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# Nanomedicines and Stroke: Toward Translational Research

Alice Gaudin<sup>1</sup>, Karine Andrieux<sup>2</sup>, Patrick Couvreur<sup>1</sup>

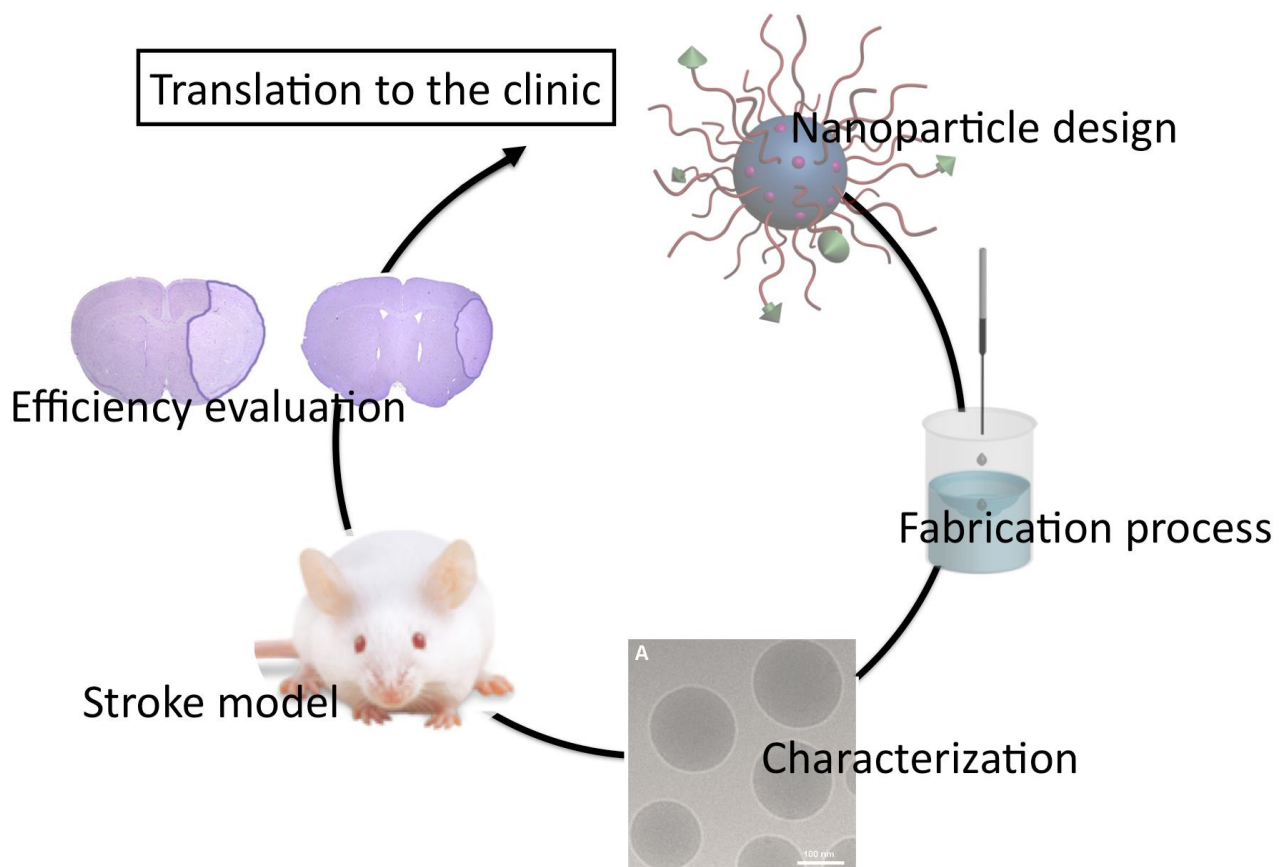
<sup>1</sup>Institut Galien Paris-Sud UMR CNRS 8612, Faculty of Pharmacy, University Paris-Sud XI, 92296 Châtenay-Malabry, France

<sup>2</sup>UTCBS UMR CNRS 8258 Inserm U1022, Faculty of Pharmaceutical and Biological Sciences, University Paris Descartes, 75006 Paris, France

## Abstract

Since their introduction in the late 60', nanotechnologies applied to medicine, called nanomedicines, are believed to have the potential to be the mankind's first "giant step" toward treatment of severe diseases. Among others, they could provide an original solution for the treatment of brain impairments, such as stroke, allowing developing and commercializing more efficient therapeutic solutions. However, despite clear improvement in pharmacokinetic properties and reductions of adverse side effects when using nanomedicines, only modest therapeutic benefits were achieved in humans. This may be due to specific issues in the nanoformulation development process and evaluation, which hampered their translation from the bench to the bedside. In this review, we aim to tackle some of the key challenges that have to be taken into account when developing and evaluating a new nanomedicines, with a special emphasis on the development of nanomedicines for the treatment of stroke.

## Graphical abstract

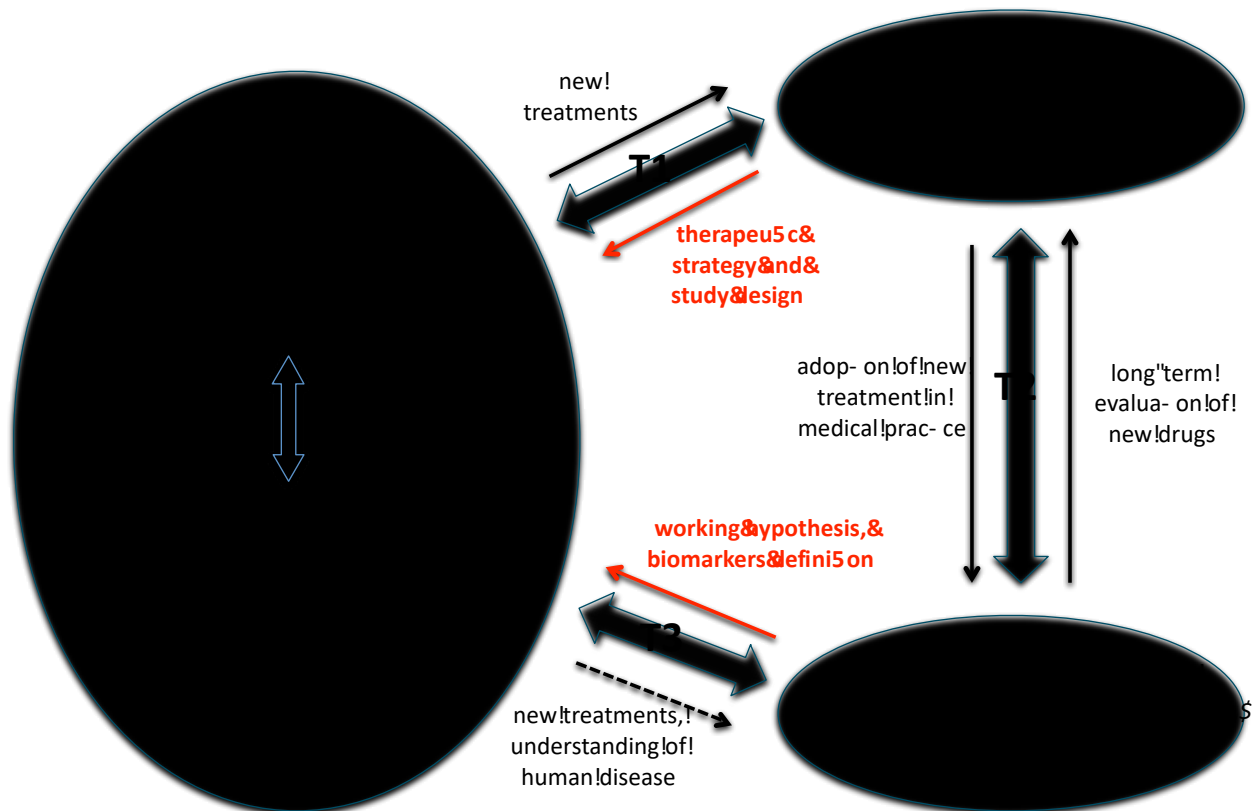


The application of nanotechnologies in the biomedical field, usually termed nanomedicines, has attracted a great deal of interest in the past decades due to its potential to revolutionize the treatment of severe diseases such as cancer, neurological disorders or cardiovascular pathologies<sup>1, 2</sup>. The early genesis of this concept sprang from the visionary idea that tiny nanorobots and related nanomachines could be designed, manufactured and introduced into the human body, to perform cellular repairs at the molecular level<sup>3</sup>. However, nanomedicines are nowadays defined as nano-scale tools (e.g. 1-1000 nm sized) for the diagnosis, prevention and treatment of diseases<sup>4</sup>, as these nanovectors should be able to guide a bioactive molecule to its desired location of action, and/or control its release to ensure an optimal concentration at the therapeutic target over a desired time frame.

Thanks to their nanometric scale, preparations with nanomaterials display physical and chemical properties that dramatically differ from those of bulk material<sup>5</sup>, together with differences in physiological interactions. Hence, nanometer-sized drug carriers including polymer-drug conjugates, dendrimers, liposomes, polymer micelles and nanoparticles have been extensively investigated for drug delivery, especially in the field of innovative cancer chemotherapies<sup>6</sup>. Lipid-based and polymeric materials are the only drug vehicles approved for clinical use and constitute the majority of nanocarriers used so far, because of their targetability, their ability to degrade under specific stimuli, and their capacity to carry a large amount of drug, thus improving the therapeutic index of the transported molecule<sup>7</sup>. Hence, nanotechnologies appear as a promising alternative to overcome the problem of the administration of new drugs coming out of the discovery pipeline<sup>8</sup>, such as proteins, peptides or siRNA, which are highly unstable in biological media, or poorly soluble molecules presenting a poor bioavailability when using traditional formulations<sup>9</sup>. Moreover, the field of nano-drug delivery has also attracted the attention of the pharmaceutical industry, because it offers a strategic tool to expand current drug market. Indeed, these new delivery technologies could repackage classic drugs, thus offering a competitive edge after the expiration of patents and preclude competition from generics<sup>10</sup>. However, despite improved pharmacokinetic properties and reductions of adverse side effects that allowed ca. 10 nanotherapeutics to reach the market<sup>6</sup>, nanocarriers for drug delivery have overall only achieved modest therapeutic benefits in humans<sup>5, 11</sup>. Hence, it is increasingly evident that many issues specific to nanoparticles exist compared to free drugs, and should be addressed in the futur<sup>4</sup>.

Translational research is a relatively new way to apprehend as a whole the development of innovative technologies (**Fig. 1**), encompassing multiple disciplines including basic research, drug development, proof-of-concept studies, physiology, physiopathology and clinical research<sup>12</sup>. It aims to optimize drug development process by a detailed understanding of why drugs fail in the clinic and to avoid misleading strategies. This should lead to a new organization of drug discovery research thanks to a more collaborative approach in which multiple stakeholders, basic scientists, clinicians, small and medium sized enterprises and Big Pharma interact, with the common objective of patient healthcare<sup>13</sup>. Hence, not only the way of

thinking research will be modified, but also the way of doing research will evolve. As for thinking, the researcher will have to early evaluate the translational potency of his project in regard with biological rationale of hypothesis and clinical settings. As for practice, translational concerns will define the way conceptual studies are run. For example, interest should be taken in the manufacturing and characterization steps that should be capable of Good Manufacturing Practice, in the choice of experimental models and administration strategies that should be in agreement with the clinical situation, and in the definition of end-points and biomarkers used to monitor safety and/or efficacy.



**Figure 1: Translational research framework.** This framework is organized around three main translational axes allowing the bench to bedside and back to bench strategy. The first axis (T1) corresponds to the constant exchange between preclinical researchers and clinicians involved in clinical trials. This should allow to bring new treatments to the clinic and also to give a feedback on clinical settings to help defining pre-clinical therapeutic strategies and study design. The second axis (T2) involves the passage from clinical trials to populations allowing the long-term evaluation of drugs and the faster adoption of new treatments in medical practice. Finally, the third axis (T3) between populations and pre-clinical researchers will help to better understand human diseases and improve working hypothesis and biomarkers definition. As for the actors, preclinical researchers will have to get their early work properly funded thanks to industrial collaborations or governmental funding, but also accept to be involved in clinical trials and regulatory processes. Industrial collaborators will have to allow publications, manage room for innovative research and equitably share financial and societal rewards. Regulatory agencies will also have to be involved in the proper definition of their expectations.

Disorders of the central nervous system (CNS) represent the largest area of unmet medical need, with more than 1.5 billion people affected worldwide, and a massive market of more than \$100 billion<sup>14</sup>. Despite this sector having the highest potential for growth due to population aging, many companies are moving away from it, largely because of the high risk of failure associated with CNS medicines, and the longer approval time<sup>15</sup>. A major contributing factor for the poor translation of neuroscience research into medicines is the high degree of complexity of the human CNS, and the presence of the blood-brain barrier (BBB), which hinders many biologically active molecules to reach the brain. Hence, a translational approach of CNS pathologies, applying the bench to bedside and back to bench principle, could allow a more integrative understanding of the diseases thus allowing a more rational and successful approach.

