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Regulatory basis for the safety assessment of nanotechnology-based drug products

Bases regulatórias para a avaliação da segurança de medicamentos à base de nanotecnologia

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ABSTRACT

Introduction: Nanotechnology is a transdisciplinary technology that is being developed and applied in several areas, including health, especially in terms of therapy and diagnosis. However, the relationship between some of their physicochemical properties and their toxicological effects remains unclear. Therefore, it is necessary to understand whether the regulatory requirements, in terms of toxicological evaluation, for the registration of a nanotechnology-based drug, are able to identify the possible risks arising from this new technology. **Objective:** To compare the regulatory approach of US Food and Drug Administration (FDA), European Medicines Agency (EMA) and Brazilian Health Regulatory Agency (Anvisa) with respect to nanomedicine evaluation compared to conventional drugs evaluation. **Method:** Qualitative bibliographic research was performed in different databases and regulatory agencies websites. **Results:** Many limitations of the currently recommended tests have been demonstrated, and several are under review for better adaptation to the effect that may suffer by the evaluated nanoparticles themselves. **Conclusions:** Toxicological tests currently recommended by the regulatory agencies of the United States of America, the European Union and Brazil, although aligned, are not specific for the evaluation of nanomedicines.

KEYWORDS: Nanomedicine; Safety; Toxicology; Regulation; Anvisa

RESUMO

Introdução: A nanotecnologia é uma tecnologia transdisciplinar que está sendo desenvolvida e aplicada em diversas áreas, dentre as quais cabe ressaltar a da saúde, principalmente no que tange à terapêutica e ao diagnóstico. Entretanto, ainda não se tem clara a relação entre algumas de suas propriedades físico-químicas e seus efeitos toxicológicos. Por isso, é necessário entender se os requisitos regulatórios, em termos de avaliação toxicológica, para registro de um medicamento com base em nanotecnologia, são capazes de identificar os possíveis riscos advindos desta nova tecnologia. **Objetivo:** Comparar a abordagem regulatória da *US Food and Drug Administration* (FDA), da *European Medicines Agency* (EMA) e da Agência Nacional de Vigilância Sanitária (Anvisa) com relação à avaliação de nanomedicamentos em comparação com medicamentos convencionais. **Método:** Foi realizada pesquisa bibliográfica qualitativa em diferentes bases de dados e agências regulatórias. **Resultados:** Foram demonstradas muitas limitações dos testes atualmente preconizados, sendo que diversos deles encontram-se em caráter de revisão para melhor adequação ao efeito que podem sofrer pelas próprias nanopartículas avaliadas. **Conclusões:** Testes toxicológicos preconizados atualmente pelas agências reguladoras dos Estados Unidos da América, da União Europeia e do Brasil, apesar de estarem alinhados, não são específicos para a avaliação de nanomedicamentos.

PALAVRAS-CHAVE: Nanomedicina; Segurança; Toxicologia; Regulamentação; Anvisa

INTRODUCTION

Nanotechnology is a field of transdisciplinary knowledge that is being enhanced and applied to several areas, like automotive, textiles, sports equipment, telecommunications, electronics, food, beauty, medical devices, diagnostic tests and pharmaceutical products[1,2,3]. Among these areas, it is worth highlighting the importance of nanotechnology for healthcare, especially for therapy and diagnosis[4], since the need for more efficient therapeutic and diagnostic systems is clear, especially regarding the risk/benefit ratio for the patients[5].

In this sense, one of the areas in which there is greater exposure to the toxicity of traditional treatments and diagnostic agents is oncology. In this area, there are often very long treatments, frequent need for imaging tests, and high doses of drugs in the treatments. Furthermore, combination therapies are common, since there are many mechanisms of resistance to conventional therapies in which drugs are distributed non-specifically in the patient's body[6,7,8].

With the progress of nanotechnology, it is expected that some shortcomings currently identified in the treatment of cancer will be resolved or, at least, mitigated, thanks to some characteristics of nanomaterials, like high surface/volume ratio (greater carry over of active ingredients), shape and size (enabling uptake by the target cell – an effect of the *enhanced permeability and retention* (EPR), introduction of targeting molecules and physicochemical improvements in the nanosystem (increased blood circulation time, evasion of the reticuloendothelial system, effective targeting and build-up at destination sites)[9,10,11].

