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Silver nanoparticles as proapoptotic drugs: pharmacological basis in non-metastatic skin melanoma

Abstract

Purpose: The incidence of different types of skin cancer increases proportionally to population aging and environmental factors, among other causes. Melanoma-type skin cancer represents a serious health problem in the world, due to its invasiveness and the high mortality associated with its development. With the rise of nanosciences, the scientific community has access to tools that facilitate cell study and the development of promising technologies and products for diagnosis and treatment.

Aims: Update research on silver nanoparticles (AgNPs) as a pharmacological alternative for the treatment of malignant melanoma-type skin lesions.

Methods: Qualitative search based on the systematic review of the scientific literature, such as the Web of Science and Pubmed, based on keywords related to the subject under study and their impact on the solution of this growing health problem. Also, advances in the research of candidates for products based on AgNPs for topical therapeutic purposes.

Results: The review carried out confirmed that nanotechnologies and, specifically, the synthesis of AgNPs from natural sources represents a source of proapoptotic active ingredients with an impact on malignant skin melanoma cells, which points to the need for further studies in this promising field for the development of future drugs for this purpose. It was found that this is a topic in constant progress and inexhaustible that supports the priority in research from multiple approaches. AgNPs obtained from natural sources activate caspase 3 by different mechanisms. Currently, the results of biochemical (enzymatic), studies in cell lines and animal tumor models are complemented with *in silico* results to demonstrate the mechanisms of action of this new generation of antitumor drugs as an alternative for the approach of melanoma skin cancer. It is evident that other studies are needed to complement the development cycle of the candidates and specifically the cytotoxicity studies on healthy cells among other preclinical and toxicological studies before reaching the target species. This review aims to draw attention to the advantages of green nanotechnologies as advantageous alternatives that could positively impact human health in the near future.

Conclusion: AgNPs stand out for their physical-chemical and biological properties that impact malignant cells through different mechanisms. The demonstrated pro-apoptosis effect makes them promising candidates for the therapeutic approach of these topical lesions with a negative prognosis. Among the most innovative research is the search for pro-apoptotic active ingredients mediated by caspase 3. These compounds are opening a promising path due to the activity demonstrated *in silico*, on cell line assays and *in vivo* studies.

Keywords: silver nanoparticles, apoptosis, caspases, skin cancer, melanoma, cell line, mode of action

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Introduction

According to the World Health Organization (WHO), Noncommunicable Diseases (NCDs) are the cause of death of approximately 41 million people each year, which is equivalent to 71% of deaths that occur in the world.[1] Of these, an estimated 10 million are caused by cancer.[2] Among the different types of cancer, skin cancer has a high incidence globally and according to WHO estimates, around 3 million new cases are diagnosed each year.[3] Within these, melanoma, although less frequent, is the most aggressive with an incidence of more than 132 thousand cases in the world.[3]

In 2017, the World Health Assembly approved resolution WHA70.12 on prevention and control of cancer disease, in which member countries and the WHO were urged to accelerate solutions to achieve the goals of the World Plan of Action for prevention and control of NCDs 2013-2030 and the United Nations 2030 Agenda for Sustainable Development for the reduction of cancer mortality.[1] Therefore, these groups of diseases are critical aspects in health programs at national and regional levels, with a view to their prevention, early diagnosis and effective treatment. The search for new cytotoxic active ingredients on cancer cells is a research priority worldwide.

With the emergence of nanotechnologies, the scientific community has access to tools that are revolutionizing approaches for the discovery of new active ingredients, due to modifications in a property as important as particle size. With the use of these technologies, nanoscale sizes are achieved, with the consequent impact on the bioavailability of these molecules at the site of action.[4]

Numerous studies point to AgNPs as drugs on tumor cells by different mechanisms that demonstrate the powerful cytotoxicity on this type of cells.[5-8] The mechanisms of action described are the induction of programmed cell death processes mediated by caspases stands out,[7,8] in addition to other cell damage at the membrane level, activation of oxidative processes, changes in signaling pathways, among others.[5]