Combining the use of nanomedicines and the treatment of CNS disorders appears to be an exciting challenge for the researcher who could use a concrete framework of translational research to run his research. We aim in this review to outline some translational key points that should be addressed at the academic bench when developing nanomedicines for CNS diseases, and to give a concrete example of the development of nanomedicines for the treatment of stroke.

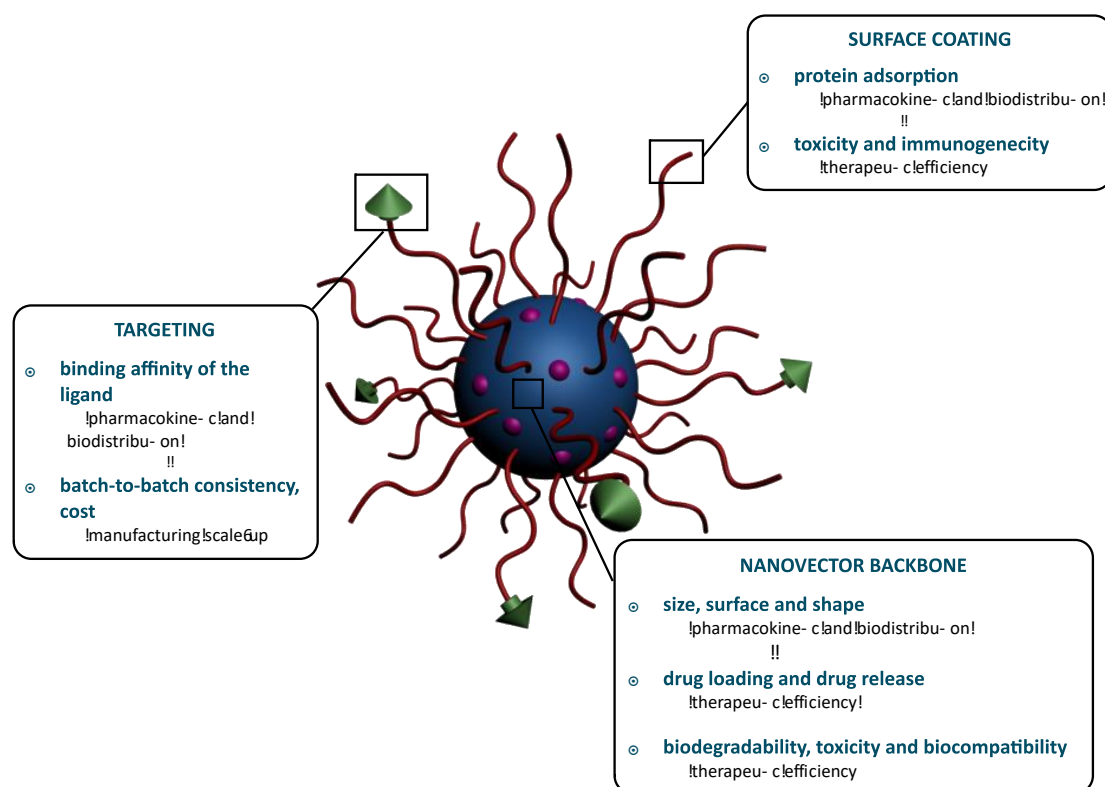
## **Translational research and nanomedicines design**

Given their inherent applied character, each specific nanomedicines has to be designed with respect to its proposed clinical use<sup>4</sup>, but also regarding material biocompatibility and possibility to establish scaling up production using good manufacturing processes<sup>16</sup>. These concerns should be addressed as soon as possible in the applied-basic research phase. Indeed, although a nanomedicines will never be useful without a strong proof of concept showing therapeutic efficiency, its inability to pass the desired pharmacokinetic, safety, and product manufacturing checkpoints may render its interesting pharmacological properties not applicable. There is currently a consensus amongst various regulatory agencies that the current regulatory guidelines for drug development could also be applied to nanoformulations<sup>17</sup>. However, it is increasingly evident that many issues specific to nanoparticles exist and will have to be addressed.

Most of the nanomaterial delivery systems that have moved into clinical trials have focused on targeting nanoparticles for cancer treatment, in order to increase the concentration of drug at the site of interest while reducing systemic side effects and toxicity<sup>18</sup>. However, therapeutic areas of applications for nanotechnologies are much wider, including among others dermal wound healing<sup>19</sup>, ocular diseases<sup>20</sup>, infectious diseases<sup>21</sup>, metabolic disorders, vaccination or neurological diseases<sup>22, 23</sup>. The understanding of the physiopathology of the target disease will help for the determination of the route and the frequency of administration. Nevertheless, the passage of the different physiological barriers to reach the systemic

circulation without losing the nanostructure's integrity remains challenging. If, in addition, the goal is to further cross the BBB in order to treat chronic neurological diseases, it seems fairly impossible that the drug will reach its target in a pharmaceutical relevant amount. That is why, so far, research has been mostly focused on intravenous administration of nanomedicines to treat acute pathologies requiring one or few administrations<sup>24</sup>. The use of nanotechnologies has also been intended to alleviate administration schedules thanks to a prolonged systemic circulation of the drug. Hence, when required, repeated administrations should allow the complete elimination of the nanocarrier in order to avoid any tissue accumulation and resulting toxicity<sup>25</sup>, as some nanomedicines demonstrated persistent in vivo deposits for months or years<sup>24</sup>. Finally, contrary to free drugs that are able to exert their pharmacological activity as soon as they have reached their biological target, nanomedicines have to release the active drug before providing any therapeutic effect. This delay in the pharmacological efficiency due to the presence of the nanostructure has to be taken into account and may be possibly tuned during rational design to reach pathology requirement. Indeed, a slow and sustained release of the drug is highly valuable for pathologies necessitating a chronic and long-term action, but may be less favorable for treatments requiring acute and extremely fast effect.

A nanomedicine is composed of a backbone, surface coating moiety and targeting ligand that are tunable and will together influence pharmacokinetic, biodistribution profiles and ultimately pharmacological efficiency, but also toxicity (Fig. 2).



**Figure 2: Different components of a nanomedicine and how they can influence its physiological behavior and translatability.** The most recent and sophisticated nanomedicines are composed of a nanocarrier backbone

incorporating the drug, covalently coated with a polymeric stabilizer and bearing a targeting moiety. Each of these components is susceptible to influence the pharmacokinetic and biodistribution profile of the nanomedicines, its therapeutic efficacy and its scale-up/manufacturing ability, and may be rationally designed during early development.

*(Courtesy of Dr Julien Nicolas)*

- *Nanovector backbone*

Nanomedicines as drug delivery systems can be formulated from diverse materials with unique architectures<sup>24</sup>. Wide ranges of structures have been proposed in the last decade including polymer-based colloidal systems (mainly nanoparticles, polymeric micelles, dendrimers and polymersomes) and lipid-based nanocarriers such as solid-lipid nanoparticles or liposomes<sup>26</sup>. So far, polymeric nanoparticles, liposomes and emulsions found themselves to be the most advanced regarding clinical development<sup>24</sup>. In particular, liposomes were the first nanomedicines to reach the clinic with the FDA approval of DOXIL<sup>®</sup>, and poly(alkyl cyanoacrylate) (PACA)-based nanoparticles (LIVATAG<sup>®</sup>) recently entered a phase III clinical trial with a FDA «Fast Track» status. However, dendrimers, carbon nanotubes, silica and metal-based nanoparticles are also subjects of many pre-clinical studies. The physical attributes of the different types of nanocarriers, including their size and shape, will first govern their pharmacokinetic and biodistribution profiles<sup>27</sup>, but also the biological transport process down to the cellular and sub-cellular levels<sup>28</sup>. Polymeric nanoparticles have received great interest due to the versatility of the available structures<sup>29</sup>, as it enables the selection of the chemistry of the building blocks to control the self-assembly process and the resultant stability, pharmacokinetics and intracellular fate<sup>30, 31</sup>. The surface properties (degree of hydrophobicity and charge) will further influence protein adsorption and drug desorption during circulation, a factor that has been shown to also dramatically influence the pharmacokinetic and biodistribution of the active molecule, by either promoting reticulo-endothelial system (RES) clearance or enhancing targeting. Recently, mathematical models have been developed in order to predict cellular interactions according to surface physico-chemistry properties and the resulting protein corona fingerprint<sup>32</sup>, opening the way of rational tuning of the nature of the nanocarrier backbone to induce a preferential adsorption of certain proteins and promote organ targeting<sup>33, 34</sup>.

The physico-chemical design of the nanostructure will also influence drug loading<sup>35</sup> and drug release capabilities<sup>36</sup>, which will both further govern pharmacological efficiency of the nanomedicines. For example, despite the fact that liposomes became the first nanomedicines to reach the bedside with the FDA approval of DOXIL<sup>®</sup>, this platform suffers from a lack of significant release and a narrow range of drug payloads<sup>37</sup>. Physical encapsulation allows controlling the final drug loading (ie. the ratio of the mass of active drug to the total mass of the nanovector) by choosing the physico-chemical properties of both the drug and the material used to form the nanocarrier backbone. As an example, liposomal and polymeric-based



nanoformulation of doxorubicin usually present a drug loading of ca. 10%<sup>38-40</sup>, or even often below. This payload may be increased up to 15% by using a remote loading via ammonium sulfate gradient, up to 75% by using a mixture of two block copolymers poly(L-histidine)-b-PEG and poly(L-lactic acid)-b-PEG<sup>41</sup>. When using a prodrug strategy before forming the nanoparticles, the drug loading will depend on both the molecular weight of the drug and that of the carrier material. Hence, using the squalenylation platform<sup>42</sup>, it was possible to reach significant drug-loadings of 57% for doxorubicin<sup>43</sup>, and 69% for paclitaxel<sup>44</sup>. Minimum threshold of drug payload may be estimated theoretically if the pharmacological action of the drug is already documented according to the targeted pathology, and hence it will help to guide the choice of the nanocarrier backbone to be used. The encapsulation strategy seems to be more adapted for pathologies requiring an easy and fast release mechanism, when the covalent linkage may be more suitable for long-term targeted release strategies. Finally, the control of the drug release has been recently addressed thanks to stimuli-responsive delivery<sup>45</sup>, with the development of on-demand processes, which aim to allow spatial, temporal, and dosage control of the release profile. These systems may be responsive to internal stimuli such as pH, redox or enzymatic activity, or to external ones such as temperature, magnetic field, light or ultrasounds. These functionalities have already been designed both in liposomal or polymeric nanoformulations, and can hence be tuned according to the desired feature when choosing the nature of the nanocarrier backbone.

While designing the nanocarrier backbone, biodegradability, toxicity and biocompatibility of the nanostructure and degradation products should be addressed early in the developmental process<sup>46</sup>. Poly(lactic-co-glycolic acid) (PLGA), poly(glycolic acid) (PGA) and poly(lactic acid) (PLA) are the three main biodegradable and non toxic polymers approved by the FDA for specific medical applications<sup>47</sup>. However, these polymers biodegradation processes may reduce local pH and thus induce dose-limitations. The use of backbones made of physiological components such as albumin or lipoproteins has also triggered interest since in this case, the nanocarrier is automatically biodegradable and non-toxic.

- *Surface coating*

To improve the stability of the colloidal systems and increase circulation time, amphiphilic or hydrophilic polymers such as poloxamer 188, polyethylene glycol (PEG), or dextran may be adsorbed or grafted onto the particles, forming a repulsive layer that generates an effective prevention of flocculation. For example, the surfactant poloxamer 188, known as its brand name Pluronic F68<sup>®</sup>, is able to stabilize and confer stealth properties to PLGA nanoparticles, thanks to steric stabilization due to bouncy blocks that are presumed to form a brush like layer around the nanoparticles<sup>48</sup>. To be noted that the choice of the surfactant should be done carefully regarding the general therapeutic strategy, and especially the acceptable toxicity<sup>49</sup>. As a FDA approved material, PEG is the most popular hydrophilic polymer for the

modification of particulate surfaces in order to improve pharmacokinetic and pharmacodynamic properties of nanomedicines<sup>50</sup>, by reducing the interaction with plasma proteins and hence avoid recognition by the RES<sup>51</sup>. However, PEG itself presents the disadvantage of a non-biodegradable main chain that is directly eliminated through kidney (PEG < 30 kDa). Moreover, although PEG has been shown to reduce immunogenicity of certain macromolecules<sup>51</sup>, it has been observed that an anti-PEG immune response was triggered when PEGylated nanomedicines were repeatedly injected<sup>52-54</sup>, inducing the production of an anti-PEG IgM together with an accelerated blood clearance. Hence, it has been suggested that in a translational perspective, the use of PEGylation for nanomedicines should be restricted to a molecular weight that can enable renal elimination (PEG < 30 kDa), and also be limited to treatments for life-threatening diseases or requiring a single administration. When multiple administrations are needed, as the induction of an immune response may not be acceptable by regulatory agencies, one could consider the development of alternative strategies such as the use of polyglycerol<sup>55</sup>.