Most of these benefits have not yet been translated into commercially available drugs. For cancer treatment we can mention: Doxil® and Caelyx®, which are the trade names of Schering-Plow's pegylated liposomal doxorubicin – Doxil® is the name registered in the United States of America (USA) and Caelyx®, in Europe and in Brazil – Abraxane®, Myocet® and Daunoxome®[12,13,14,15]. In this scientific pursuit of nanomedicines and nanodevices, one must take into account – with equal importance – the adequate characterization of the toxicity profile inherent in these new materials. Although some publications have shown the toxicological effects of nanoparticles (NPs) on cells, the nature of this cytotoxicity is still unclear[16].

In fact, the population is already exposed to the beneficial effects and potential risks of this new technology. Therefore, it is important to understand and characterize these materials properly, as well as to compile and make this information available to the scientific community, industry, regulatory bodies and society as a whole.

The justification for choosing this topic is given by the importance of nanomaterial toxicity for patients. Consequently, we must understand whether the toxicological assessment regulatory requirements for approving a nanotechnology-based drug can identify the possible risks arising from this new technology.

Overview of nanotechnology in the regulatory processes of the USA, Europe and Brazil

The evolution of the regulatory methodology to deal with emerging technologies is not a new issue. Lessons learned from previous technological revolutions, including *in vitro* fertilization, genetically modified organisms and cloning, have shown the need to strike a balance between industrial innovation, risk reduction and public debate on the regulation of these technologies. This is even more important when it is not clear whether or not the potential risks of the technology can be qualified and quantified by the methodology recommended by the legislation in force[2]. Along the same lines, the fast growth of nanotechnology in recent years has led to the scientific questioning of the current methods for analyzing and monitoring the risks these new materials pose to the society[2].

Considering the US Food and Drug Administration (FDA), the European Medicines Agency (EMA) and the Brazilian National Health Surveillance Agency (Anvisa), we note that these regulatory agencies have been increasingly interested in understanding the adequacy of the current regulation for medicines, in order to assess the possible risks arising from nanomaterials. This concern can be seen in the articulation of specific working groups to assess the needs of this new technology, discussion forums on the topic, and government investment in research on the toxicity and benefits of these materials.

For this reason, regulatory agencies continue to work to understand how effective their regulation and toxicological tests are, so as to evaluate the impact of nanotechnology on human health[17,18,19,20,21,22,23,24].

It is, therefore, important to compare the regulatory approach of EMA, FDA and Anvisa to toxicity assessment for conventional and nanotechnological drugs in order to identify shortcomings in the required tests and, whenever possible, suggest strategies to improve them and strengthen the regulatory dossiers for nanomedicines.

METHOD

This study was conducted based on a literature review, with a qualitative focus. It should be noted that the language of the area is not yet fully standardized. To corroborate the justification of the methodology used here we have the precariousness of the documentary language, which fails to provide an information retrieval that is consistent with the informational needs of this paper. The low degree of specificity of the language adopted by the information systems in this area hinders the appropriate indexing/retrieval of information and reduces the accuracy of the results obtained through the quantitative approach.

Thus, we made queries in the following databases: Scientific Electronic Library Online (SciELO), Scopus, Pubmed, Embase,

Cochrane Library, CAPES Journals TrialTrove and Clinical Trials. Whenever necessary, bibliographic data were added to information retrieved from websites belonging to governmental and intergovernmental organizations, whose purpose is to share studies, newsletters and data related to the topics addressed in this article. Among them, we can mention: FDA, EMA, Anvisa, Ministry of Science, Technology and Innovation (MCTI), National Institute of Metrology, Quality and Technology (Inmetro) and Organization for Economic Cooperation and Development (OECD).

The following terms were used as primary descriptors: *Nanotecnologia*/Nanotechnology, *Nanomedicina*/Nanomedicine, *Nanopartícula*/Nanoparticle, *Nanocarreadores*/Nanocarriers, *Nanodispositivos*/Nanodevices and *Câncer*/Cancer. As secondary descriptors: *Testes toxicológicos, toxicologia*/Toxicological analysis, toxicology, *Tolerabilidade*/Tolerability, *Estudos de fase IV*/Phase IV studies, *Evento adverso*/Adverse event, *Observacional*/Observational, *Aprovação regulatória, dossiê regulatório*/Regulatory approval, regulatory dossier, *OCDE*/OECD.