However, many of the technologies applied to obtain AgNPs, mainly based on physical and chemical processes, have disadvantages such as economic (high costs), technological (need for specialized equipment), and environmental (requirement to use highly toxic organic solvents and other aggressive chemical compounds). So, efforts are currently being made towards more feasible and environmentally friendly technologies. Such is the case of methods based on biological processes for obtaining AgNPs, with the bioreduction of metal cations standing out among the most used and promising methods for the formation of biogenic nanoparticles with antitumor activity,[5] with metal salts being (groups 10, 11 and 12) the most used as sources of the metallic cations of silver (Ag^{+}), gold (Au^{3+}), copper (Cu^{2+}) and zinc (Zn^{2+}).[5,9,10]

Dissimilar sources of reducing compounds have been reported in recent years. Botanical sources standing out due to the great diversity of phytochemical compounds and reducing secondary metabolites.[9,10] However, their industrial applicability largely depends on the sustainability of these bioresources. For this reason, numerous studies evaluate various sources that guarantee the required levels, such as microorganisms,[10] insects,[11] among others.

Our team recently reviewed the potentialities of AgNPs (synthesized by biological methods) as antimicrobial agents, as well as the many challenges to reach the end of the road that is clinical practice and really impact health.[4] Although it is a topic in constant progress and reviewed in recent years,[5,6] this review aims to update research on AgNPs as an alternative for the treatment of malignant melanoma-type skin lesions and exemplify this aspect of nanotechnological field.

Cancer incidence: Factors involved in its origin and development

The WHO defines Cancer as a broad group of diseases that can affect any part of the body, also named malignant tumors or malignant neoplasms, and that are characterized by the rapid multiplication of abnormal cells and whose growth extends beyond its limits, being able to invade adjacent parts of the body or spread to other organs (metastasis).[2]

Environmental or external factors include physical carcinogens, such as ultraviolet and ionizing radiation; chemical carcinogens, including asbestos, tobacco smoke toxins, arsenic, aflatoxins, among others; and biological carcinogens as bacteria, fungi, and some viruses such as human papilloma (cervical cancer), among others.[12]

Genetic factors determine the origin of the disease, since the malignant transformation of a cell takes place due to the accumulation of mutations in specific genes. These genes are grouped into two families: proto-oncogenes and tumor suppressor genes.[13] In this sense, numerous studies have demonstrated the role of specific genes in the development of different types of cancer.[14-19] The genetic atlas of cancer is a priority for cell and molecular biology with a view to discovering the different signaling pathways involved, and identifying new targets for the development of antitumor diagnostics and drugs.

Skin cancer

The skin is the largest organ in humans, accounting for approximately 15% of body weight in average-sized adults. It is made up of different types of tissues and of mixed embryological origins (ectodermal and mesodermal), which is why it is classified as a complex organ.[20]

The main functions of the skin is the protection of the body against external factors, the regulation of body temperature, the maintenance of fluid and electrolyte balance, endocrine and exocrine functions, the perception of stimuli, synthesis of vitamin D, among others.[20] This organ consists of three layers with specific functions: the epidermis, which is the outermost layer and is formed mainly by keratinocytes and melanocytes (synthesis of melanin), as well as Langerhans and Merkel cells;[20] the dermis, formed by a fibrous and elastic tissue, mainly made up of collagen and to a lesser degree of elastin; and the fatty or subcutaneous layer that helps to insulate the body from heat and cold, as well as a source of energy.[23]

Due to the exposure of this organ to dissimilar external factors, mainly solar radiation, its cells are prone to alterations in their reproductive cycle and frequently generate changes in cell division processes associated with proliferative processes that trigger some type of cancer.[21] Among the different types of cancer, skin cancer has a high incidence globally and according to WHO estimates, around 3 million new cases are diagnosed each year.[3]

Types of skin cancer

Skin cancer types are divided into two groups: melanoma or non-melanoma.[21] In this second group, basal cell carcinoma is described, which is the most frequent (approximately 75% of all cases in the world) and squamous cell carcinoma (approximately 20% of all cases in the world), other less frequent such as Merkel cell carcinoma, Kaposi's sarcoma, cutaneous lymphoma, etc.[21]