- *Targeting*

Targeting strategies have evolved together with the progresses of the nanomedicines field, leading to the apparition of different generation of nanoparticles, able to transport their drug payload to specific organs or tissue where the disease resides and even to deliver materials to the right cell in those tissues. Introducing a subcellular localization of the targeted drug within the cell also starts to be investigated<sup>56</sup>. Such active targeting requires the conjugation of receptor specific ligands at the surface of the nanoparticles. In this case, the binding affinity and specificity of the ligand have to be evaluated. As nanoparticles are usually large enough to engage in considerable non-specific surface interactions with cells<sup>57</sup>, the complete characterization of the targeting benefit may be challenging. Moreover, this kind of strategy may become an obstacle to manufacturing translation, as complex systems are usually expensive, and raise the analytical challenge of ensuring a consistent batch-to-batch specification. Consequently, the clinically approved nanotechnology products and the products currently under clinical development are relatively simple and generally lack active targeting<sup>8</sup>.

## **Toward industrial manufacturing of nanomedicines**

Early preclinical physicochemical characterization studies should include measurement of size/size distribution, shape, charge, surface chemistry and aggregation/agglomeration state, in the "as-dosed" form in biological media, as several of these parameters may vary with dispersion medium and microenvironment. Nanoparticles size characterization is often complicated by the polydispersity of the samples, but characterization using multiple methods such as transmission electronic microscopy (TEM)

and dynamic light scattering (DLS) can provide size information relevant to the therapeutic performance. TEM and cryogenic TEM (cryo-TEM) are also used to investigate nanoparticles shape and provide great details at the atomic scale, such as information about the granularity of the sample.

Having a complete knowledge of the composition, purity and stability of the sample is another critical point. Knowing how much active drug is encapsulated into the nanocarrier and how much is free is of great importance to avoid dosing miscalculation. Moreover, multifunctional nanoparticles including functionalized groups, coatings and targeting moieties have to be characterized much more rigorously, as these several parts must work in concert to achieve functionality. Hence, meaningful physicochemical characterization of such a multipart entity includes assessment of the individual part purity, the stoichiometry and connections between the parts, and the chemical stability of those connections<sup>58</sup>. By the same way, purification strategies intending to remove manufacturing components such as surfactants or targeting agents should be designed to be 100% effective, as these components may exhibit unwanted toxicity<sup>59</sup>. Finally, when it comes to nanotechnology-based platforms, the «process is the product», that is to say, if the process changes, whether due to alternate production schemes or as part of scale-up, it is likely that the finished product is going to change as well, and thus industrial translation will also imply to find bridging biological studies to establish pharmacological consistency between batches<sup>60</sup>.

In order for new nanomedicines to have a place in the pharmacy, their cost of manufacture must be affordable for national health providers. Currently, courses of drugs costing more than 10,000€ a year are considered too expensive, so the cost of the raw materials must be significantly less<sup>61</sup>. Hence, exotic drug-delivery devices made from synthetic nucleic acid or decorated with highly specific peptides as targeting moieties are unlikely to compete with cheaper materials, unless they offer very significant advantages. The issue of the cost is often interlinked with that of manufacturability. Indeed, multicomponent systems are usually expensive, and ensuring a consistent batch-to-batch specification is an analytical challenge: the more the nanosystem is complex, the more its industrial development will be difficult and thus its chances to reach the clinic will be low<sup>62</sup>. Several strategies can be employed to produce nanocarriers for drug delivery and the choice of the preparation method mainly depends on the employed material<sup>1</sup>, which will also influence the scale-up ability. The lipidic film rehydration method for liposomes, or the emulsion-solvent evaporation process/nanoprecipitation technic for polymeric nanoparticles are the most often described in the literature<sup>63</sup>. These methods have been shown to present scaling-up capabilities<sup>64</sup>. However, scaling-up may influence the characteristics of the resulting nanoparticles<sup>65</sup> and may face industrial challenges such as the impossibility to use toxic organic solvents during all manufacturing steps or the necessity of short-time processes.

## Translational research and CNS nanomedicines

Because of their complexity, our knowledge of most brain disorders is largely rudimentary and makes the therapeutic strategy definition as well as cellular and molecular target identification a real challenge<sup>14, 15</sup>. Given this inherent intricacy, the use of a single compound selectively modulating individual genes or molecular targets may not be sufficient to efficiently treat such multifaceted disorders<sup>66</sup>. Hence, the integrative comprehension of the whole pathophysiological cascade of the disease represents a starting point for the development of polytreatments, using several drugs each of them attacking specific aspects of the disease and specific symptoms. In this context, the use of delivery platforms such as nanomedicines constitutes a major step forward<sup>67</sup> with the ability to deliver multiple drugs in the cerebral parenchyma<sup>68, 69</sup>. One could even imagine using such nanovectors where both the drug and the carrier are neuroactive compounds. However, as for all neuroprotective drugs, the development of CNS targeted nanomedicines presents hurdles, such as the presence of the BBB, the possible apparition of side effects and the translation to the clinic<sup>14</sup>.

Crossing BBB represents a real challenge for CNS nanomedicines, as, unlike blood vessels in every other part of the body, the endothelial cells composing the walls of the brain vasculature form tight junctions through the interaction of cell adhesion molecules<sup>70</sup>. These joined endothelial cells, together with astrocytes, pericytes and macrophages, form a physical and enzymatical barrier, that prevents the passage of more than 98% of therapeutic drugs<sup>71</sup>. The paradox is that more than 99% of the global CNS drug development effort is devoted to CNS drug discovery, while less than 1% is devoted to CNS drug delivery<sup>72</sup>, restricting new CNS drugs only to lipid-soluble compounds with a molecular weight inferior to 500 Da. In this context, the selection of compounds or drug delivery systems with properties favorable for the transport of compounds from blood into the CNS has the potential to increase the chances of drug candidates to reach the market and to reduce the time to get there. Numerous nanosystems have been developed for CNS therapeutic applications<sup>73</sup> and a significant number has reached clinical trials<sup>74</sup>. In the most recent studies involving CNS nanomedicines, the main targeted pathology is the glioma<sup>75-78</sup>. However, numerous other applications are investigated such as the treatment of Alzheimer disease<sup>22, 79</sup>, stroke<sup>23</sup>, HIV<sup>80</sup> or epilepsy<sup>81</sup>. Nanoparticles are also considered as a mean to assess BBB permeability<sup>82</sup>. Most of these studies involve polymeric nanoparticles (PLGA NPs, PLA NPs, PACA NPs and PCL NPs) and include a surface coating with PEG or polysorbate 80. Noteworthy, the nature of the surfactant<sup>83</sup> and the density of the coverage<sup>84</sup> have been shown to strongly influence brain entry in in vitro models of BBB and in vivo. Moreover, in order to ensure drug delivery across the BBB, several targeting strategies have been developed such as the use of transferrin to target transferrin receptors<sup>69, 85</sup>, the use of Angiopep-2<sup>75</sup>, a recombinant peptide from human apoB100<sup>86</sup> or lactoferrin<sup>76</sup> to target lipoprotein receptors, or 2-deoxy-

D-glucose to target glucose receptors<sup>78</sup>. All these systems have demonstrated significant brain targeting in vivo and subsequent therapeutic efficacy, although limited. Indeed, the effective quantity of active drug found in the brain is always very low (< 1% of the injected dose), that is why the gain of targeting strategies to cross the BBB remains debatable regarding the manufacturing cost that they would imply.

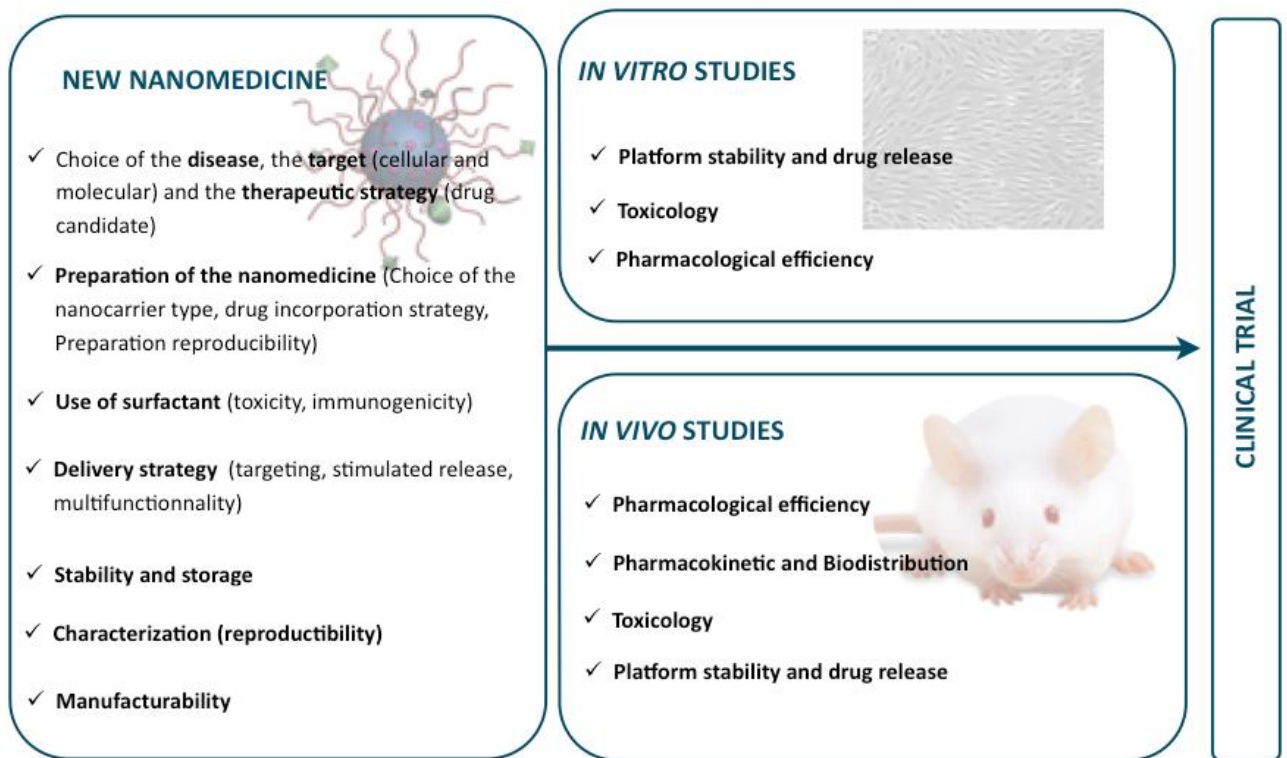
The comprehension of the cellular and molecular actors of the disease, and the interdependence between these actors should allow the development of therapeutic strategies with reduced side effects. Indeed, CNS-mediated side effects associated with off-target interactions and even with uncontrolled action at the target can lead to dramatic consequences<sup>14</sup>. As for cancer therapy, the reduction of side effects for CNS treatments is essential and could be achieved by the use of targeting strategy. However, where cancer therapy deals with two types of cells, one that should be killed (the cancerous cell) and one that should not be killed (the healthy cell), the CNS treatments have to deal with a possible action on all the cellular subtypes of the brain parenchyma (i.e. endothelial cells, astrocytes, pericytes, microglia and neurons), and with the fact that one single treatment could have synergic or opposite actions on two different cellular subtypes<sup>87</sup>. Moreover, the network structure and operating of the brain implies that it is often difficult to pharmacologically influence a single neuronal circuit or subcircuit.