No time limit was established for the research and it ended in June 2019. The articles and/or documents we found were selected based on the analysis of their relevance to the chosen topic.

RESULTS AND DISCUSSION

To provide an overview of the development stage and the initiatives in this area in the USA, Europe and Brazil, Chart 1 introduces the results that will be discussed below.

The regulatory requirements for submitting applications of nanotechnological drugs to the regulatory agencies in the USA (FDA), Europe (EMA) and Brazil (Anvisa) were compared. Although the progress of nanomedicine is gaining momentum worldwide, we can notice that regulatory agencies are adopting conservative criteria for the evaluation of these new drugs. Both the FDA and the EMA make their position on the nanomedicine regulation available on their websites. In the case of the FDA, two official documents are worth mentioning: the *Guidance for Industry Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology*, published in 2014, and a preliminary version for comments of *Drug Products, Including Biological Products, that Contain Nanomaterials Guidance for Industry DRAFT GUIDANCE*, published in December 2017. In these two documents, the FDA makes it clear that there is no distinguished process for nanotechnology-based drugs, but one must take extra care in relation to the submitted data. It is important to remember that these guidelines are not of a regulatory nature, but rather they help the industry prepare their submissions following recommendations that facilitate the review and approval process[25].

In the light of the publications of its scientific and advisory committees and of its independent risk assessors, the European Union has published a definition of nanomaterials and the confirmation that nanotechnology-based drugs follow the standard process for the assessment of any other medication, as well as its toxicological assessment, even though studies on specific aspects of the risk assessment of these new materials are still necessary[26]. Like the FDA, the EMA, since 2011, has released documents (reflection papers) to assist the industry in the content of their submissions, namely: a) *Joint MHLW/EMA reflection paper on the development of block copolymer micelle medicinal products* (EMA/CHMP/13099/2013)[27]; b) *Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product* (EMA/CHMP/806058/2009/Rev. 02)[28]; c) *Reflection paper on surface coatings: general issues for consideration regarding parenteral administration of coated nanomedicine products* (EMA/325027/2013)[29]; d) *Reflection paper on non-clinical studies for generic nanoparticle iron medicinal product applications* (EMA/CHMP/SWP/100094/2011)[30]; e) *Reflection paper on the data requirements for intravenous iron-based nano-colloidal products developed with reference to an innovator medicinal product* (EMA/CHMP/SWP/620008/2012)[31]. The latter addresses a controversial topic that will begin to emerge as soon as the patents for nanomedicines that are already approved expire: the regulatory approval of nanosimilar products. Like the

Chart 1. Map of processes and activities focused on nanotechnology in regulatory agencies.

Processes and activities	USA (FDA)	EU (EMA)	Brazil (Anvisa)
Financial investment for the development of nanotechnology in the country	Yes	Yes	Yes
Specific regulations for nanomedicines	No	No	No
Guides to orient the regulated sector on the submission of nanomaterials	Yes	Yes	No
Specific form for declaring the presence of nanomaterials in the final medicine	Yes	Yes	No
Position on the applicability of toxicological tests for nanomedicines	Yes	Yes	No
Federal investments are directed to the validation and improvement of toxicological tests for nanomedicines	Yes	Yes	Yes
Metrology laboratories are adapted to nanotechnology	Yes	Yes	Yes
Unified database available to store information about nanomaterials	Yes	Yes	No

Source: FDA[17,18,19], EMA[22], Anvisa[32].
USA: United States of America; EU: European Union; FDA: US Food and Drug Administration; EMA: European Medicines Agency; Anvisa: National Health Surveillance Agency.

FDA guide, these documents are not of a regulatory nature. They are educational documents to guide manufacturers and other stakeholders.