Melanoma, incidence, types, treatment

The generic name of melanoma comes from the Greek mélas "black" and oma "tumor", and has been used to name the malignant transformation of the pigmented cells of the skin: the melanocytes. In general, it is a highly invasive skin tumor due to its ability to generate metastases.[22,23]

The incidence of this serious malignant disease is increasing worldwide.[24] Melanoma represents 4% of all malignant skin tumors, although it is responsible for 80% of deaths from this type of tumor. It has an incidence of more than 132 thousand cases in the world.[3] The most common localization of melanomas are on the skin (95%) and less frequently (5%) on mucous membranes (oral, digestive tract, genital), retina or meninges.[25]

Melanoma has two phases of growth: radial and vertical. In the radial growth phase, neoplastic cells grow limited to the epidermis or superficial dermis.[22] This is an early stage in the development of the disease, and early diagnosis and appropriate treatment (eg. surgery) facilitate the patient's cure. In the vertical growth phase, in some types of melanoma, after a period that can vary between 1 and 2 years, the characteristics of the proliferation of cells in the dermis change, with the appearance of new cells (different clones) that spread, becoming arranged in nodules spheroidal that expand faster than the rest of the tumor. The resulting growth direction tends to be perpendicular to the radial growth phase; hence it receives its name of vertical growth phase.[22] In these cases where vertical growth appears, the prognosis is generally negative due to the infiltration of the lower layers of the skin that allow the dissemination of neoplastic cells through the lymphatic vessels to the regional lymph nodes, or through from blood vessels to any organ.[23]

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There are several ways of classifying the different types or subtypes of melanoma. Based on their genetic origin, they are classified as hereditary or familial melanoma and non-familial melanoma.[26] Based on the clinical and histological features, five main subtypes are currently recognized:

Superficial spreading or flat melanoma is the most common (about 70%) is the which is often flat and irregular in shape and color, with variable shades of black and brown; predominates in white skin and young population.[21,22] Nodular melanoma is the most aggressive (between 10 or 15%) and usually begins as a raised area of dark blue-blackish or red-bluish color; although some have no color at all.[21,22] Lentigo maligna melanoma is more common in elderly people (between 10 or 15%), or in sun-damaged skin, on the face, neck and arms. Abnormal skin areas are usually large, flat, and brown with tan areas.[21,22]

The acral lentiginous melanoma is the least frequent form of melanoma and they are usually located on the palms of the hands, the soles of the feet or under the nails, it is more common in black people.[21,22] Desmoplastic melanoma is a rare malignant melanoma (0,4 -4%) marked by non-pigmented lesions on sun exposed areas of the body.[27] Also, there are several rarer variants of melanoma, such as amelanotic and polypoid melanomas, which constitute less than 5% of cases.[24]

Due to the aggressiveness of this type of malignant neoplasm, diagnosis is essential in clinical dermatology. In general, the primary diagnosis is made in specialized dermatology consultations or in primary care.[22] In this sense, morphometric studies are necessary due to the value they provide in histological diagnosis to improve the prognosis of the disease.[26]

Treatment

Treatment in patients with malignant melanoma is a priority issue in the specialties of Dermatology and Oncology due to the high associated mortality. For this reason, interdisciplinary evaluation between specialists in surgery, pathology, nuclear medicine, and oncology is recommended.[28] Early diagnosis is essential for the cure of melanoma. The treatment consists of three variants: surgery; adjuvant treatment with immunotherapy and treatment with chemotherapy or immunotherapy for metastatic melanoma.[25]

The effective treatment in the case of primary melanoma is excision, after defining the margins of the lesion with precision, which is essential for the success of the surgical procedure, to avoid local recurrence and the outcome in metastasis. In this sense, a prospective study by the World Health Organization showed that melanomas up to 2 mm thick can be safely resected with a margin of 1 cm without affecting patient survival.[22]

Adjuvant treatments after surgery are recommended when there is a poor prognosis in high-risk patients, with recurrence rates between 50 and 80%. These treatments include: chemotherapy, nonspecific immunotherapy (treatment with bacillus Calmette-Guerin, levamisole), active specific immunotherapy, immunochemotherapy, isolated chemotherapy infusion in one limb for melanomas of the extremities, and radiation therapy; however, authors agree that these therapeutic options have not improved patient survival.[30]

