injectable long-acting in situ forming systems

224 for Radix Ophiopogonis polysaccharide. Int. J. Biol. Macromol. 72, 553-559.

225

226 Zhou, J., Chai, F., Lu, G., Hang, G., Chen, C., Chen, X., Shi, J., 2013. TREM-1 inhibition attenuates

227 inflammation and tumor within the colon. Int. Immunopharmacol. 17, 155-161.

228

229 **FIGURES**

230

231 **Figure 1:** A) Murine sequence of LR12 monomer peptide (Mr 1341 g/mol, calculated pI ~ 3.6)

232 and B) main pathway of degradation by dimerization.

233

234 **Figure 2:** *In vitro* release profiles of LR12 (monomer and dimer) obtained from *in situ* implants
 235 made of TA and of either PLGA (A) or PLA (B). Results are presented as mean $\pm$ sd of three
 236 experiments.

Figure 3: Blood concentrations of LR12 (monomer form, panel A and dimer form, panel B) after administration of LR12 monomer (unformulated, 5 mg/kg, intraperitoneally vs formulated into *in situ* implants, 80 mg/kg, subcutaneously). Limit of quantification of the method was 50 ng/mL. All experiments were performed in accordance with the European Community guidelines (2010/63/EU) for the use of experimental animals, and protocols were approved by the regional and national ethical committees (project “Slow-release”, APAFIS#1146-2015071313458604 v3).

TABLE 1

Pharmacokinetic parameters of LR12 after administration to male Wistar rats.

		Intraperitoneal injection of LR12 monomer	Subcutaneous injection of LR12 monomer- <i>in situ</i> implants	
			PLGA	PLA
Monomer	AUC _{0→7 days} (ng. mL ⁻¹ .h)	18	17 467	17 388
	AUC/Dose (L ⁻¹ .h.g body weight)	4	218	217
	T _{max} (h)	0.08	1	1
	C _{max} (ng/mL)	181 ± 35	261 ± 95	468 ± 252
Dimer	AUC _{0→7 days} (ng. mL ⁻¹ .h)	197	38 414	57 499
	AUC/Monomer dose (L ⁻¹ .h.g body weight)	39	480	719
	T _{max} (h)	0.25	24	24
	C _{max} (ng/mL)	300 ± 162	379 ± 74	367 ± 39

Supplementary data

(S1): Representative photograph of the extraction of one *in vivo in situ* forming implant (here, PLA implant, one week after injection).