With regard to the regulation of this new technology in Brazil, as mentioned earlier, only in 2013, ten years after the beginning of the Brazilian Federal Government's initiatives to encourage and develop nanotechnology, was Anvisa's Internal Nanotechnology Committee established (officially only in 2014)[32]. Although it is a subject of fierce debate, there is still no information on Anvisa's website about its position in relation to the need to adapt the regulation to nanomedicines. However, it can be noted that society is seeking guidance on this discussion. The concern with the topic stands out when we analyze the bills (PL) submitted by congresspeople for the appreciation of the government (Chart 2).

Guides for the toxicological analysis of nanomedicines

After analyzing the pre-clinical tests recommended by the FDA and the EMA, we can notice that these agencies adopt a very flexible approach, which enables the choice of tests according to the characteristics of the drug to be tested and its clinical development plan. The aforementioned agencies are based on the guidelines of The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), which consider the recommendations of the OECD and international working groups on the topic.

Brazil has prepared a guide for medicines in general (it is not nanomedicine-specific either) based on the same guidelines and agencies mentioned above (ICH, OECD, FDA and EMA) – the so-called Guide for conducting non-clinical studies on toxicology and pharmacological safety for drug development[40]. This guide is a guideline for conducting non-clinical safety studies during the development process of a drug. It is not regulatory in nature and is flexible when it comes to the inclusion of other tests that are not listed in the document, as long as they are validated and accepted internationally. It covers the following areas: single dose (acute) toxicity studies, repeated dose toxicity, reproductive toxicity, genotoxicity, local tolerance and carcinogenicity, as well as studies of interest in the assessment of pharmacological and toxicokinetic safety (Administration, Distribution, Metabolism and Excretion – ADME).

Although the aforementioned tests are widely used for the assessment of conventional drugs and have demonstrated their importance for correlating the toxicological profile of conventional drugs in preclinical tests and clinical practice, based on the information currently available we notice that this

Chart 2. Bills submitted by congresspeople on nanotechnology.

Project	Topic	Situation
PL n. 880/2019[33], being debated	Creates the Legal Framework for Nanotechnology and establishes incentives for scientific development, research, training and innovation in nanotechnology.	April/3/2019 - Constitution, Justice and Citizenship Commission (Secretariat of Support to the Constitution, Justice and Citizenship Commission) April/3/2019 - Matter with the rapporteur
Complementary PL n. 23[34], of February 4, 2019	Allows the inclusion in the Simples Nacional category of companies whose activity is support, technical and technological analysis, research and development of nanotechnology.	March/13/2019 - Commission for Science, Technology, Innovation, Communication and Informatics (Secretariat to Support the Commission for Science, Technology, Innovation, Communication and Informatics) March/13/2019 - Matter with the rapporteur
PL n. 683[35], of July 2, 2019	Confers the title of "National Capital of Nanotechnology and New Materials" on the city of Florianópolis (SC).	June/6/2019 - Plenary of the Federal Senate (Legislative Secretariat of the Federal Senate) June/6/2019 - Awaiting appeal
PL n. 6.741[36], of November 11, 2013	Provides for the National Nanotechnology Policy, research, production, the disposal of waste and the use of nanotechnology in the country and provides other measures.	April/5/2017 - Join PL n. 6.741/2013 with PL n. 5.133/2013 January/31/2019 - Archived
PL n. 5.133[37], of March 13, 2013	Regulates the labeling of nanotechnology products and products that use nanotechnology.	April/5/2017 - Join PL n. 6.741/2013 with PL n. 5.133/2013 January/31/2019 - Archived
PL n. 5.076[38], of April 18, 2005	Provides for the research and use of nanotechnology in the country, creates the National Technical Commission on Nanosafety (CTNano), institutes the Fund for the Development of Nanotechnology (FDNano) and other measures.	November/5/08 - Rejected February/18/2009 - Archived "The Finance and Taxation Committee, in an ordinary meeting held today, unanimously concluded that Law n. 5.076-B/05 is incompatible and inadequate in both financial and budgetary terms, pursuant to the rapporteur's opinion"
PL n. 131/2010[39], being debated	Amends Decree-Law n. 986, of October 21, 1969, which establishes basic rules on food, and Law n. 6.360, of September 23, 1976, which provides for health surveillance to which medications, drugs, pharmaceutical and related supplies, cosmetics, sanitizing products and other products are subject and takes other measures to determine that labels, packaging, tags, package inserts and advertising materials for products made using nanotechnology contain information about this fact.	August/1/2013 - Rejected "Finally, in addition to creating confusion and alarm, the project under analysis may increase the price of products, due to the imposition of more bureaucratic requirements"

Source: Prepared by the authors based on the aforementioned legislation, 2019.
PL: Bill of Law.

correlation is not necessarily true when it comes to nanomedicines. Therefore, there is a need to evaluate the applicability of these tests for nanomedicines.