Authors recommend that high-risk patients (stages IIB, IIC and III) should be evaluated for adjuvant treatment with high doses of interferons, to avoid relapses in these patients.[25] Patients with metastatic melanoma should be evaluated individually.[25] One of the products applied is chemotherapy with dacarbazine (also known as DTIC), which consists of a standard systemic treatment that provides a 20% favorable response; In addition, there are other combinations of cytotoxics and immunotherapy with DTIC that increase the adequate reaction rate, but not survival, since they produce greater toxicity, so they are not recommended.[25]

Among these, in different phases of development are Ipilimumab, Vemurafenib, Temozolomide, among others. Likewise, numerous signal transduction inhibitor candidates are being studied, such as: BRAF inhibitors (vemurafenib or dabrafenib), MEK inhibitors (trametinib or cobimetinib), as well as combinations of these.[25]

Among the Immunotherapy options, different strategies have been described, such as: non-personalized immunotherapy such as monoclonal antibodies against tumor antigens (anti-CD19, CD20); cytokines that enhance antitumor responses (IL-2, IFNa); and inhibitory receptor blocking antibodies (PD-1, PD-L1, CTLA-4).[24]

Melanoma genetics

Knowing the advances in genetic studies and the cellular processes involved is of great importance for the design of safer and more effective drugs, and to anticipate their possible modes of action.

Gen BRAF

Numerous genes have been studied with involvement in the development of melanoma.[31] The BRAF gene is a proto-oncogene located on chromosome 7q34 made up of 18 exons. It codes for a serine/threonine kinase of 84kDa and 766 amino acids. The BRAF protein belongs to the RAF family. This family is made up of three kinase isoforms, ARAF, CRAF (RAF-1), and BRAF, which activate the MAPK/ERK signaling pathway. The constitutive activation of the MAPK pathway by these oncoproteins induces abnormal growth and resistance to proapoptotic signals.[32] Authors, almost twenty years ago, detected four types of point mutations in BRAF analyzing 15 cancer cell lines.[33] Two are located in exon 15 that affect the activation segment of the protein: T1796A, which gives rise to the V600E mutation, and C1786G, which generates the L596V substitution. The other two are located in exon 11, located in the G loop: G1388T, which gives rise to G463V, and G1403C, which gives rise to G468A.[33]

Mutation V600E

It consists of substituting valine for glutamate at position 600 of the BRAF protein.[34] Specifically, it is an alteration that occurs in exon 15 at nucleotide position 1796. It is a transversion of thymine to adenine that at codon 600 gives rise to a substitution of a valine for a glutamine.[33]

The V600E mutation induces constitutive activation of BRAF and generates constitutive signaling of the RAS/RAF/MEK/ERK pathway, activating the transcription of genes related to telomerase induction, growth factor secretion, the ability to invade and metastasize, evasion of apoptosis and resistance to chemotherapy. This mutation is also associated with transcriptional inactivation of the hMLH1 repair gene due to hypermethylation of its promoter, but not with its germline mutation.[35]

Melanomas carrying the V600E mutation are characterized by lower expression of the cyclin-dependent type 2A kinase inhibitor (CDKN2A, p16 or MTS1),[36] for which the function of p16, which is to block the transcription of important regulatory proteins of the

cell cycle and cause its rest, preventing the cycle from progressing from G1 to S, that is, inhibiting the proliferation of the cell that is affected.[37] For this reason, authors consider that the loss of expression of the p16 protein correlates with invasive and metastatic melanoma,[38] hence tumors that present the BRAF mutation generally have a worse prognosis.[39]