Once it has moved from the bloodstream to CNS interstitial fluid, a neuroactive compound must be available to interact with its molecular target, which is usually an extracellular membrane receptor or transporter. Once again, this membrane site of the target poses a complete new perspective for the nanomedicines compared to cancer therapy. Indeed, in cancer treatment the molecular target is more often inside the cells<sup>88</sup> and the main challenge for a nanotechnology based therapy results from its 2R capability, ie. drug Retention in circulation versus its Release inside the cytoplasm of cancerous cells<sup>16</sup>. This allows the use of different triggered release strategies depending on biological differences between the intercellular fluid and the cytoplasmatic environment such as pH or enzymatic activity. In the case of CNS disorders, the nanomedicines should protect the drug during circulation into the blood stream but also during the passage of the endothelial cells of the blood-brain barrier and thus be resistant to any pH or enzymatic activity found in intracellular compartments. It should then ensure the release of the drug once in the interstitial fluid of the brain parenchyma to allow its interaction with the cell membrane target. Hence, one big challenge in translational development of nanomedicines for CNS application is the comprehensive understanding and control of the nanostructure integrity along the passage of the BBB, the determination of the different molecular species present in the different brain compartments, and the investigation of the release mechanism of the active drug in the interstitial fluid. The key point will then be the establishment of a pharmacokinetic-pharmacodynamic (PK-PD) relationship for the neuroactive compound<sup>89</sup>. Such kind of relationship is essential to link preclinical and clinical data, as the PK-PD profile

in the blood and brain interstitial fluid (ISF) of experimental animals can be used together with the PK profile in human blood to infer the concentration of the drug in human ISF. This will allow the determination of clinical starting doses ensuring a sufficient drug exposure to trigger a pharmacological activity devoid of side effects.

As for the development of any drug, an important component of CNS translational research is the use of experimental models and appropriate biomarkers<sup>90</sup>. Key requirements for experimental preclinical models are strong phenomenological similarities (face validity), similar physiopathology (construct validity) and accurate prediction of the therapeutic efficacy in humans (predictive validity). Predictive validity and the establishment of relevant biomarkers in animals and human are challenging goals that have not been reached yet for many CNS drugs.

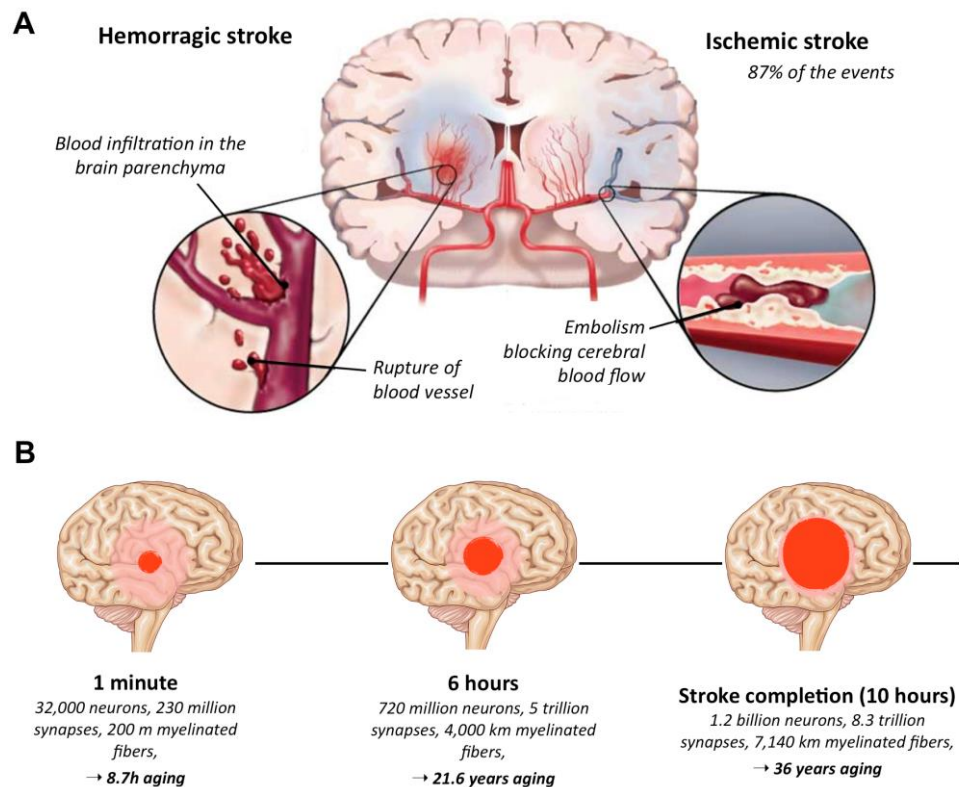
**Figure 3** aims to summarize the different points discussed previously and which could help to rationalize the development of new translatable nanomedicines for the treatment of CNS diseases.



leading cause of death in developed countries and a real public health concern<sup>91</sup>. Among 4,000,000 people experiencing a new or recurrent stroke each year worldwide, 19% will die in short terms after the event, and without treatment more than 30% will remain moderately or severely disabled at 3 months. Being the main cause of long-term disability in most industrialized countries, stroke also represents an economic burden, as its total cost in the United States is estimated at \$36.5 billion per year, while the indirect costs from lost productivity and other factors are estimated at \$15 million per year.

Although these statistics are staggering, stroke is highly preventable. Indeed, modifiable cardiovascular risk factors account for much of the proportional risk of stroke, and it is estimated that up to 80% of strokes may be prevented<sup>92</sup>. In particular, hypertension is the most important modifiable risk factor for stroke. Smoking, diabetes, alcohol consumption, overweight and obesity, cardiovascular diseases such as atherosclerosis, and lifestyle factors also increase risks for occurrence of a first event.

Among all stroke cases, more than 87% result from a blood clot in an artery, and are called ischemic strokes<sup>91</sup>. An ischemic stroke (**Fig. 4A**) induces a transient or permanent reduction in cerebral blood flow, caused in most cases by an embolus or by local thrombosis, which is restricted to the territory of a major brain artery. To date, the integrative comprehension of stroke pathobiology mainly relies on the observation of neuroprotective or deleterious effects of different treatments, and on the use of transgenic animal allowing protein over-expression or deletion. It is now widely accepted that ischemic brain injury results from a complex sequence of pathophysiological events that evolves over time and space<sup>93</sup>, leading to the notion that for a stroke patient, «Time is Brain-Quantified»<sup>94</sup> (**Fig. 4B**). Indeed, after the completion of a stroke, it is estimated that the patient will have lost 1.2 billion neurons, 8.3 trillion synapses and 7,140 km of myelinated fibers, corresponding to the equivalent loss of 36 years of normal aging.



**Figure 4: Physiopathology of stroke and its evolution with time.** Stroke can be either hemorrhagic or ischemic. The latter represents 87% of the events and corresponds to a transient or permanent reduction in cerebral blood flow, caused in most cases by an embolus or by local thrombosis (A). Due to a rapid evolution of the ischemic area and the dramatic loss that it implies in term of neurons, synapses and myelinated fibers, it is widely accepted that for a stroke patient, «time is brain quantified»<sup>125</sup> (B). (This figure was produced using Servier Medical Art, courtesy of Dr Ebeline Bihel (A))

Thanks to prospective randomized clinical trials and other studies performed over the past decade, the general approach to the management of acute stroke has evolved from nihilism to active intervention. Previous management of stroke was mainly based on natural recovery capabilities of the brain, as patients that have experienced a stroke often exhibit continued functional recovery for many years following their initial injury. Natural recoveries include varying degrees of behavioral compensation and cerebral plasticity<sup>95</sup>, neurogenesis<sup>96</sup> and to a less clear extend, angiogenesis<sup>97</sup>. Nevertheless, post-stroke capacities are unlikely to be identical to pre-stroke ones owing to the loss of neurons that present specific functions. This leads to chronic disabilities which severity is directly linked to the volume of the infarct<sup>98</sup> and infarct localization, raising the urgent need for therapeutic intervention.

The current gold standard treatment for stroke patients is thrombolysis, via the use of recombinant tissue plasminogen activator or rt-PA. The aim of thrombolytic therapy is to lyse the occluding thrombus or embolus by disruption of fibrin, usually by enhancing the endogenous formation of plasmin from plasminogen, and thus reducing the volume of cerebral tissue irreversibly damaged<sup>99</sup>. When given to stroke victims within three hours of blockage caused by a clot, at a dose of 0.9 mg/kg, the rt-PA increased the proportion of patients free of disability at three months by 30 to 35%<sup>100</sup>, and this benefit was sustained to one year after the event<sup>101</sup>. More



recently, the time-window guideline of rt-PA administration has been liberalized to 4.5 hours, primarily because very few patients were able to present for treatment within 3 hours<sup>102, 103</sup>. Nevertheless, rt-PA is still given to less than 5% of stroke patients<sup>104</sup>, showing a lag between positive clinical trials results and routine medical practice. This attitude stems, at least partly, from concern over a 10-fold increased risk of intracerebral hemorrhage in patients receiving the thrombolytic agent<sup>105</sup>, and a neurotoxic potential within the parenchyma if the molecule is not confined to the vasculature<sup>106</sup>. Finally, the effectiveness of intravenous rt-PA may be poor in certain type of occlusion, and early reocclusion occurs in 34% of rt-PA-treated patients, leading to neurologic deterioration and higher in-hospital mortality<sup>107</sup>. Therefore, research currently focuses on different therapeutic approaches for acute ischemic stroke such as the use of ultrasounds<sup>108</sup>, possibly with concomitant administration of gaseous microbubbles or lipid microspheres<sup>109</sup> to enhance thrombolytics activity, or the use of devices designed specifically for the cerebral vasculature allowing endovascular angioplasty, stenting or mechanical clot retrieval. Given the advance of neuroendovascular techniques in parallel with the development of new devices, several neurothrombectomy strategies are now available, three of which having received clearance from the FDA to treat patients with acute ischemic stroke<sup>110</sup>.

Neuroprotective therapies aim at structurally preserve the intact tissue until adequate blood supply can be reestablished, either through spontaneous or therapeutic recanalization, or via collateral blood flow. The elucidation of biochemical and molecular events and the identification of intracellular mediators of the ischemic injury allowed the development of pathophysiological rational for extended classes of neuroprotectants<sup>111, 112</sup>. Most of these strategies tend to: (i) decrease neuroexcitotoxicity using calcium channels blockers, glutamate antagonists or GABA agonists, (ii) to decrease oxidative stress with anti-oxidants and NO down-regulatory molecules, (iii) to decrease the inflammatory response, (iv) to avoid the no-reflow phenomenon by inhibiting leukocytes and neutrophils adhesion and activation, and (v) to inhibit apoptosis pathways with caspase-3 inhibitors and modulation of the Bcl-2 family. Other neuroprotective strategies include neural stem cells therapy, exploiting the ability of these cells to differentiate and regenerate in response to both internal and external stimuli, and thus being possibly able to restore loss of brain functions<sup>113</sup>. Finally, pre-clinical studies have shown the protective effects of therapeutic hypothermia in models of focal ischemia, and early-phase feasibility studies recently showed its safety and efficacy in patients with acute ischemic stroke<sup>114</sup>. However, amongst 400 neuroprotective drugs sent to clinical trials, none ever demonstrated real pharmacological efficacy in man. These neuroprotective strategies consisting in a single target therapy suffer from the fact that modulation of one pathway alone may not be sufficient to tip the balance of molecular activity in an extremely complex injury<sup>115</sup>. Thus, the use of multitargeted pleiotropic molecules able to modulate several pathways in all cells of the neurovascular unit may be an interesting strategy for the treatment of acute ischemic injury<sup>66,</sup>

<sup>116</sup>.

## Stroke translational research

As a matter of fact, despite the idea of protecting brain tissue from injury using neuroprotective agents

has been investigated for years and appear quite effective in preclinical studies, none of these have proven conclusively to be effective in humans<sup>111</sup>. In this context, to overcome barriers in the translation of animal studies to human clinical trials, the Stroke Therapy Academic Industry Round Table (STAIR) has published recommendations<sup>117, 118</sup> for ways to improve preclinical development of neuroprotective and restorative drugs in acute ischemic stroke. In a translational perspective, these recommendations directly stem from the clinical status and management of stroke patients.

- *Animal models*

The number and diversity of experimental focal ischemia models have increased over the recent decades and animal studies have provided most of the knowledge on pathophysiological mechanisms of stroke. There are numerous models of focal ischemic stroke involving both permanent and transient techniques, for small and large animals. No ideal standard model has yet been established and there will certainly not be since human stroke itself is a highly diverse condition<sup>119</sup>. However, human ischemic stroke usually results from middle cerebral artery occlusion (MCAo), and so techniques that occlude this artery are considered as closest to the clinical picture<sup>120</sup>. The most commonly used model is the intra-luminal suture model<sup>121</sup>, which involves the introduction of a surgical filament via the internal carotid artery to occlude the MCA at its origin. Several alternatives have been proposed in the past decades such as the thromboembolic model, the endothelin-1 model, the electrocoagulation model or the photothrombosis model. Hence, the first aspect to consider in the choice of an experimental model is «how much does the model match with the clinical problem that will be taken under investigation (according to the putative drug mechanism), including invasiveness, occlusion site, reperfusion possibility and lesion variability?». Preclinical studies are generally first conducted in small animals (mouse and rat), which are less expensive, have easier husbandry and handling conditions, and are more ethically acceptable compared to larger species, while being close enough to human in term of phylogenetic. Rats are usually preferred as their cerebrovascular anatomy and physiology offers good similarity to that of humans<sup>122</sup>, when mouse models are usually kept for transgenic approaches. However, it is highly recommended to confirm positive results from a small animal study in higher species such as cats or primate prior to clinical evaluation in humans<sup>117</sup>, as these species offer better similarity to humans in term of behavior and sensorimotor integration, but also concerning cerebral anatomy and organization as represented by the ratio of neocortex to basal ganglia and the volume of white matter. The most salient discrepancy between human clinical stroke and animal models is that experimental ischemia is usually induced in otherwise young and healthy animals, whereas human stroke is most of the time occurring in the context of aging and underlying diseases or risk factors, with the use of concomitant medication<sup>123</sup>. So far, studies have shown that standard models can be performed in middle-aged rats<sup>124, 125</sup> and species as Fischer 344 are available for studies in very

old animals. Gender differences may also influence the effect of both stroke mechanisms and responses to therapy, therefore, it is important to examine neuroprotective drugs in both male and female animals, separately<sup>126</sup>.

