Advances and challenges in retinoid delivery systems in regenerative and therapeutic medicine

Running title: RA delivery systems

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Highlights

- Recent discoveries of retinoid action in cancer, stem cell and immune cell biology have been translated into a high number of clinical trials
- Controlled release systems are important for clinical translation of retinoids because they increase its aqueous solubility and lifetime in the bloodstream, while decreasing RA photo-sensitiveness, cytotoxicity and side effects
- The properties of retinoid-controlled release systems (i.e. physicochemical, release, targeting and uptake/intracellular trafficking properties) vary according to their ultimate biomedical applications
- Further developments in retinoid controlled-release systems are needed to increase organ/tissue/cell targeting, as well as capacity to release retinoids in combination with other drugs

Abstract

Retinoids regulate a wide spectrum of cellular functions from the embryo throughout adulthood, including cell differentiation, metabolic regulation and inflammation. The development of retinoid delivery systems offers several advantages for clinical translation of retinoid-based therapies, including improved solubilization, prolonged circulation, reduced toxicity, sustained release and improved efficacy. In this Review, we discuss advances in preclinical and clinical tests regarding retinoid formulations, specifically the ones based in natural retinoids, evaluated in the context of regenerative medicine, brain, cancer, skin and immune diseases. Advantages and limitations of retinoid formulations as well as future prospects will be presented.

Keywords

Retinoid, retinoic acid, delivery systems, cancer, inflammation, brain pathologies, skin diseases

1. Introduction

Retinoic acid (RA) signaling is one of the most important biological pathways in nature, triggered by RA interaction with nuclear receptors that control gene expression. The chemical structure of retinol (vitamin A, a RA precursor) was first described by Paul Karrer in 1931[1], who was awarded a Nobel prize in 1937 for the discovery. The use of RA for skin disorders[2] and cancer treatment (acute myeloid leukemia[3] and cervical neoplasia[4,5]) started in the 1960´s and 1980´s (**Figure 1**). In 2000´s, RA had been incorporated in many tissue engineering scaffolds as a stem cell differentiation agent[6,7]. Over the last 10 years, many discoveries related to the biological role of RA in controlling the biology of hematopoietic stem cells[8,9], tumor-initiating cells[10-12], immune cells[13], intestinal mucosa wound repair[14], cancer resistance[15] and cell reprogramming and differentiation[16,17], have further stimulated interest in this drug for many other biomedical applications. This interest is confirmed by more than 50 active clinical trials (according to ClinicalTrials.gov) evaluating the effect of RA in cancer (28 trials), mostly in hematological (16 trials) and brain tumors (8 trials), skin pathologies (e.g. acne, photoaging, eczema) (5 trials), as well as in other conditions such as inflammation, olfactory loss and neuropsychiatric diseases (**Table 1**).

Clinical applications of RA have highlighted three main limitations of its pharmacological use. First, RA is poorly soluble in aqueous solutions[18] and photosensitive[19], which makes its administration challenging. Secondly, RA induces irritation when applied onto skin and increases its catabolism, when it is administered intravenously, reducing its therapeutic efficacy[20]. Lastly, RA is involved in many biological processes and thus the systemic delivery of RA causes side effects. All these limitations motivated researchers to synthesize novel and better tolerated synthetic retinoid compounds and to develop retinoid delivery formulations based on gels, liposomes, microparticles, nanoparticles, and micro-/nanofibers which, in some cases, have been modified to target specific tissues and cells of interest [21].

Recent developments in the use of retinoid for cancer treatment[22,23] and differentiation studies using stem cells[8,24], as well as in the development of more advanced formulations to control its bioactivity[25,26] makes this review timely. In addition, with the exception of a limited number of reviews, with a restricted scientific scope in retinoid formulations[27] or highlighting the importance of retinoids in development[28] or for specific therapeutic applications[29], no study has fully covered the application of retinoid formulations for therapeutic and regenerative medicine applications. In this review, the role of retinoid formulations in the context of the brain, skin, immune system and cancer applications will be discussed. For each application, the pathological context will be briefly presented as well as the effect of retinoid administered without any controlled release system. The reasons behind the development of retinoid formulations in each application will be presented, as

well as their benefits and limitations, considering retinoid solubility, photostability, biocompatibility, release profile, tissue/cell availability and targeting, and therapeutic efficacy.

2. Retinoid chemistry and general overview on RA signaling

2.1. Retinoid chemistry

Retinoids are a class of compounds composed by 3 regions: a hydrophobic, a central polyene and a polar (usually a carboxyl group)[29]. There are 3 natural retinoids: all-*trans* RA (ATRA), 9-*cis* RA (alitretinoin) and 13-*cis* RA (isotretinoin). To decrease the toxicity of the natural retinoids as well as to increase their stability and selectivity against a specific RAR subtype, several semi-synthetic and synthetic retinoids (including "atypical retinoids") have been developed[29,31]. Some of them have been tested in clinical trials and approved for therapy (**Table 2**). At least 4 molecules have reached phase 4 clinical trials: bexarotene, tazarotene, adapalene and trifarotene[29,31,32]. Bexarotene has optimal RXR binding, which effectively causes cancer cell death, particularly in cutaneous T cell lymphoma. However, high levels of serum aminotransferase and liver injury have been reported[33]. Tazarotene and adapalene formulations have high affinity and selectivity for RARβ and RARγ, although the first one showed more toxicity than the second in dermatological applications[34]. Trifarotene is a selective RAR-γ agonist, approved in late 2019 in the USA, for the topical treatment of acne in patients as young as 9 years old[32]. Despite the progresses made in the last years in the synthesis of novel retinoids to increase natural retinoid efficacy while reducing their toxicity, it is evident that natural retinoids are still under intense scrutiny as demonstrated by a considerable number of clinical trials (**Table 1**). It is likely that the reasons are combinatorial: RA is a natural drug, blocks multiple disease-signaling pathways[12], in opposition to some synthetic retinoids that are very selective to a single receptor-mediating signaling pathway, and it has been used for many years in combinatorial therapy and thus well known by clinicians. Because it would be difficult to cover all advances made in the area of retinoid delivery systems in a single review, the authors have chosen to cover only natural retinoids in the present manuscript. This focused approach is also supported by recent progresses in RA biology and many formulations developed in the last years for the delivery of RA.