The MAPK (mitogen-activated protein kinase) intracellular signaling pathway, also known as the RAS/RAF/MEK/ERK pathway, is involved in the genesis and progression of melanoma.[40] This pathway is responsible for translating signals from the activation of a variety of growth factor receptors, to convert them into cellular events. The signal starts after the binding of cell membrane receptors with their ligand. After binding of the ligands (growth factors to their respective tyrosine kinases), receptor dimerization triggers its intrinsic tyrosine kinase activity, autophosphorylating specific tyrosine residues in the intracellular portion of said receptors. The receptors actívate RAS by recruiting small cytosolic adapter proteins Grb-2 that associate with SOS (guanine nucleotide exchange factor RAS) and convert the RAS protein from inactive RAS-GDP to active RAS-GTAP. Activation of RAS (small G protein) occurs, which has three isoforms (NRAS, HRAS, KRAS). These form complexes with RAF proteins and their activated form phosphorylates MEK, which is a serine/threonine kinase present in two isoforms (MEK1 and MEK2).[40]

Most of the mutations that occur in BRAF increase the function of the kinase (hyperphosphorylation of MEK/MAPK), although there are other less common forms whose activation capacity is reduced to phosphorylate. These mutations imply the permanent activation of this pathway. Mutations in BRAF are essential to initiate the development of melanoma, but they are not sufficient to justify the definitive transformation of melanocytes.[41]

Diagnostic implications and treatment of the mutation of BRAF V600E

Some years ago, Uruguayan authors developed an investigation in which the V600E mutation was determined by ASO-PCR (allele specific oligonucleotide-polymerase chain reaction) in 28 samples; obtaining as results when amplifying the deoxyribonucleic acid (DNA) in 27 of the 28 samples and the mutation was detected in 21 of them.[42] Two years later, Argentine researchers demonstrated in the analyzed population of patients with cutaneous melanoma (CM) a frequency of the mutated BRAF V600E oncogene of 77%, higher than previously reported, which ranged from 17-72%.[43] Among patients with BRAF V600E mutated tumors, a prevalence of BRAF V600E mutation (98%) was observed, followed by BRAF V600K (2%). The results obtained in the study population indicate that the mutational status of the BRAF oncogene presents an association with clinical-pathological parameters related to tumor progression.[43]

Likewise, no significant associations were found with other clinicopathological characteristics or with clinical evolution in the study population, so the authors opened the possibility of considering the mutational status of the BRAF oncogene as a prognostic factor, in order to improve the staging of cutaneous melanoma. Other more recent studies on the BRAF V600E mutation in primary and metastatic cutaneous melanoma confirmed the involvement of this gene in the progression to more severe forms of this type of skin cancer.[44,45]

Apoptosis in the skin cancer

The term apoptosis was used, for the first time, to describe a form of cell death that is morphologically different from necrosis and is a process mediated by a set of enzymes called caspases. It is a balancing process that enables a physiological balance of cells and is essential in numerous processes from embryogenesis, neuronal synaptic connection, the development of the immune response, the elimination of cancer cells, infected or damaged by toxic agents, among others.[46]

Caspase 3 as a therapeutic target in the search for pro-apoptotic drugs

In the cell cytoplasm, apoptosis is mediated through caspase proenzymes and when these are activated, a proteolytic cascade is initiated that leads to cell death. There are three pathways of activation of apoptosis:[5]

a) Extrinsic or through cell death receptor,

b) Intrinsic or mitochondrial, and

c) Granzyme

As caspases, a large family of cysteine protease-type enzymes are identified, which around 14 are known, divided between initiator caspases (caspases 8, 9 and 10) and effector caspases (caspases 6 and 7). Initiator caspases converge on caspase 3 which activates effectors leading to cell death. The main target of effector caspases is the enzyme poly-adenosyldiphosphate-ribose polymerase (PARP), which is involved in DNA repair processes, cell survival, proliferation and differentiation, among others.[5,48-51]

Caspases and melanoma

There is considerable evidence linking the activation of caspase pathways with melanoma control. Pharmacological variants that combine inhibitors of the BRAF and MEK genes are used in the clinic to treat melanoma. In this sense, studies in melanoma cell lines have shown that these drugs induce the activation of caspase 3.[48-51] It was recently shown that the intrinsic apoptotic pathway is related to a new form of cell death called pyroptosis[52,53] in which caspase 3 is also involved.[48] Pyroptosis is a recently discovered form of programmed inflammatory necrosis and is characterized by caspase 1-mediated and gasdermin D-dependent cell death that is involved in the release of inflammatory cytokines such as interleukin-1 beta (1L-1β).[54] The discovery of substances that activate apoptotic pathways lead to antiproliferative agents.[55]