One of the topics on the agenda of the Working Party on Manufactured Nanomaterials (WPMN), established in 2006, is the review of toxicological tests recommended in the OECD guidelines (Chart 3), bearing in mind the emerging needs arising from nanotechnology. The objective of this project is to identify the need for new guidelines, as well as points of improvement and shortcomings of the existing guidelines for the assessment of nanomaterials.

After analysis, the OECD considers that part of its current guidelines is applicable to nanomaterials. In some cases, adjustments to the methodology are necessary. In other cases, the design of a new methodology will be necessary, since the available guidelines are inadequate. This inadequacy is primarily related to the lack of standardization and validation of qualitative and quantitative analysis methods for nanomaterials[41].

In addition to the review done by the WPMN, it is worth mentioning the studies by Nel et al.[42] and Jones and Grainger[43], where one can identify some critical points that influence the analysis of the results of *in vitro* toxicological tests. Among the characteristics to be analyzed, we can mention: particle size; size of the formed aggregate and/or agglomerate, size distribution in the formulation; area, chemistry and surface charge; *zeta* potential; structure/format; formulation stability; solubility; surface reactivity; purity; porosity, among other characteristics[44,45,46,47].

One of the most important discussions about nanomedicines is the establishment of criteria for assessing their dosage. Even though mass is used by many of the published studies, it may not be the most appropriate measure for assessing exposure in relation to the effects on the patients' health. Considering that there is still a knowledge gap regarding what the best alternative is, some proposals available in the literature can be discussed, but without the hope of reaching a consensus, at least not in the short term.

The dose expressed in mass/volume has the advantage of being easier to quantify. However, this does not guarantee relevance to the dose-response correlation that must be analyzed, since nanomaterials are considered "different" from materials on a macro scale, especially because of their high surface/volume ratio, among other reasons. In addition, the need for further investigation of the relevance of this measure in relation to the observed response stands out when we consider the results of toxicological studies that demonstrated greater toxicity of nanomaterials when compared to the material on a macro scale using the same dose in relation to mass/volume[48,49]. Some researchers, like Wittmaack (2007), consider the number of particles/volume ratio to be the most relevant for their studies, but others, like Oberdörster[48], have demonstrated that the measure that would have the best dose-response correlation would be the surface area/volume. That is because we know that the toxicological response depends on the surface properties of the nanomaterial and that the surface area increases exponentially with the decrease in the size of the NP. Therefore, since there is still no consensus on the criteria to be used, it may be necessary to take into account that different nanomaterials are likely to need different criteria and, therefore, it is very important to invest in studies in this area.

There is still a lively discussion in the literature about *in vitro* tests based on cell cultures[43,50,51]. For example, 3D cultures produce a more complex and dense extracellular matrix and their cells are distributed in an inhomogeneous manner, which translates into a greater challenge for the transport and uptake of nanomaterials by the deeper cells in relation to the most superficial cells of the culture. Moreover, this variation in the penetrating power of nanomaterials is also related to the size of the nanomaterial and how long the cells are exposed to it. This fact could be better observed after the advent of 3D cell cultures, since in 2D cell cultures there was no demonstrated difference in the penetrating power of NPs, which, despite their different sizes, were homogeneously distributed in the cells. The study by Huang et al. has shown that in a 3D cell culture there was a significant increase in the uptake of smaller NPs (2 and 6 nm), with an increase in the incubation period from 3 to 24 h, which was not observed in bigger NPs (15 nm). These data demonstrated that, in general, the NP-induced toxicity was lower in the 3D culture than in the 2D culture[52,53,54].