- *Drug administration*

In numerous studies the drug is given before the onset of the ischemia, or at the time of reperfusion, with little attention given to administration at various times after reperfusion. In humans, however, given the fact that the time of stroke onset is rarely precisely known and that the patient undergoes several diagnosis tests, pharmacological agents are not routinely administered until hours after the beginning of the ischemic event. Thus, a careful assessment of the therapeutic window, that is the interval after the onset of ischemia or reperfusion when the drug can be administered with successful outcome, should be determined before performing expensive clinical trials. For this purpose, rodent studies have appeared to be relevant to address a therapeutic window in humans for both thrombolytic and neuroprotective drugs. The route of administration should be carefully considered too in evaluating a stroke recovery drug. In the case of acute neuroprotective drugs, the administration will likely occur at the hospital, with the assistance of clinical staff. Hence, the semi-invasive administration route which is the intravenous route is very likely appropriate, avoiding the difficulty of the passage of the gastro-intestinal barrier. To circumvent the presence of the BBB, one could consider the intrathecal route that may be performed simultaneously with a lumbar puncture. However, more invasive ways of administration such as intracisternal, intraventricular or intracerebral injections are not likely feasible approaches for clinical development. As for recovery drugs, which will have to be taken for a longer time period, the oral route should be favored in order to increase the patient compliance.

- *Therapeutic outcome evaluation*

One of the main hurdle for clinical translation remains that endpoints are imperfectly defined both in animal models and in humans. The major preclinical outcome measures for stroke pharmacotherapy studies are final infarct size and neurological deficit. The latter is the more clinically relevant but also the more challenging to accurately assess in animal models. It is recommended that at least these two outcome measures should be reported during preclinical studies, and that they should be monitored during the acute phase (1 to 3 days) but also for longer time periods (7 to 30 days) to demonstrate a sustained benefit of the drug<sup>117</sup>.

- *Experiment quality*

Because of the frustrations engendered by the failure of translation of apparently efficacious animal neuroprotectants into human stroke therapies, there is a need to improve the quality of preclinical stroke studies<sup>127, 128</sup>. Indeed, systematic review and meta-analysis of the data from 13 putative neuroprotectants revealed that the presence or absence of randomization to a treatment group, blinding to drug administration during stroke induction, and blinding for outcome assessments were among the most powerful determinants of outcome<sup>129</sup>. More and more importance should also be given to sample size calculation when it is possible to evaluate the magnitude of the effect expected following the drug administration. Inclusion and exclusion criteria should be clearly defined, before the beginning of the study, and all mortalities be reported. Finally, basic physiological parameters such as blood pressure, temperature, blood gases and blood glucose should be routinely monitored. Corporal and cerebral temperatures should be maintained within normal range to avoid variability due to hypo- or hyperthermia. It is also important to monitor the cerebral blood flow using Doppler flow or perfusion imaging to document adequate sustained occlusion and to monitor reperfusion in temporary ischemia models, when it is not visually accessible. Consistency and quality of pre-clinical trials will be necessary for selection of appropriate study design, therapeutic approaches and treatments, and to carry forward assessment in human population<sup>120</sup>.

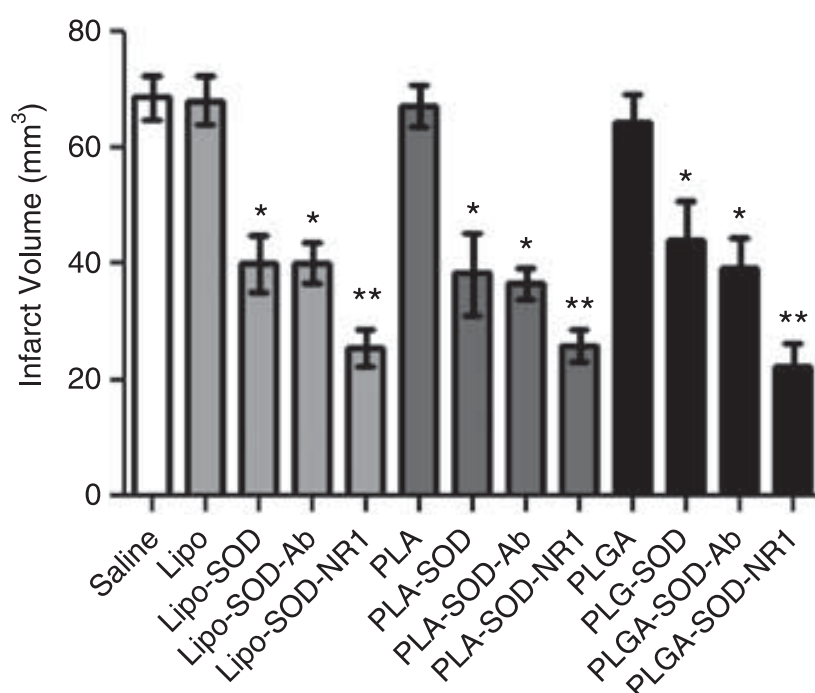
## **Translational research and CNS nanomedicines for stroke**

Given the repeated failures of classical drug formulations in clinical trials, the use of nanomedicines for the treatment of stroke has raised interest in the past decades<sup>67</sup>, and has been widely investigated for the delivery of active drugs, including growth factors<sup>68, 130</sup>, erythropoietin<sup>131</sup>, hemoglobin<sup>132</sup>, antioxidant molecules<sup>133</sup> or siRNA<sup>134</sup>. Platinum<sup>135</sup> and ceria<sup>136</sup> nanoparticles or carbon nanotubes<sup>137</sup> have also been investigated because of their free radical scavenger properties. The part of the present review will discuss - in terms of nanocarrier design and characterization, pre-clinical stroke model and experiment quality - the use of nanotechnologies for the treatment of cerebral ischemia (total 40 publications). The reader will find a complete comparison of these studies in Tables 1 and 2.

- *Nanocarrier design and characterization*

Out of 40 publications, 21 justify the use of nanocarriers to improve the entry of the transported drug into the brain, usually thanks to an improved bioavailability (increased blood half-life time and increase of BBB translocation). Only four papers intend a reduction of both the injected dose and the frequency of the

administration to avoid side effects and systemic toxicity. The remaining studies look at nanotechnologies to increase the drug oral bioavailability, to enhance the drug solubility, to transport a gas or to augment neuronal uptake. The most used nanosystems are liposomes and polymeric nanoparticles, especially PLGA nanoparticles. Their use is justified by their biodegradability, low toxicity and biocompatibility, and because they are well known drug carriers, which have previously been shown to effectively cross the BBB. Moreover, their scaling-up ability was already proven, especially in the case of liposomes. One investigation by Yun et al.<sup>23</sup> compared the pharmacological efficiency of three different types of nanocarriers (PEGylated liposomes, PLA NPs and PLGA NPs) encapsulating superoxide dismutase (SOD) as an antioxidant agent, with and without a targeting modality against NMDA receptor 1 (NR1). No significant differences were observed in term of infarct volume reduction between the different nanocarriers when the animals were injected with the same quantity of encapsulated drug and the nanovectors were functionalized with the same ligand (**Fig. 5**). Hence, the difference between these nanosystems may only arise from their drug loadings and encapsulation efficiencies, which will govern their relative systemic toxicity and manufacturability, together with their specific ADME profile. This illustrates that the pharmacological outcome may not be the only parameter involved when comparing the translatability of nanocarriers.



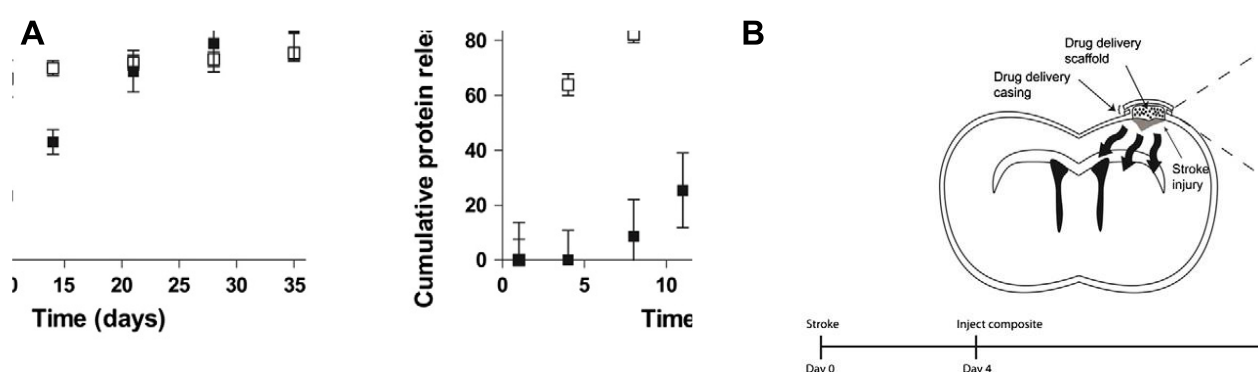
**Figure 5: Comparison of different nanocarriers incorporating the same drug on stroke outcome (Yun et al., reprinted by permission from Macmillan Publisher Ltd: *Journal of Cerebral Blood Flow and Metabolism*, 2013).**

Three types of nanocarriers (PEGylated liposomes, PLA NPs and PLGA NPs) incorporating superoxide dismutase (SOD) were tested in a 1 h ischemia, followed by 24 h of reperfusion in an intra-luminal suture model in mice. Treatments (containing an equal dose in SOD) were administered immediately after the onset of ischemia via an intra-carotid injection, and infarct volume was assessed at the end of the reperfusion period (24 h after ischemia). No significant differences were observed on infarct volume quantification between the different types of nanocarriers. This illustrates that the translatability difference between the three nanosystems will likely arise from their drug loadings and

encapsulation efficiencies, which will govern their systemic toxicity and manufacturability, rather than from their pharmacological efficiency.

Only few nanoformulations<sup>23, 138-140</sup> involved the covalent linkage between a drug and a component of the nanocarrier material. All other strategies used the encapsulation or the electric complexation of the drug. Hence the question of the drug loading and drug release should be addressed in order to properly characterize the nanosystem. However, these parameters are not frequently reported (only 10 times upon 40 publications concerning the drug loading and only 8 times concerning the drug release profile). Noteworthy, several drug release profiles have been realized in physiological conditions (in human plasma<sup>141</sup> or CSF<sup>68</sup> at 37°C), whereas the other were performed in PBS at 37°C. Encapsulation efficiency is more often reported and is usually high, ensuring a limited loss of raw material during the preparation process and thus allowing to consider the industrial scaling-up with reasonable cost.