Dysregulation of apoptosis in melanoma

Something distinctive in human melanoma is the tendency of malignant cells to evade programmed cell death, due to the ability to resist the induction of cell death, provoking a survival advantage for malignant cells.[56] One theory explains that resistance to programmed cell death may be caused by loss of expression or function of pro-apoptotic molecules and/or high expression of proteins that inhibit programmed cell death.[57]

Silver nanoparticles: Studies in melanoma cell lines

Metal nanoparticles (MPNs) are considered among the most efficient for biomedical applications due to their use as an imaging resource and their multifunctional theranostic capabilities, such as their antibacterial, antitumor, and drug-carrying properties. In recent years, interest in the development of NPM has grown due to its high chemical activity and specificity in the interaction. Currently, the NPs of iron, silver, gold, zinc and copper are the most studied due to their properties.[55] These are structures with sizes less than 100 nanometers (ie $1x10^{-7}$ meters), which can be synthesized from different materials.[56,57]

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Authors consider that the green synthesis of AgNPs opened a new era for the diagnosis and treatment of cancer.[5] In terms of treatment and diagnosis, nanoparticles are used due to their unique shape, size, and optical and thermal characteristics.[17,18] These exceptional properties of metallic nanoparticles, which are due to a particular size and high surface area to volume ratio, make them ideal for many biological applications, including theranostics.[58,59]

The use of silver nanoparticles as a potential drug carrier in cancer treatment has recently received considerable attention, as remarkable nanotechnology research has opened new avenues for drug, treatment, and diagnosis[60] been focused on obtaining other forms of AgNPs with different physical-chemical and biological properties that make it possible to enhance their biological activities (cytotoxic, antimicrobial, etc.), while reducing their toxic effects on cells of healthy tissues.[61]

This is possible due to the cytotoxic activity of these nanoparticles. The cytotoxicity of nanoparticles is defined as the extent to which interaction with cells alters cellular structures and/or processes essential for cell survival and proliferation. Cytotoxicity assays are a quick and easy way to perform initial assessments of acute toxicity. Combining nanoparticle cytotoxicity data with other safety test data can help predict nanoparticle biocompatibility.[62]

In recent years, studies of the cytotoxicity of AgNPs on cell lines of different types of cancer, including investigations in melanoma-type skin cancer (Table 1), have taken off, placing them among the new therapeutic alternatives with a promising future for the treatment of this type of topical lesions.

Table 1 Examples of cytotoxic AgNPs evaluated in melanoma cell lines

Type of AgNPs evaluated/ Source	Particle size	Cytotoxic activity assays	Melanoma cell line	Effective dose
AgNPs/Carboxymethyl-cellulose hydrogel Doxorubicin	10±3 nm	In vitro cell viability	A375	Synergistic effect[63]
		mitochondrial activity		
		DNA staining assay		
		Cellular uptake bioimaging and DOX-tracking		
		Charged Hybrid Hydrogels		
AgNPs polyvinylpyrrolidone	35±15 nm	cell viability	B16-F10	42µg/mL[64]
		Induction of apoptosis and necrosis		
		ERO generation		
AgNPs/Aloe vera	50-100nm	In vitro cell viability	F10B16	100µg/mL[65]
AgNPs/Olax scandens	55-85 nm	In vitro cell viability	B16	2.5µg/mL[55]
AgNPs/hojas de Annona muricata leaves	19.63±3.7 nm	In vitro cell viability	A-375	8.404 µg/mL[66]
AgNPs/Peel of Annona muricata	16.56±4.1 nm	In vitro cell viability	A-375	2.943µg/mL[66]
AgNPs/Galium aparine	35-110 nm	In vitro cell viability	A375	77.03µg/mL[67]
AgNPs/sodium borohydride/collagen	140.7±7.8nm	In vitro cell viability e in vivo	MV3	4,35µg/mL[68]
Commercial AgNPs	10nm	In vitro cell viability	B16F10	1.5 to 5mg/mL[69]
AgNPs/Butea monosperma	20-80nm	In vitro cell viability	B16F10	dose dependent[70]
AgNPs/Zinnia elegans	95nm	In vitro (Transwell Migration, Cell Cycle, Apoptosis) In vivo (Biodistribution Study in Tumor Model, Hemolysis)	B16F10	dose dependent[71]
Ag@TiO_2 core–shell NPs/ two-step method and coated with TiO_2 to obtain Ag@TiO_2 NPs by a facile sol-gel method	5nm	In vitro (cytotoxicity B16-F10) In vivo (melanoma tumor model in mice C57BL/6J)	B16F10	dose dependent[72]