Another important aspect that must be taken into account when it comes to the applicability of toxicological tests for nanomedicines is the possible interference of nanomaterials with the components and in the testing processes. A literature review by Ong et al.[55] demonstrated that in 2010, approximately 84% of publications on nanotoxicology used at least one type of colorimetric or fluorescence test. Of these analyzed tests, 95% were published without information about the use of appropriate controls to identify this interference. The same researchers did an identical analysis with the publications of 2012, to understand whether greater access to information about this type of interference could improve the planning of these tests. However, the results have shown that, of the publications from 2012, 90% did not report the use of controls for this purpose. That study also reported that the most commonly used control was the addition of the NPs alone with the test components (2010: 5%, 2012: 8%), followed by fluorescence/intrinsic absorbance analysis of the NPs (2010: 2%, 2012: 5%) and then the concomitant use of the NP with an analyte (2010: 1%, 2012: 4%). Regarding the procedures adopted as control, the study highlighted that, although the addition of NPs to the test components was the most frequent procedure, this method is not completely reliable for the control of NP interference. That is because in real conditions there will be interference from other factors, like proteins, which will affect the results, eliminate or potentiate the interference. Therefore, there is a clear need to characterize the action of every component in the chosen test.

Chart 3. OECD guidelines for testing chemicals[41].

Test number	Title	Opinion	
420	*Acute Oral Toxicity - Fixed Dose Procedure*	Adequate	It would be appropriate for an initial investigation. It must be recognized that the extent of the pathological assessment at autopsy is limited. • Expanded assessment of pathology/histology is required.
423	*Acute Oral toxicity - Acute Toxic Class Method*	Adequate	
425	*Acute Oral Toxicity: Up-and-Down Procedure*	Adequate	
436	*Acute Inhalation Toxicity - Acute Toxic Class Method*	At first they are not suitable for nanomaterials	• It is likely that they cannot contribute much to the toxicity profile of nanomaterials. • Materials of low intrinsic toxicity should be tested up to a dose of 5,000 mg/m^3, which would lead to death due to airflow obstruction and not intrinsic toxicity. • TG 403 - Includes only very limited histological examination at autopsy.
403	*Acute Inhalation Toxicity*		
412	*Subacute Inhalation Toxicity: 28-Day Study*	Revised and adequate	Review: • Specific measurements of bronchoalveolar lavage fluid (BALF) to be performed for all test chemicals, dividing the lung for histopathology and BALF analysis. Any planned recovery group must also include the BALF analysis. • Measurement of pulmonary deposition and retention of particles. • Consider the mean aerodynamic diameter of the mass ≤ 2 µm with a geometric standard deviation of 1–3.
413	*Subchronic Inhalation Toxicity: 90-day Study*	Revised and adequate	
402	*Acute Dermal Toxicity*	Inadequate	Requires only minimal pathology; improved pathology is desirable when investigating nanomaterials.
430	*In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test (TER)*	No mention	It can be used but bearing in mind that measuring cell viability using MTT may not be appropriate due to inactivation of the marker.
431	*In Vitro Skin Corrosion: Human Skin Model Test*	No mention	It can be used but bearing in mind that measuring cell viability using MTT may not be appropriate due to inactivation of the marker. Some critical issues related to the protocol were identified, for example: lack of circulation in the subcutaneous region, the duration of exposure for a relevant time, area of exposure, the compatibility of the receiving fluid for nanomaterials, which suggest the need to make this test more adequate.
435	*In Vitro Membrane Barrier Test Method for Skin Corrosion*	No mention	It can be used but bearing in mind that measuring cell viability using MTT may not be appropriate due to inactivation of the marker.
404	*Acute Dermal Irritation/Corrosion*	No mention	It may be appropriate to assess the irritability of nanomaterials.
405	*Acute Eye Irritation/Corrosion*	No mention	It may be appropriate to assess the irritability of nanomaterials.
429	*Skin Sensitisation*	No mention	TG 429 is more appropriate than TG 406 because of the well-being and number of animals used in the test, objectivity of the outcome, estimation of the potency of sensitizing agents, less compound is necessary.
406	*Skin Sensitisation*	No mention	Less appropriate when compared to TG 429.
407	*Repeated Dose 28-day Oral Toxicity Study in Rodents*	Adequate	Provides general information about a range of potential toxic effects, including neurotoxicity.
408	*Repeated Dose 90-Day Oral Toxicity Study in Rodents*	Adequate	
409	*Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents*	Adequate	Additional studies may be necessary to characterize neurotoxic, immunological or reproductive organ effects.
471	*Bacterial Reverse Mutation Test*	Not recommended	Justification: the bacterial cells used are not able to perform endocytosis and the diffusion of nanomaterials through the bacterial cell wall can be limited; both of these factors limit nanomaterial uptake; some nanomaterials also have antibacterial properties. TG 476 is considered as an alternative to TG 471.
473	*In vitro Mammalian Chromosome Aberration Test*	Adequate	No interference of nanomaterials with the test was reported.
476	*In vitro Mammalian Cell Gene Mutation Test*	Adequate	Potential influence on the conduction of the test when assessing high concentrations of ZnO-based nanomaterial (increased turbidity).
474	*Mammalian Erythrocyte Micronucleus Test*	Adequate	
475	*Mammalian Bone Marrow Chromosome Aberration Test*	Adequate	
486	*Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells in vivo*	Adequate*	
421	*Reproduction/Developmental Toxicity Screening Test*	Adequate*	For oral administration.
422	*Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test*	Adequate*	For oral administration.

