Ultrasound Molecular Imaging: How to develop clinical products?

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The introduction of targeted contrast agents among agents eligible for ultrasound molecular imaging (USMI) has reinforced the interest for this method and significantly broadened the scope of CEUS but in the same time, has raised significant issues with regard to the agent to be used clinically (1,4-8,11-14,16).
When entering the USMI domain, the need to add a ligand to target a specific molecular marker/signature implies the validation of the targeting moiety and more precisely, the compatibility with regulatory requirements for a human use.
The bubble size is not significantly modified by the presence of a ligand meaning that the specific characteristic of these agents as strict markers of the vascular bed is still a property which can be considered as a great advantage for quantification in some indications, such as therapeutic treatment monitoring.
The specific issues considered for such agents are strictly related to the nature of the ligand itself and the mode of attachment to the shell membrane. Whereas preclinical tests have been performed with a biotin-streptavidin linker, the impossibility to translate this construct into clinics due to possible immunogenicity has conducted scientists to propose alternative methods compatible with human use.
From a regulatory point of view, the gas microbubble is considered as the active entity meaning that each of the microbubble components should be fully characterized. The manufacture of clinical material should be carried out in compliance with the GMP guidelines.
With respect to the formulation characteristics, the selection of the ingredients is of paramount importance since the use of specific components should be validated for these new drug delivery systems for parenteral administration. In that perspective, the retained formulation for clinical trials must be challenged before finalization as changing any of the components at a late stage could be difficult and costly, even impossible.
Once the formulation is finalized, many steps must be accomplished before any clinical use: robustness of the manufacturing process, stability of the product, validation of the test methods. Another requirement is completing a pharma-toxicology package according to the International Conference on Harmonization (ICH) guidelines. These different steps are time-consuming and relatively expensive.
Finally, when the steps above have been completed, the agent is suitable for clinical testing pending Investigational New Drug (IND) submission and Institutional Review Board (IRB) or ethical committee approval for the selected indication.
At present, literature is rich of papers reporting good results with targeted UCAs in many animal models. However, only one agent BR55 (Bracco Imaging, Milan, Italy) entered clinical testing so far. This illustrates the difficulties to develop a suitable approach for clinical use. The development time of such agents does not differ significantly from what is currently reported for therapeutic drugs, i.e. at least 10 years.
The translation to clinics targeted agents requires high level expertise to develop suitable agents according to the various constraints.

What makes a difference for targeted agents?
It has been proposed to exploit the specific property of UCA as strict vascular bed marker to be used for molecular imaging when targeting receptors or proteins of interest are expressed at endothelial level. It is well-known that diseases are accompanied by the expression of various markers at tissue and endothelial levels, the latter being the specific target of targeted UCAs. This is particularly the case for inflammation and angiogenesis (tumor angiogenesis and wound healing) in which the luminal surface of endothelial cells within capillaries and vessels express various well-identified receptors such as selectins, Vascular Cell Adhesion Molecule 1 (VCAM-1), integrins and Vascular Endothelial Growth Factor Receptor (VEGFR).
Therefore, microbubbles need to be functionalized with appropriate ligands that have relevant affinity and specificity for the selected target in order to observe binding and retention of UCA on the target in physiologic flow conditions. The selection of the ligand is a critical step either for the sensitivity required for USMI or for a possible clinical use. Antibodies, antibody fragments, recombinant proteins, peptides, aptamers and carbohydrates have been proposed for such purpose (14).
In many cases, a spacer arm like polyethylene glycol (PEG) can be conjugated to the surface of the microbubble, and then an avidin-biotin link can be used to attach a disease-specific ligand. Avidin-biotin interaction is among the strongest noncovalent bonds and is widely used in biomedical research and analytic practice, as in immunoassays. Commercial biotinylated antibodies are abundant, and can readily be linked to pre-manufactured streptavidin microbubbles (14). While really

advantageous for preclinical testing, this cannot be translated to clinical use due to the immunogenicity of avidin (13).

Two methods have been proposed for covalent protein coupling. In the early method, a carboxyl group on a UCA is activated with carbodiimide in the presence of N-hydroxysulfosuccinimide (NHS), forming active ester. This NHS ester reacts with the protein amino group, forming an amide bond. Alternatively, a maleimide on the shell is coupled with a thiol group on the ligand, forming a thioether bond. The advantage of the latter approach is oriented coupling—if a ligand protein has a single thiol (e.g., a cysteine residue placed far from the binding segment), then a single point attachment to the microbubble shell will retain affinity of the ligand to its target. This latter approach is favored when clinical use is foreseen (14).

Proteins possess multiple lysine residues, so coupling via amide bond is random and may interfere with ligand-receptor interaction. This specific point must be addressed during the development phase to fully characterize the conjugation and to be able to identify the precise interaction mechanism between the ligand and the endothelial marker. Another disadvantage of the NHS approach is low coupling yield at the reactant concentrations typical for UCA preparations. Loss of expensive humanized antibodies or other recombinant protein ligands is undesirable. Overall, region-selective coupling of thiol protein with a maleimide-carrying UCA looks more attractive (14).