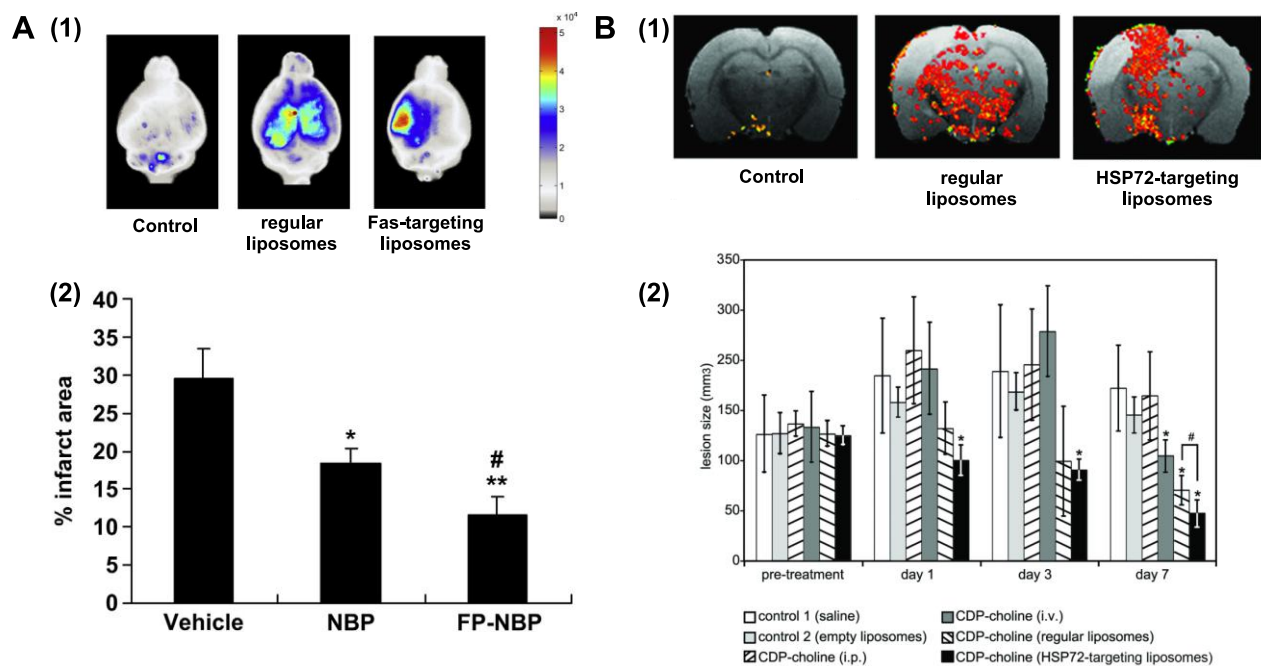
The study by Wang et al.<sup>68</sup> was the only one to report an original system including the delivery of two drugs, erythropoietin (EPO) and epidermal growth factor (EGF). In this formulation, EPO was encapsulated in PLGA NPs, which were then incorporated into bigger PLA microparticles (PLA MPs), whereas the EGF was also encapsulated in PLGA NPs but not incorporated into MPs. Both particulate formulations (EPO-PLGA NPs-PLA MPs and EGF-PLGA NPs) were then embedded together in a hyaluronan and methylcellulose (HAMC) hydrogel (**Fig 6A**). It allowed the sequential release in vitro and in vivo (**Fig 6B**) of first EGF, which promotes neurogenesis, followed by EPO, which prevents the apoptosis of the newly formed cells. Regarding the clinical application, the main obstacle results from the need to implant the device at the cortical surface (epicortical delivery) (**Fig 6A**) and from the short diffusion length of the proteins into the brain tissue (typically 1 mm), which limits the volume of impregnated brain tissue.



**Figure 6: Composite drug delivery system allowing the sequential release of EGF and EPO in vivo** (*reprinted from Journal of Controlled Release, 172, Wang Y. et al., "Bioengineering sequential growth factor delivery stimulates brain tissue regeneration after stroke", 1-11, 2013, with permission from Elsevier*). EPO was encapsulated in PLGA NPs, which were then incorporated in PLA microparticles (PLA MPs). PEGylated EGF (EGF-PEG) was encapsulated in PLGA NPs but without incorporation in microspheres. The two particulate formulations (EPO-PLGA NPs-PLA MPs and EGF-PEG-PLGA NPs) were then embedded together in a hyaluronan and methylcellulose (HAMC) hydrogel. For drug

delivery, the gel was implanted at the cortical surface (A). The in vivo drug release profile of EGF-PEG and EPO in the stroke-injured brain tissue (B) showed that EGF-PEG was released first, with the peak concentration detected after 1 week, followed by EPO accumulation over 25 days. This should allow EGF to promote neurogenesis, followed by EPO, which will prevent the apoptosis of the newly formed cells.

The design of the nanocarrier is in general relatively simple, as only few of them included a targeting moiety or a surface coating agent (mostly with PEG 2 kDa). Lu et al.<sup>142</sup> and Agulla et al.<sup>143</sup> clearly demonstrated the targeting ability of, respectively, surface functionalized solid lipid nanoparticles and liposomes respectively (**Fig. 7**). However, the resulting benefit on the infarct size when compared to the non-targeted nanocarriers was only ca. 10%, and may not be highly relevant with regard to the multiple steps of preparation and characterization required, and the cost that they imply. Nevertheless, in the case of the Agulla and collaborators' strategy<sup>143</sup>, the main goal was to develop a theranostic nanoformulation. In this case, even if the targeting moiety may not be profitable from the therapeutic point of view, it will definitely be essential concerning the diagnostic aspect.



**Figure 7: Targeting of the ischemic area ((A) reprinted from *Biomaterials*, 35, Lu Y.M. et al., "Targeted therapy of brain ischaemia using Fas ligand antibody conjugated PEG-lipid nanoparticles", 530-537, 2014, with permission from Elsevier and (B) reprinted with permission from *Theranostics*, 4, Agulla J. et al., "In vivo theranostics at the peri-infarct region in cerebral ischemia", 90-105, 2014 (B)).** The conjugation of Fas ligand (protein belonging to the tumor necrosis factor family which receptor is a member of the death receptor family) to the surface of PEGylated liposomes clearly induced the targeting of the ischemic area 24h after intravenous administration, compared to non-targeted liposomes (A - 1). When encapsulating Butyrylcholinesterase, these targeted liposomes significantly reduced the infarct size by ca. 8% compared to the non-targeted ones (A - 2). Similar results were obtained with liposomes targeted against HSP72, which is a member of the Heat Shock Proteins. Regular (non-targeted) liposomes appear to be

randomly distributed along both hemispheres when targeted liposomes concentrate in the ischemic hemisphere (B - 1). This targeting allowed a significant reduction of the infarct size compared to non-targeted liposomes, 7 days post-injury (B - 2). Noteworthy, in this study, the authors designed their targeting strategy by conducting a complete proteomic, immunoblot and immunohistological study, ensuring the relevance of the target with the pathological setting.

Finally, most of the investigations reported the use of only one characterization technique (DLS or microscopy), whereas few of them reported the use of both DLS and microscopy, usually TEM. Only four publications<sup>140, 144-146</sup> provided a complete description of the nanocarrier characteristics (size, surface charge and polydispersity) whereas a significant number of studies did not give any characterization parameters at all, without referring to any previous study where these parameters could be found. The lack of systematic and consistent characterization of the nanoformulations used represent for obstacle to further translation to higher animal species or humans, as the pharmacological efficiency of a nanomedicines is deeply linked to its physico-chemical properties.

**To summarize, among 40 publications, most of them used liposomes or polymeric nanoparticles (especially PLGA NPs) because of their biodegradability and biocompatibility. They aimed to increase the brain penetration of the drug, thanks to an improved bioavailability. Nanosystems were mostly simple as only few of them presented a PEG coating and/or a targeting moiety. Overall, the developed nanomedicines were rarely fully characterized with two different techniques (DLS and TEM), and usually drug loading and drug release profiles were not reported.**

- *Pre-clinical stroke study*

The majority of the pre-clinical studies were conducted in rats (Wistar or Sprague-Dawley strains), as only seven investigations were performed in mice, one in gerbil<sup>147</sup> and one in macaque<sup>148</sup>. This is consistent with the STAIR recommendations<sup>117</sup>, advising that in a first approach, the privileged rodent species should be the rat as it presents the highest similarity with human in term of cerebrovascular anatomy and physiology, the mice being preferred for transgenic approaches. The use of gerbil, however, has been completely unadvised given the fact that numerous molecules have shown a therapeutic effect in this species but not in others. The experimenters commonly reported the sex and the age of the animals. Noteworthy, only one experiment used female rats to assess the pharmacological efficacy of liposomal luetonin<sup>149</sup>. Since stroke fatality is higher in women than in men<sup>91</sup>, and because an interaction between female hormones and drug is often likely<sup>150</sup>, the use of female animals should be much more common. One group has been interested in the effect of aging and thus has compared the pharmacological



efficiency of their nanosystems in young (2 months) and old (20 months) rats<sup>151-153</sup>. Finally, as a comorbidity factor, only one study on liposomal citicoline assessed the effect in spontaneous hypertensive rats<sup>154</sup>.

Clearly, the more frequently employed experimental model was the intra-luminal suture model (or filament model) to induce a transient MCA occlusion of various durations (between 40 min and 2 h). Two studies performed occlusions of less than 1 h<sup>142, 149</sup>, when it has been shown that obtaining reproducible infarct volumes required at least 60 to 90 min of ischemia<sup>119</sup>. The variability in the occlusion times did not allow an easy comparison between the infarct size and the pharmacological efficacy of the different tested nanosystems. Six groups performed bilateral clamping of different time-length (5 min to 2 h), two others used the endothelin-1 model<sup>68, 155</sup> and one employed the thromboembolic approach with an autologous clot<sup>156</sup>.

Most of the papers assessed the pharmacological outcome with both functional outcome (using a general sensory-motor scale and/or other tests) and the measure of the infarct volume by histological staining (TTC, cresyl violet, Nissl staining, H&E staining) or imaging techniques (MRI, PET, CT). The functional outcome assessment was predominantly performed using general sensory-motor scales of various number of points (4 to 22 points), even if some studies used more specific tests such as the adhesive removal and the cylinder tests<sup>156</sup>, the beam-walking test<sup>141, 149</sup>, the open-field test<sup>145, 157</sup>, the grip strength test<sup>145</sup>, or the rotarod test<sup>131, 135, 137, 157, 158</sup>. Most of the functional outcome evaluations and infarct volume measurements were done 24 h post-ischemia, but eight groups performed long-term evaluations (up to 7 days, 14 days or 21 days post-ischemia) on functional outcome or on infarct evolution thanks to imaging techniques. Long-term studies allowed assessing the sustained benefit of the nanomedicines. For example, Reddy et al.<sup>133</sup> used PLGA NPs encapsulating SOD, and followed the neurological score and the infarct volume of the animals up to 28 days post-injury. In this investigation, even if the functional improvement was not important between 6 h and 28 days post-injury, more than 75% of the treated animals survived 28 days when 100% of control animals died within 3 days post-injury.

The routes of drug administration were also diverse. The majority of the treatments were administered intravenously, either via caudal injection, intra-femoral injection or intra-jugular injection. Two studies performed intra-carotid injections which ensure a direct access to the brain vasculature avoiding the first passage through the systemic circulation, and hence increasing brain capture as demonstrated by Reddy et al. with SOD-PLGA NPs. To be noted that intra-arterial injections are not likely used in clinic as they can lead to severe complications<sup>159</sup>. Few groups performed intra-peritoneal (IP) injections, which is usually not a privileged route for nanomedicines, as nanostructures hardly diffuse through peritoneum to reach systemic circulation in their intact form. One of these studies utilized a crystal nanodrug just to allow

better drug dissolution and improve administration<sup>147</sup>. Thus, in this case, the nanostructure was not kept intact into the blood circulation. Another experiment required IP injections since it involved neo-natal rats on which intravenous injections were technically difficult<sup>131</sup>. The use of intra-cortical (IC) injections mainly concerned nanocarriers transporting siRNA<sup>134, 155</sup>. Indeed, given the position of their molecular target inside the nucleus of the neuronal cells, the use of a nanovector was mandatory to improve cellular uptake. Hence, the IC injections were preferred, as it appeared difficult to simultaneously protect the siRNA into the circulation, cross the BBB and allow specific delivery inside the cytoplasm of the neurons.

As for the administration schedule, many groups injected the nanomedicines before inducing cerebral ischemia, rendering the translation to the clinic highly unlikely. Especially in the case of a treatment with siRNA, the administration was performed before the ischemic episode to give a chance to the gene to be silenced during a sufficient period of time in order to obtain a significant pharmacological effect. Indeed, it has been shown that the post-injury intra-cortical injection of carbone nanotubes complexing anti-caspase 3 siRNA was unable to reproduce the significant effect obtained when the injection was performed 24 h before inducing the infarction<sup>155</sup>. This result illustrates that the occurrence of a pharmacological effect with administration performed before injury does not systematically implies efficacy when the nanovector is given after injury. Numerous investigators also performed the treatment just after inducing the MCAo or at reperfusion, but only two studies established a real therapeutic window assessment. These reports investigated the use of liposomal FK506<sup>160</sup> (an immunosuppressor) and PBCA NPS loaded with SOD<sup>23</sup>. It was observed that the sooner the treatment, the better was the outcome whereas delayed administrations at 3 h or 24 h failed to significantly decrease the infarct volume. Hence, this kind of experiment is of primary importance to determine the optimal time period for patient treatment.

Finally, some studies included two or more drug dosages. In particular, a complete dose investigation by Kim et al.<sup>135</sup> using ceria nanoparticles emphasized the importance of this parameter. In this report, the minimal dose to observe a pharmacological efficiency (0.5 mg/kg) and the optimal dose (0.7 mg/kg) to reach the highest pharmacological efficacy were clearly identified.