Bioinformatics for mode of action studies

Bioinformatics is a science that arose from the need to interpret the information contained in the sequences of DNA, RNA and proteins. Advances in computing technologies and DNA and protein sequencing techniques increased the volume of sequences in data banks, for which the need arose to develop algorithms to catalog these sequences, analyze the similarities between them, as well as discover its structural and functional properties.[73]

Bioinformatics is an interdisciplinary science and is based on biology and computer science. However, it draws on other sciences such as physics, chemistry, mathematics, statistics and probability.[73]

In the field of pharmacology, in which in vitro experiments are costly and in vivo experiments encounter more and more bioethical limitations, bioinformatics, although still limited, is a valuable tool for validating hypotheses and/or creating new hypotheses of the interaction of ligands (inhibitors, modulators, activators) with therapeutic targets (enzymes), and thus streamline research and rule out unpromising candidates.

As part of the studies of the cytotoxic mode of action of AgNPs on tumor cell lines, numerous results have been reported in recent years (Table 2) demonstrating in silico the effect of these nanostructures on targets involved in proliferative, anti-inflammatory and anti-inflammatory processes. programmed cell death (apoptosis).[74]

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Table 2 Examples of in silico mode of action studies of AgNPs

Type of nanoparticle/ Source	Enzyme target(s)	Cellular process involved/ Disease	Positive control	E (kcal/mol) o Kapp (L mol^{-1})
Silver oxide nanoparticle (Ag 2 O-NPs)/ Lagerstroemia indica	Caspase 3	Apoptosis/ Human cell lines	isatin sulfonamide	0.96931 kcal/mol[74]
AgNPs/Coral Nephthea	COX-1 (pdb code: 5WBE)	anti-inflammatory/ Cancer	Mofezolac Rofecoxib	COX-1 (- 7.6- - 4.7) kcal/mol
	COX-2 (pdb code: 5KIR)			COX-2 (- 7.6- - 4.6) kcal/mol[75]
AgNPs/*Andrographis peniculata*	caspase-3 and caspasa-3	Apoptosis/ Human cell lines	NR	Caspase 3 (+ 1.62 kcal/mol)
				Caspase 9 (+0.29 kcal/mol)[76]
AgNPs	DNA	DNA damage/ Cancer	NR	1.6×104 L mol^{-1} [77]
AgNPs/*Acalypha wilkesiana*	Topoisomerase I-Human DNA	Cancer	NR	−8.90 a −7.80 kcal mol^{-1} [78]

NR, no reported

Other bioinformatic strategies applied to caspase 3 are the prediction of cleavage (cleavage) sites.[79,80] A broader understanding of caspase substrates is essential for a more detailed understanding of the biological functions of these enzymes. The identification of the cellular targets of caspase-3 is crucial to delve into the cellular mechanisms that have been implicated in various diseases such as cancer, neurodegenerative diseases and immunodeficiencies.[81]

The relatively high variability in the cleavage site recognition sequence often complicates identification of these. The Peptid Cutter program provided by the ExPasy server (http://www.expasy.org/tools/peptidecutter) takes into account the queried sequence with the possible cleavage sites mapped on it and/or a table of cleavage site positions. Lohmüller et al.,[82] implemented a bioinformatics tool that is limited to caspase 3 and cathepsin B and -L substrates. Subsequently, GraBCas emerges, which provides a prediction based on the score of the possible cleavage sites for caspases 1-9, which includes an estimate of the size of the fragment.[83-86]