Continue

Continuation

Test number	Title	Opinion	
415	*One-Generation Reproduction Toxicity Study*	Adequate**	
416	*Two-Generation Reproduction Toxicity*	Adequate**	
414	*Prenatal Development Toxicity Study*	Adequate**	
428	*Comet Assay (Single-Cell Gel Electrophoresis)*	Adequate	TG 427/428 in combination with complementary tests are, in general, suitable for the evaluation of nanomaterials. Some changes, for example those related to longer observation periods with nanomaterials, should be mentioned in its next update.
427	*Skin Absorption: In Vivo Method*	Adequate	
437	*Bovine Corneal Opacity and Permeability Test Method for Identifying: i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage*	Adequate	Adaptation is necessary to include the complete characterization of nanomaterials (powders or suspensions).
487	*In Vitro Mammalian Micronucleus Test*	Adequate	Adaptations to the test are necessary, including: the application of CytoB and nanomaterial to cells separately, exposure time (24 h seem to be enough), attention to serum concentration to avoid false positives and the use of stable genetically modified cell lines and competent P53.
489	*In Vivo Mammalian Alkaline Comet Assay*	No mention	

Source: OECD, 2009.
MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.
* Except for analysis of the respiratory tract as the target organ.
** For oral use only.

Tests that use colorimetric or fluorescence detection, in general, depend on redox reactions. These reactions occur in the presence of cellular activity, however, the study noted that some metallic NPs can also interact with the dye/marker (for example: alamar blue, 2.7-dichlorodihydrofluorescein – DCF) and cause its reduction[55].

The optical properties vary both according to the chemical composition of the material and its physical properties (particle size, shape, crystallinity, among others). Overall, both the alamar blue test and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) are affected by this interference. In these tests, fluorescence indicates cell viability and, since some NPs are also capable of generating fluorescence, false positive results eventually appear and lead to an underestimation of the toxicological impact of these NPs. On the other hand, NP interference in tests that measure cellular oxidative stress may overestimate their toxicological impact[55,56,57,58,59,60,61].

NPs have also been shown to interfere with the conformation of some proteins and, thus, decrease their enzymatic activity. For example, there is the interference of NPs with the activity of lactate dehydrogenase (LDH), an enzyme used in the test to assess cell viability. There is also some information about the catalytic activity of NPs in the reduction of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chlorine (INT), similar to the catalytic action of LDH[62]. In addition, it is also worth highlighting the effects of adding proteins to the test and their influence on the stability of NPs and, consequently, on their activity in the assay[55].

Other interactions may arise from the existence of electrostatic interactions between the NPs and the test materials. We must understand how much the NP charge (positive or negative) leads to the observed interference. We must also understand whether the observed interaction comes only from this criterion or whether other characteristics of the NP could be potentiating the interaction. NP behavior does not always follow a steady interaction pattern. There is data showing that both positively and negatively charged NPs can interfere with the tetrazolium marker. In addition, the same NP can interfere or not with the same marker in different tests. That is why it is important to use as much information as possible about the characteristics of the NPs to interpret the results[55].