**To summarize, among 40 publications, most of the investigators used the intra-luminal suture model in male rats to induce a transient MCA occlusion of various durations. The use of female animals or animals presenting comorbidities (age, hypertension) was rarely reported. Intravenous injection was the privileged route of administration. Half of the studies assessed the outcome with both functional evaluation (mostly using general sensory-motor scales of various point) and infarct volume measurement. Only few papers reported the long-term outcome evaluation, and even less assessed a therapeutic window and a dose-response profile of the nanomedicines.**

- *Experiment quality*

As for classical formulations, nanomedicines should be tested with great experiment quality. According to STAIR recommendations, randomization, blinding to treatment administration and outcome evaluation, and physiological monitoring are the four main requirements in order to validate a stroke pre-clinical study<sup>118</sup>. However, among 40 pre-clinical investigations, only 16 reported randomization, 12 reported blinding during outcome evaluation and only two recent studies by Agulla et al.<sup>140, 143</sup> reported blinding during treatment administration. Concerning physiological monitoring, the majority of the experiments included the monitoring and maintenance of corporal temperature at 37°C thanks to heating pad, but less comprised the complete monitoring of the animals (blood pressure, PaO<sub>2</sub>, PCO<sub>2</sub>, blood pH, blood glucose and heart rate). Finally, only few studies reported the measure of the cerebral blood flow to assess MCA occlusion, which is mandatory to ensure a quantitative reproducibility of the infarct induction between animals. **To summarize, randomization and blinding for outcome assessment were not systematically reported, when blinding to treatment administration almost never occurred. Complete physiological monitoring and assessment of occlusion measuring cerebral blood flow is rarely presented.**

## Conclusion

Nanomedicines represent highly promising opportunities for the treatment of CNS diseases and especially the treatment of stroke. They open the way of enhanced drug delivery to the brain and controlled release of one or several drugs into the brain parenchyma. As an emerging pharmaceutical field, translatability of nanodrug formulations will have to be assessed as soon as possible during design, characterization and development process. This is especially true in the case of CNS treatments, for which translation from bench to bedside is known to be a real challenge. Recent pre-clinical studies using nanotechnologies for the treatment of stroke illustrate the lack of proper characterization of the used nanosystems in terms of pharmaco-chemical properties and pharmacological efficacy. Hence, although drug loaded nanoformulations are expected to have a huge impact in the treatment of neurological disorders, a great deal of effort is still required in the design of experiments ensuring the demonstration of their ability to reach the clinic.

**Table 1: Comparison of studies using nanotechnologies for the treatment of cerebral ischemia in vivo in terms of nanocarrier design and characterization**

(NS: Not Specified ; PEG: poly-ethylenglycol ; DLS: dynamic light scattering ; TEM: transmission electronic microscopy)

Reference	Drug and therapeutic rational	Nanocarrier material	Rational to use nanoformulation	Surface coating & targeting	Preparation method & drug incorporation	Characterization methods (size, ZP, Pdl)	EE & DL	Nanostructure stability	Drug release pattern
143	Citicoline (protects against cell membrane damage)	liposome	to improve brain penetration	PEG (2000) Anti-HSP72 Ab	lipid film rehydration encapsulation	DLS, cryo-TEM (100 nm, NS, NS)	NS 8%	stable for 4 days when kept at 4°C	NS
160	FK506 (immunosuppressant)	liposome	to improve brain penetration and reduce frequency of administration	PEG (2000) No	freeze-drying method encapsulation	NS (110 nm, -7 mV, NS)	NS	NS	NS
138	Asialo-EPO (enhance oxygen delivery and promote cell survival without hematopoietic toxicity)	liposome	to increase blood half lifetime, reduce frequency of administration and injected dose	PEG (2000) No	lipid film rehydration formation of DSPE-PEG-AEPO conjugate	NS	NS	NS	NS
156	Dexamethasone (decrease secondary risks of rtPA, multiple mechanisms)	liposome	to improve brain penetration and reduce injected dose	PEG (2000) No	lipid film rehydration encapsulation	DLS (100 nm, NS, <0.1)	NS	NS	NS
130	VEGF gene (multiple mechanisms)	liposome	to improve brain penetration	PEG (2000) Transferrin	lipid film rehydration followed by freeze-drying encapsulation	DLS, TEM (100 nm, 30 mV, NS)	86% NS	stable for 1h in PBS and PBS+FBS at 37°C	NS
149	Luteolin (multiple mechanisms)	liposome	to improve brain penetration	No No	lipid film rehydration encapsulation	DLS (150 nm, NS, NS)	98% NS	NS	NS
161	Citicoline	liposome	to improve brain penetration	PEG (NS) No	lipid film rehydration	DLS (100 nm, NS, NS)	NS 8%	NS	NS

	<i>(protects against cell membrane damage)</i>				encapsulation				
<b>141</b>	Xenon <i>(NMDA receptor antagonist)</i>	liposome	to transport and deliver xenon	No No	lipid film rehydration followed by pressurization freeze method encapsulation	NS	NS	NS	release profil in PBS and human plasma at 37°C for 18h, w and w/o US
<b>151</b>	Antisens oligonucleotide against synaptotagim I <i>(reduce glutamate release)</i>	liposome	to enhance neuronal uptake	No Hemagglutinating virus of Japan	lipid film rehydration encapsulation	NS	NS	NS	NS
<b>148 – 132 - 162</b>	Hemoglobin <i>(oxygen delivery)</i>	liposome	to transport and deliver oxygen	PEG (5000) No	lipid film rehydration encapsulation	NS (230 nm, NS, NS)	NS	NS	NS
<b>163</b>	Quercetin <i>(free radical scavenger)</i>	liposome	to improve brain penetration and oral bioavailability	No Mannoside	lipid film rehydration encapsulation	Light micrometry (50 nm, NS, NS)	NS	NS	NS
<b>154</b>	Citicoline <i>(protects against cell membrane damage)</i>	liposome	to increase blood half lifetime and improve brain penetration	No No	lipid film rehydration followed by freeze-drying encapsulation	DLS NS	23% NS	NS	NS
<b>152</b>	Quercetin <i>(free radical scavenger)</i>	PLGA NPs	to improve brain penetration and oral bioavailability	No No	emulsion-diffusion-evaporation encapsulation	TEM, AFM (20-50 nm, NS, NS)	NS 80%	NS	NS
<b>164</b>	Triiodothyronine <i>(multiple mechanisms)</i>	PLGA NPs	to improve brain penetration	PEG (NS) glutathione	nanoprecipitation encapsulation	DLS, TEM (327 nm, -2 mV, < 1)	NS	NS	NS
<b>165</b>	Puerarin <i>(multiple mechanisms)</i>	PLGA NPs	to improve brain penetration	No No	double emulsion solvent evaporation	DLS, SEM (165 nm, NS, NS)	43% NS	NS	Release profil in PBS

					Formation of puerarin-cyclodextrin complex encapsulated in PLGA NPs				at 37°C for 7 days
131	EPO (enhance oxygen delivery and promote cell survival)	PLGA NPs	to improve brain penetration and reduce injected dose	No No	double emulsion solvent evaporation encapsulation	NS	NS	NS	NS
133	SOD (antioxidant enzyme)	PLGA NPs	to increase blood half lifetime and improve brain penetration	No No	multiple emulsion solvent evaporation encapsulation	TEM, DLS (291 nm, -25 mV, NS)	75% 8%	NS	NS
68	EGF + EPO (stimulate neurogenesis and reduce apoptosis of the newly generated cells)	PLGA NPs embedded in PLA MPs embedded in HAMC hydrogel	to improve brain penetration and ensure temporal controlled release	pegylation of EGF (5000) No	PLGA NPs: double-emulsion PLA MPs: single emulsion encapsulation	DLS, SEM, XPS (PLGA(EGF) NPs 800 nm ; PSA-PLGA MPs (EPO) 18 µm)	EGF 54% EPO 23% NS	NS	release profil in aCSF at 37°C for 35 days
166	Panax notoginsenoside (multiple mechanisms)	PLGA NPs embedded in liposomes	to increase oral bioavailability and cellular uptake	PEG (5000) No	PLGA NPs: double solvent evaporation liposomes: film rehydration encapsulation	DLS, TEM (338 nm, -45 mV, NS)	R1 56% Rb1 83% Rg1 41% NS	stable for 12 months when kept at 4°C	Release profil of R1, Rb1 and Rg1 in PBS at 37°C for 35h
23	SOD (antioxidant enzyme)	PBCA NPs	to enhance brain targeting	No anti-NMDA R1 Ab	Nanoprecipitation + o/n polymerization covalent link at the surface of the NPs	DLS (326 nm, -47 mV, NS)	NS	NS	NS
167	Transhinone IIA (multiple mechanisms)	PLA NPs	to increase blood half lifetime and improve brain penetration	PEG (2000) cationic BSA	emulsion solvent evaporation encapsulation	DLS, TEM (118 nm, -20 mV, NS)	83% 6%	NS	NS

153	Quercetin (free radical scavenger)	PLA NPs	to improve brain penetration and oral bioavailability	Ps80 No	emulsion solvent evaporation encapsulation	NS	NS	NS	NS
144	Puerarin (multiple mechanisms)	PBCN NPs	to improve brain penetration	No No	nanoprecipitation encapsulation	DLS (201 nm, -8 mV, 0.387)	NS	NS	Release profil in PBS at 37°C for 24h
145	Curcumin and derivatives (multiple mechanisms)	PNIPAM NPs	to increase blood half lifetime and improve brain penetration	No No	nanoprecipitation + sonication encapsulation	DLS, TEM, SEM (93 nm, -16 mV, 0.291)	85% 39%	NS	NS
168	TEMPO (free radical scavenger)	PS micelles	to increase blood half lifetime and reduce systemic toxicity	PEG (NS) No	covalent link to the PS-PEG chain	DLS (40-60 nm, NS, NS)	100% NS	NS	release profil in acidic environment (pH sensitivity)
140	Adenosine (multiple mechanisms)	Squalenoyl NPs	to increase blood half lifetime and reduce systemic toxicity	No No	nanoprecipitation Covalent link between adenosine and squalene	DLS, cryo-TEM (120 nm, -20 mV, 0.15)	100% 37%	stable for at least one month at 4°C stable for 2h in mouse plasma at 37°C	NS
142	Butylphtalide (preservation of neurovascular components)	solid lipid NPs	to improve brain penetration	PEG (2500) Fas ligand Ab	solvent diffusion method encapsulation	DLS, TEM (61 nm, NS, NS)	94% 16%	NS	release profil in PBS at 50°C for 24h
157	Curcumin (multiple mechanisms)	solid lipid NPs	to increase blood half lifetime and improve brain penetration	No No	crystallization of oil droplets encapsulation	DLS (135 nm, NS, NS)	82% 92%	NS	NS
146	Daidzen (multiple mechanisms)	solid lipid NPs	to increase oral bioavailability	PEG (2000) No	hot homogeneization method	DLS, TEM (126 nm, -34 mV, 0.226)	83% 3.5%	NS	release profil in PBS containing

					encapsulation				4% BSA at 37°C for 120h
169	Anti-Ask1 shRNA ( <i>silence apoptosis signal regulating kinase</i> )	dendrigraft poly-L-lysine (DGL)	to improve brain penetration	PEG (3500) dermorphin	DGL commercial electric interaction	DLS, TEM (104 nm, 10 mV, NS)	NS	NS	NS
134	Anti-HMGB1 siRNA ( <i>silence inflammatory cytokine-like molecules</i> )	arginine PAMAM-ester dendrimer	to enhance neuronal uptake	No No	synthesis	NS	NS	NS	NS
85	Z-DEVD-FMK ( <i>caspase-3 inhibitor</i> )	chitosan NPs	to improve brain penetration	PEG (5000) Transferrin receptor	ionic gelation encapsulation	DLS (650 nm, 20 mV, NS)	25% $0.01 \times 10^{-3} \%$	NS	Release profil in PBS at 37°C for 24h
155	Anti-caspase-3 siRNA ( <i>silence apoptosis signal-regulating kinase</i> )	carbone nanotube	to enhance neuronal uptake	amine modification of the surface No	commercial NT modified by cycloaddition reaction complexation	TEM (20-30 nm diameter, 0.5-2 $\mu$ m length)	NS	NS	NS
137	/	carbone nanotube	to reduce apoptosis and inflammation	amine modification of the surface No	commercial NT modified by N <sub>2</sub> /H <sub>2</sub> plasma	NS (4-10 nm diameter, 0.5-1.5 $\mu$ m length)	/	NS	/
147	PX-18 ( <i>PLA2 inhibitor</i> )	drug nanocrystal	to increase drug solubility	No No	high pressure homogeneization	DLS (41 nm, NS, 0.4)	100% 100%	Stable for 180 days when kept at 4-6°C	NS
136	/	ceria NPs	ROS and radical scavenger	PEG (2000) No	Modified reverse micelle method	DLS, TEM, SAED, X-ray diffraction (18-30 nm, NS, NS)	/	Stable for 10 days in PBS and plasma	/
135 - 158	/	Pt NPs	ROS and radical scavenger	No No	ethanol reduction	NS	/	NS	/
170	/	gold NPs	anti-inflammatory agent	No No	citrate reduction method	TEM, UV-Vis spectra (20 nm, NS, NS)	/	NS	/