Future perspectives

Nanotechnology and specifically phytonanotechnology constitute relatively young lines of materials science, which could positively impact the pharmaceutical field and health in general, so there are still many answers to be found in this promising field. Current and future studies directed in search of scientific evidence that supports its introduction in clinical practice for human use, focused on: pre-clinical studies (Pharmacokinetic- pharmacological- toxicological) in cell lines and animal models; clinical trials; optimization of clean technologies and full cycle; study of sources of sustainable reducing compounds; action mechanisms; bioinformatics and mathematical modeling of the interaction NP-cellular proteins and NP-cell signaling molecules; nanoparticle size-bioavailability ratio; design and development of poly-action formulations; eco-toxicology; characterization studies (physical, chemical, morphological); regulatory framework; among others.

Toxicological considerations

Although the reduction of particle size in active substances, and even in excipients and other auxiliary substances that are part of pharmaceutical formulations, have been historical goals for pharmaceutical technologists and researchers, due to the implications it has on the bioavailability, there is currently no consensus on the advantages of nanotechnologies and the substances derived from their use, which points to the need for further studies in this field.

The registration of a pharmaceutical product entails, depending on the intended purpose, the demonstration of requirements that guarantee its safety and efficacy, and that complement the knowledge of the delivery system of the active ingredient at the site of action (local or systemic). In this sense, toxicological studies are mandatory requirements within the drug development cycle, and in the case of nanoparticles in general, and NPM in particular, these studies are in their "infancy".

The demonstration that nanoscale structures have different physical and chemical properties requires the demonstration of their behavior in biological systems. Theoretically, the smaller the particle size (greater the surface area per unit volume), which leads to a greater impact on the site of action. It suggests a lower dose to achieve the therapeutic effect. However, this rule is not always fulfilled in active nanostructures, so bioavailability, bioequivalence, and pharmacokinetic studies are essential to complement the dose/response findings under the influence of particle size.

On the other hand, although the use of formulations with metallic elements as active drug ingredients is ancient (eg, ferrous fumarate for the treatment of anemia, silver sulfadiazine as a topical antibiotic, copper salts against bacteriosis in seeds, etc.), there are currently few scientific results on toxicological and eco-toxicological studies of active ingredients and formulations based on metallic nanoparticles.

The benefit-risk analysis, the dose, the specificity of action of the drug and the individualization of the therapy must constitute essential principles in the therapeutic approach to this serious health problem that is currently unresolved and that constitutes a challenge for the scientific community.

Final considerations

The synthesis of metal nanoparticles (NPM) from plants constitutes a current and future line of research with a view to complementing other conventional approaches for the discovery of new effective and safe active ingredients for the treatment of life-threatening tumor diseases.

Another aspect of vital importance is the need for verifiable regulatory requirements that guarantee the design and development of quality products that have demonstrated their safety and efficacy. The existence of a great variety of promising botanical species for this purpose should lead to the use of renewable sources that guarantee sustainability on an industrial scale.

Several authors reviewed the state of the art on this fascinating topic, and new scientific evidence is provided every day on new horizons and strategies for the therapeutic approach of this complex

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family of diseases through the design and development of more effective and safer therapeutic agents based on plants and other natural sources rich in valuable phytochemical compounds for these purposes[5] and the methods of synthesis and characterization of these structures. In our opinion, there is still a lack of evidence to reach the safe systemic administration of the many candidates that are currently under study. However, the possible local administration by topical route (antimicrobial and antitumor) (Example: melanoma of the skin), once the studies that guarantee its safety and efficacy are completed individually, is closer.

Accessing unknown levels of matter will always be a driving force for science makers. In this sense, too, nanotechnology represents a gateway to this endeavor. Nanotechnologies and the products derived from their use have shown that they constitute promising variants to contribute to the solution of health problems not resolved with the available means, and that they can complement other previous approaches, but their introduction in clinical practice will have to go through the path that elucidates the unknowns related to the safety of man and the environment.

Ethics approval and consent to participate

All authors have read and agreed the ethics for publishing the manuscript.

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Conflicts of interest

There are no conflicts of interest to be declared.

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