DARPin is a small sized (16 kDa) protein derived from the naturally occurring protein ankyrin, which is engineered to exhibit high affinity for target proteins. The epithelial growth factor receptor 2 (Her2/neu) is a target of interest for targeted therapies as it is overexpressed in epithelial tumours such as breast and gastric cancers. Antitumour activity and biodistribution of five novel biparatopic Her2-targeting DARPin (CME114, CME115, CME118, CME119 and SPA28) were evaluated in mice bearing Her2-positive human breast tumours. Biparatopic DARPin bind simultaneously to two different epitopes in Her2 and trigger apoptosis in Her2-expressing tumour cells. The tested molecules are free DARPin variants containing different amino acid sequences, relative domain orientations, and PK-extension technologies.

Introduction
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Material and Methods
Antitumour activity of DARPin in BT-474 tumour bearing mice
Healthy female Balb/c Nude mice were obtained from Charles River. Animal experiments were performed according to the principles and guidelines of animal experimentation (2) and were approved by Oncodesign’s internal ethical committee (Comité d’éthique). The four CME compounds had a similar PK in both the treated versus control groups.

Tumour induction
BT-474 tumour cells were subcutaneously injected (with matrigel) in the right flank of Balb/c Nude mice on 0D. Randomisation was performed on 255 (mean tumour volume at randomization: 264 ± 57 mm3).

Treatments
All treatment groups were composed of 8 mice. The four CME compounds had a similar PK in both the treated versus control groups.

Histological analysis
Lungs, kidneys and heart were collected and embedded in paraffin. The histology of organs was investigated after hematoxylin and eosin staining. Evaluation of antitumour activity
Tumour growth inhibition (T/C%) was calculated as the ratio of the median tumour volumes of treated versus control groups.

Radioiodination of DARPin with technetium-99m (99mTc)
The DARPin were radioiodinated with 99mTc, using the tricarbonyl technique (Isobiquik kit). The radiiodination purity (RP) of the 99mTc-labeled DARPin was assessed by thin layer chromatography (TLC). Quality control of the 99mTc-labeled DARPin was also performed by exclusion chromatography. The ability of the 99mTc-labeled DARPin to bind albumin and Her2 was confirmed by both ELISA and BT-474 tumour cell binding assay.

Results
Quality control of 99mTc-radiolabelled DARPin (target binding assessment)
All tested DARPin was composed of a cone anti-Her2 biparatopic DARPin (red and orange) and a PK extension moiety. The latter consists of either a genetic fusion to an albumin binding DARPin (grey) or a conjugation to PEG40B.

Safety profile
No body weight loss was observed during the course of treatment. No lesion was observed by histology in kidneys, heart and liver (A – C).

Antitumour activity
No tumour was observed at the end of treatment. All treated mice were cured and survived longer than the control group (D).

Biodistribution of 99mTc-radiolabelled DARPin
Intraperitoneal injection of 99mTc-[albumin: DARPin] complexes showed a similar biodistribution pattern between the tested complexes and albumin in the organs analyzed.

Conclusions
DARPin is a flexible platform allowing easy conjugation to payloads, including radiolabelling. Tumour models allow ranking of CANe molecules according to their in vivo antitumour properties. All tested DARPin, except CME118, induced tumour regression. DARPin showed promising antitumour and penetration. CME115 showed the best tumour to blood/liver ratios. No side-effects related to treatment with DARPin were evidenced. PK extension via albumin provided longer PK and easier DARPin production than via PEG40B conjugation.