In view of all the information compiled and discussed, here are some important recommendations for the regulation of this technology in Brazil:

1. More assertive and active participation in international working groups that are validating toxicological tests for nanomaterials.

2. Better control over the progress and publication of results achieved in research on nanotoxicology sponsored by federal government programs.

3. Invest in the proper training of Anvisa's staff to ensure that the regulatory analyses of these new products are done with the appropriate depth and time to guarantee Brazil's competitiveness in the area of nanomedicines, but also the population's right to safety and information.

4. Implement a procedure to ensure the proper labeling of drugs containing nanomaterials. That does not mean we need specific regulations for nanotechnology or a symbol on the label, as is the case of genetically modified organisms, but something that can guarantee that the labels and package inserts of medicines include enough information to protect the population's right to information and informed decisions.

5. Improve Anvisa's communication plan about its initiatives and positioning in relation to the regulation of nanomedicines.

6. Ensure that the federal government's investments in research and development in nanotechnology are allocated to the generation of knowledge in the area of pre-clinical tests that are necessary to:

    a. assess the safety and efficacy of nanomaterials;

    b. obtain data on the impacts of nanomaterials on the absorption, distribution, metabolism and elimination of conventional drugs;

    c. obtain data to better understand the structure-activity relationship of these new materials.

7. Regarding the adaptation of the requested toxicological tests:

    a. The minimum group of requested tests should be constantly aligned with international guidelines.

8. Regarding regulatory submission, in addition to the conventional process, at least the following information should be required:

    a. Appropriate bio-physical-chemical characterization of the nanomedicine, considering the factors that may influence this analysis (means, different exposure conditions, aggregation and agglomeration potential, manufacturing residues, formulation stability, surface binders, possible interactions with characterization procedures).

    b. Make sure there is a rationale specifically mentioning the nanotechnological characteristics that may impact the selection of tests for the pre-clinical assessment of nanomedicines (chosen cell line, cell culture model, analyzed outcomes, test exposure time, etc.), as well as an explanation of every adaptation made to the tests, seeking alignment with the published/available methodologies, whenever possible. If this is not possible, the rationale behind the adaptation should be recorded.

    c. Rationale for choosing reference materials for toxicological tests.

9. Inclusion of all nanomedicine data in a database designed for this purpose.

Given that nanotechnology is comprehensive in scope and interdisciplinary in nature, ensuring the participation of those involved in its application and regulation is essential to improve the technical training in this area, reduce information asymmetry and streamline the process of knowledge incorporation in the country.

The late start of the regulation review for nanomedicines in Brazil, when compared to the FDA and EMA, can be used to our advantage. In view of all the information that is already available, the working groups already established and the experience of the countries that have already implemented some initiatives to regulate the approval of nanomedicines, it is expected that Anvisa's development speed in this area will be fast and that in a short time it can be well established and producing results.

CONCLUSIONS

With this study, we can conclude that the toxicological tests currently recommended by the regulatory agencies of the USA, the European Union and Brazil, albeit aligned, are not specific for the assessment of nanomedicines. In this sense, based on the available information, it cannot be taken for granted that the data generated by the requested tests are reliable for the establishment of a robust risk/benefit ratio for nanomedicines. Furthermore, many of the limitations of these tests and some suggestions for improvements in their conduction have already been demonstrated. However, this "homemade" process of adapting tests that should be "standardized" distorts the results and, consequently, makes it difficult to understand and correlate the generated data with those available in the literature, even though this process is useful to increase the suitability of the available guidelines.

On the same note, the importance of the bio-physical-chemical characterization of every nanomedicine submitted to analysis stands out, since, as demonstrated, one of the greatest challenges is the alignment between the definitions used by the research group for the classification of its nanomaterials – which also negatively impacts the data compilation process for the generation of evidence. We believe that the suggestions elaborated in the present study can strengthen the regulatory assessment process for nanomedicines.

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Authors' Contribution

Tobler JP - Planning (study design), data acquisition, analysis and interpretation and writing of the paper. Rocha HVA - Conception and writing of the work. All authors approved the final draft of the paper.

Conflict of interest

The authors report that there is no potential conflict of interest with peers and institutions, nor political or financial conflicts in this study.