**Table 2 : Comparison of studies using nanotechnologies for the treatment of cerebral ischemia in vivo in terms of pre-clinical stroke model and experiment quality** (NS: Not Specified ; IV: intravenous ; IA: intra-arterial ; ICV: intra-cerebroventricular ; IP: intra-peritoneal)

Reference	Drug and therapeutic rational	Animal (sex, species, weight/age)	Ischemia model	Route of drug administration	Time of drug administration	Different doses	Functional outcome assessment	Infarct volume assessment	Randomization Blinding to treatment administration Blinding to outcome evaluation	Physiological monitoring
<b>143</b>	Citicoline (protects against cell membrane damage)	male Sprague-Dawley rats, 350 g	ligation (permanent)	IV (jugular)	+45 min and +6h and +12h and +24h and +30h	No	No	MRI +24h, +3 days, +7 days	Yes/Yes/Yes	corporal temperature, blood glucose, cerebral blood flow
<b>160</b>	FK506 (immunosuppressant)	male Wistar rats, 170-210 g	filament (1h)	IV (caudal)	at reperfusion or +1h or +2h or +3h or +6h (therapeutic window)	Yes	general scale (21 points), +24h	TTC, +24h	NS	corporal temperature
<b>138</b>	Asialo-EPO (enhance oxygen delivery and promote cell survival without hematopoietic toxicity)	male Wistar rats, 170-210 g	filament (1h)	IV (caudal)	at reperfusion	No	general scale (21 points), +24h	TTC, +24h	NS	corporal temperature
<b>156</b>	Dexamethasone (decrease secondary risks of rtPA, multiple mechanisms)	male Wistar rats, 350 g	thromboembolic (autologous clot)	IV (caudal)	+2h30 (following rtPA administration)	No	general scale (22 points), adhesive removal, cylinder test, +24h, +3 days, +7 days	MRI +24h, +7 days	NS	corporal temperature

130	VEGF gene (multiple mechanisms)	male Sprague-Dawley rats, 230g-250 g	filament (permanent)	IV (femoral)	+48h	No	general scale (18 points), + 24h, +7 days, + 14 days, +21 days	TTC, +21 days	Yes/NS/Yes	corporal temperature, cerebral blood flow
149	Luteolin (multiple mechanisms)	female Sprague-Dawley rats, 200g-220 g	filament (40 min)	IP	+6h and then everyday for 13 days	Yes	general scale (5 points), beam walking test, +10h, +7 days, +14 days	TTC, +14 days	Yes/NS/Yes	NS
161	Citicoline (protects against cell membrane damage)	male Sprague-Dawley rats, 260 g	ligation (permanent)	IV (jugular)	+ 30 min and +6h and +12h and +18h and +24h and +30h	No	No	MRI, +30 min, +24h, +3 days, +7 days	Yes/NS/NS	corporal temperature
141	Xenon (NMDA receptor antagonist)	male Sprague-Dawley rats, 260-280 g	filament (2h)	IV (caudal)	after MCA occlusion	No	Beam walking, grid walking, limb placement, +24h, +2 days, + 3days	TTC, +3 days	Yes/NS/Yes	corporal temperature, blood pressure, PO <sub>2</sub> , PCO <sub>2</sub> , pH, glucose, heart rate
151	Antisens oligonucleotide against synaptotagim I (reduce glutamate release)	male Sprague-Dawley rats, 2 months (160-180 g) and 20 months (415-440 g)	bilateral clamping (30 min)	IV (caudal)	- 3h	No	No	No	NS	NS
148	Hemoglobin (oxygen delivery)	cynomolgus monkey, 3.5-6.7 g	transorbital microvascular clip (3h)	IV	after MCA occlusion	Yes	No	PET, +2h, +3h, +6h HE, +8h	Yes/NS/Yes	corporal temperature, blood pressure, PO <sub>2</sub> , PCO <sub>2</sub> , pH, glucose, heart rate
132	Hemoglobin (oxygen delivery)	male Sprague-Dawley rats, 270-300 g	photochemical thrombosis	IV (caudal)	after MCA occlusion	Yes	No	TTC, +24h	NS/NS/Yes	NS

162	Hemoglobin (oxygen delivery)	male Wistar rats, 200-250 g	Arachidonic acid model (permanent)	IV (caudal)	after MCA occlusion	No	general scale (4 or 18 points), + 24h	TTC, +24h	NS	corporal temperature
163	Quercetin (free radical scavenger)	neonatal Wistar rats (7 days)	filament (2h)	ICV	- 72h	Yes	No	Nissl staining, +24h	NS	corporal temperature
154	Citicoline (protects against cell membrane damage)	male spontaneous hypertensive rats (SHR), 250-275 g	filament (1h)	IV (fermoral)	at reperfusion and +3h	Yes	No	TTC, +24h	NS	corporal temperature, blood pressure, PO <sub>2</sub> , PCO <sub>2</sub> , cerebral blood flow (Doppler)
152	Quercetin (free radical scavenger)	male Sprague-Dawley rats, 2 months (150-170 g) and 20 months (415-440 g)	bilateral clamping (30 min)	oral	- 2h	No	No	No	NS	NS
164	Triiodothyronine (multiple mechanisms)	CD-1 male mice, 2-3 months	filament (1h)	IV (jugular)	- 30 min	No	No	TTC, +24h	NS/NS/Yes	corporal temperature, cerebral blood flow
165	Puerarin (multiple mechanisms)	male Wistar rats, 270-300 g	filament (1h)	oral	NS	No	No	CT scan, +24h, +3 days, + 7 days / TTC	Yes/NS/NS	NS
131	EPO (enhance oxygen delivery and promote cell survival)	neonatal Wistar rats (10 days)	suture ligation (4 min) + hypoxia (75 min)	IP	+ 1h and + 24h and + 48h	Yes	rotarod, +21 days	TTC, +72h	Yes/NS/NS	NS
133	SOD (antioxidant enzyme)	male Sprague-Dawley rats, 250-300 g	filament (1h)	IA (carotid)	at reperfusion	Yes	general sensory-motor scale (14 points), + 6h, +	TTC, +6h, +7 days, +14 days, +28 days	NS	corporal temperature, blood pressure, PO <sub>2</sub> , PCO <sub>2</sub> , pH,

							7days, +14 days, +28 days			glucose, cerebral blood flow (Doppler)
<b>68</b>	EGF + EPO <i>(stimulate neurogenesis and reduce apoptosis of the newly generated cells)</i>	male C57Bl6 mice, 9-11 weeks	endothelin-1	placement of the gel at the cortical surface	+ 4 days	No	No	cresyl violet, +18 days	NS	NS
<b>166</b>	Panax notoginsenoside <i>(multiple mechanisms)</i>	male Sprague- Dawley rats, 220 g	bilateral clamping (2h)	oral	- 10 days	No	No	TTC, +4h	Yes/NS/NS	corporal temperature
<b>23</b>	SOD <i>(antioxidant enzyme)</i>	male C57Bl6 mice, 22-25 g	filament (1h)	IA (carotid)	at reperfusion or +2h or +4h or +8h or +24h (therapeutic window)	No	general scale (4 points), +24h	TTC, +24h	Yes/NS/Yes	corporal and cerebral temperature, blood pressure, PaO <sub>2</sub> , PCO <sub>2</sub> , pH, cerebral blood flow (Doppler)
<b>167</b>	Transhinone IIA <i>(multiple mechanisms)</i>	male Sprague- Dawley rats, 220 g	filament (2h)	IV (caudal)	at reperfusion	No	general scale (4 points), +24h	TTC, +24h	Yes/NS/Yes	cerebral blood flow
<b>153</b>	Quercetin <i>(free radical scavenger)</i>	male Sprague- Dawley rats, 2 months (150- 170 g) and 20 months (415- 440 g)	bilateral clamping (30 min)	oral	- 24h	No	No	No	NS	corporal temperature
<b>144</b>	Puerarin <i>(multiple mechanisms)</i>	male Wistar rats, 220-250 g	filament (1h30)	IV (caudal)	- 10 min and at reperfusion	No	general scale (5 points), +24h	TTC, +24h	Yes/NS/NS	NS
<b>145</b>	Curcumin and derivatives <i>(multiple mechanisms)</i>	male Wistar rats, 250-300 g	filament (2h)	intranasal	- 1h	No	open-field activity, grip strength, +24h	TTC, +24h	NS	NS

168	TEMPO (free radical scavenger)	male Sprague-Dawley rats, 280-350 g	filament (1h)	IV (femoral)	+ 30 min	No	general scale (3 points), +24h	TTC, +24h	NS	corporal and cerebral temperature, blood pressure, PaO <sub>2</sub> , PCO <sub>2</sub> , pH, cerebral blood flow (Doppler)
140	Adenosine (multiple mechanisms)	male SWISS mice, 25-30 g	filament (2h)	IV (caudal)	just before MCA occlusion or at reperfusion	Yes	general scale (3 points), +24h	Nissl staining, +24h	Yes/Yes/Yes	corporal temperature, blood pressure, cerebral blood flow (Doppler)
142	Butylphtalide (preservation of neurovascular components)	male C57Bl6 mice, adult	filament (45 min)	IV (caudal)	- 1h	Yes	general scale (4 points), +24h	cresyl violet, +24h	Yes/NS/NS	corporal temperature
157	Curcumin (multiple mechanisms)	male Wistar rats, 250-300 g	bilateral clamping (10 min)	oral	- 5 days and + 3 days	Yes	general scale (8 points), Morris water maze, open-field test, rotarod, +24h	No	Yes/NS/NS	corporal temperature
146	Daidzen (multiple mechanisms)	male Wistar rats, 215 g	filament (2h)	IV (caudal)	+ 1h	No	general scale (5 points), +24h	TTC, +24h	NS	NS
169	Anti-Ask1 shRNA (silence apoptosis signal regulating kinase)	male Sprague-Dawley rats, 200-240 g	filament (1h)	IV (caudal)	- 48h	No	No	TTC, +24h	NS	NS
134	Anti-HMGB1 siRNA (silence inflammatory cytokine-like molecules)	male Sprague-Dawley rats, 250-300 g	filament (1h)	intracortical	6, 12, 18 or 24h before ischemia	No	No	TTC, +48h	Yes/NS/NS	corporal temperature, blood pressure, PO <sub>2</sub> , PCO <sub>2</sub> , pH, glucose, cerebral blood flow (Doppler)

85	Z-DEVD-FMK ( <i>caspase-3 inhibitor</i> )	male SWISS mice, 18-22 g	filament (2h)	IV (caudal)	just before MCA occlusion or at reperfusion	Yes	general scale (3 points), +24h	HE, + 3 days	NS/NS/Yes	corporal temperature, blood pressure, pulse rate, oxygen saturation, cerebral blood flow (Doppler)
155	Anti-caspase-3 siRNA ( <i>silence apoptosis signal-regulating kinase</i> )	female C57Bl6 mice, 6-8 weeks adult male Sprague-Dawley rats	endothelin-1	intracortical	- 24h or + 1h	No	skilled reaching test (rats), + 7 days to + 10 days	No	NS	corporal temperature
137	carbone NT	male Sprague-Dawley rats, 250-300 g	filament (1h30)	ICV	- 1 week	No	rotarod, + 24h and + 7 days	TTC, +24h	NS	NS
147	PX-18 ( <i>PLA2 inhibitor</i> )	male Mongolian gerbil, adult	bilateral clamping (5 min)	IP	at reperfusion	Yes	No	No	Yes/NS/NS	corporal temperature, regional cerebral blood flow
136	ceria NPs	male Sprague-Dawley rats, 200-220 g	filament (1h)	IV (caudal)	subsequent to ischemia	Yes	No	Nissl staining, + 24h	NS	corporal temperature
135	Pt NPs	male C57Bl6 mice, 8 weeks	filament (1h)	IV (caudal)	at reperfusion, just after tPA administration	No	general scale (4 points), rotarod, +48h	HE, + 48h	NS/NS/Yes	corporal temperature, regional cerebral blood flow
158	Pt NPs	male C57Bl6 mice, 8 weeks	filament (1h)	IV (caudal)	at reperfusion	No	general scale (4 points), rotarod, +48h	HE, + 48h	NS/NS/Yes	corporal temperature, regional

										cerebral blood flow
170	gold NPs	male Sprague-Dawley rats, 220-260 g	filament (1h30)	IP	Within 15 min after reperfusion	No	general scale (4 points), + 6h, +12h, +24h +48h, +72h	TTC, +24h and +72h	Yes/NS/Yes	corporal temperature

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