Even very convenient for clinical use, antibodies have to be humanized to avoid an immune response while this is not needed for preclinical testing. However, these antibodies are not always available and the species specificity is problematic when performing preclinical imaging. Indeed, the results obtained with one antibody in one species might differ significantly from another antibody in another species even directed towards the same epitope. This is of utmost importance when dealing with a targeted agent intended for clinical use. There is a need to first establish animal models using such agent to validate the sensitivity as well as imaging conditions before initiating clinical dosing. This limitation must be carefully considered when proposing antibodies for clinical application in addition to other constraints.

Small molecules such as peptides can be attached to the shell-forming material before UCA generation. Targeted UCA BR55, which has reached clinical trial stage, has a combination peptide ligand attached to the UCA shell via a PEG spacer arm. One great value of BR55 is its capacity to be used in many animal models and humans, this being a major advantage when developing a new agent (13).

Therefore, the most critical issue with regard to possible clinical use is the selection of the ligand and the mode of attachment to the shell. The objective is to avoid any allergic reaction and to check for the absence of safety concerns with the ligand of interest. This implies a careful selection during the formulation development to ensure a proper selection of the compounds according to the requirements for an approval down the road. For this purpose, the underlying mechanism of interaction between the ligand and the spacer arm must be carefully explored to identify how and how many ligands are attached to the shell in order to envision the binding mechanism of this agent in patients. In that perspective, the proposed multi-targeted agents (i.e. interacting with multiple receptors) will remain only a tentative approach as there is very little chance to validate such agents for clinical use despite claimed improved detection (15). This explains the need for an extensive characterization of the ligand itself at very early stages.

Even though adverse events cannot be considered as a limiting factor for the use of UCA and targeted UCA, the introduction of small molecules in addition of other materials in the bubble requires specific toxicology assessment. For conventional agents, it is generally admitted that the rate of these events (around 0.01% for serious adverse events based on post-marketing safety data, with no significant differences between agents) is below what is reported for iodinated compounds and MR agents (2,9,10). Some key points need to be investigated such as allergic reactions to foreign materials, maximal tolerable dose in animals to determine the maximal dose to be used in patients and the absence of compromised blood flow after injection due to sticking of UCA to endothelial cells.

Last but not least, the translation of animal results into clinics cannot be straightforward and requires a specific validation in humans. It is well-known that the expression of some receptors could differ significantly between animals and patients. In that perspective, performing an exploratory phase in patients could satisfy the validation step by ensuring the possible detection of this targeted microbubble in humans according to the receptor's expression. In some cases, the absence of animal model or the impossibility to use the selected ligand in animal models due to species specificity could require to perform this clinical phase for agent validation. The exploratory phase, if positive, will precede a full clinical development as for therapeutic drugs (13).

WHERE DO WE STAND NOW ?

At present, literature is rich of papers reporting good results with targeted UCAs in many animal models. However, only one agent BR55 (Bracco Imaging, Milan, Italy) entered clinical testing so far. This illustrates the difficulties to develop a suitable approach for clinical use. All these UCAs are considered as drugs, and so have to fulfill specific criteria. The development time of such agents does not differ significantly from what is currently reported for therapeutic drugs, i.e. at least 10 years. The time required as well as the human resources needed

imply that only few companies have been ready to invest in this domain so far. Moreover, there is a significant increased complexity when entering the molecular imaging domain with the need to validate the target and the targeted agent, this being negatively impacted by a more limited market than for a conventional blood pool agent.

It is mandatory to demonstrate the real value of USMI versus other imaging modalities not as a competition between modalities but as a convergence for a better diagnostic chart with an improved cost/benefit ratio. The increased demand from various medical specialties will definitely reinforce the interest from imaging companies in developing such agents.

There is a need to reinforce the network between UCA manufacturers and medical imaging ultrasound scanner companies to adapt the machines to this new modality. Indeed, the current scanners are suited for the use of blood pool agents but the introduction of targeted agents requires different imaging techniques with longer time for detection of bound UCA and additional post-treatment tools to exploit the specific properties of these targeted agents. In that perspective, a strong partnership is needed to modify the current sequences for an easy use and improved detection of these agents. This will strengthen the place of these agents in the imaging palette to be used by physicians according to their specific demands.

CONCLUSION

CEUS with conventional agents has proved its efficiency in many indications, this being reinforced by guidelines established by scientific societies. The introduction of targeted agents will enlarge the capabilities of US techniques in molecular imaging domain with promising indications and results based on preclinical imaging results. The translation to clinics requires high level expertise to develop suitable agents according to the various constraints e.g. selection of the raw materials, validation in selected animal models, chemistry analysis, toxicology assessment and finally test in small groups of patients to confirm the imaging potential before a complete clinical development. This long development stage has been performed for only one agent so far, but new agents might reach the clinical phase in the future.

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