2.2. RA signaling

RA is the main biologically active metabolite of vitamin A[28]. In humans, the only source of vitamin A is obtained through diet, as lipophilic retinol (or its more stable form, retinyl ester) or as carotenoids. The transport of these retinoids to cells occurs when blood circulating retinol is bound to retinol-binding protein (RBP) 4 (**Figure 2**). This complex interacts with membrane transporter

and receptor *stimulated by retinoic acid 6* (STRA6) facilitating entry into the cytoplasm, where retinol binds to Crbp1 (encoded by RBP1). A two-step process converts retinol into ATRA. RA binds to cellular retinoic acid binding-proteins (CRABP) , assisting autocrine and paracrine signaling[35]. The mechanisms underlying paracrine signaling remain unclear while autocrine signaling requires CRABP2 for nuclear entry[28,35]. In the nucleus, RA triggers gene transcription by binding to heterodimers formed by RA receptors (RARα, RARβ and RARγ) and retinoid X receptors (RXRα, RXRβ, and RXRγ). RAR-RXR heterodimers interact with a deoxyribonucleic acid sequence known as the retinoic acid-response element (RARE)[36], which facilitates the binding of co-activators with histone acetylase activity, ultimately leading to the transcription of target genes (**Figure 2**). Recent data showed that RXRα homodimers can also regulate gene transcription[37]. Another isomer of RA, isomer 9-*cis* RA (alitretinoin), binds to retinoid X receptors[28,36], while 13-*cis* RA (isotretinoin), has negligible affinity for retinoic acid receptors (RAR or RXR) or for cellular RBP[38]. However, 13-*cis* RA may be converted into molecules that act as agonists for nuclear RAR and RXR. Importantly, RAR also regulates non-nuclear and non-transcriptional effects, namely the activation of kinase signaling pathways[39] (**Figure 2**). Finally, RA is catabolized by monooxygenases of the cytochrome P450 superfamily[35].

3. Formulations for the delivery of RA

RA has very low solubility in aqueous solution (0.21 μM at pH 7.3)[18] and thus requires specific binding proteins (e.g. CRABPs) to be transported within cells to act at nuclear receptors. In addition, it has a short (few hours) lifetime, due to its degradation by a cytochrome P450-dependent monooxygenase system[40]. Moreover, it induces undesirable side effects (congenital malformations[41] as well as mucocutaneous dryness, headache, and hypertriglyceridemia[42]) when administered at high concentrations. Therefore, for more than 35 years, several groups have developed different RA delivery systems to overcome these limitations. Delivery systems based in polymeric scaffolds (e.g. hydrogels, nanofibers), nanoparticles (liposomes, micelles, polymeric, dendrimers), microparticles, among others, are described and summarized in **Table 3**.

3.1. Type and strategies for the delivery of RA

Several strategies have been used to prepare RA delivery systems: namely, by complexation of RA with proteins (e.g. transthyretin)[43] or cationic polymers (e.g. poly(ethyleneimine)(PEI))[44,45], by physical encapsulation in polymeric[25,45] or inorganic[46] nanoparticles, microparticles[47], micelles[48,49], liposomes[50] or films[44], by covalent attachment of RA to a carrier[51], or by immobilization of RA on surfaces of nanoparticles[46], among others. Despite several approaches

having been reported of RA delivery systems based on polymeric scaffolds or electrospun fibrous meshes, the most common strategy for RA delivery has been based on liposomal or polymeric nanoparticles formed by polyesters, polyimines, polysaccharides and proteins (**Table 3**). A significant number of formulations have allowed the RA encapsulation in the core of the nanoparticles. This RA encapsulation was either obtained by (i) physical or (ii) chemical interaction with the components of the nanoparticle or (iii) by physical entrapment. Regarding physical interaction, RA is complexed with positively charged polymers such as chitosan, or PEI[44,52]. The carboxylic acid of RA interacts electrostatically with the amine group located in the polymer, forming a complex that can be stabilized by addition of a polyanion and divalent ions[44,45]. Concerning chemical interaction, RA is typically conjugated chemically to one of the components of the nanoparticle by biodegradable ester[51,53], amide[26,54-56] or disulfide[57] linkages. These bonds are susceptible to degradation during specific pH conditions, in presence of proteases, or reducing agents leading to the RA release. Concerning physical entrapment, RA is captured during nanoparticle formation[58,59] or in the pores of the nanoparticles[60]. In a relatively low number of formulations, RA was immobilized not in the core but on the surface of the nanoparticle[22]; however, the immobilized concentration of RA is lower than the ones having RA in the core. Formulations with high (above 100 μg/mg of formulation)[52,57,61], medium (between 100 and 25 μg/mg of formulation)[45,46] and low (below 25 μg/mg of formulation)[7,59] loading have been described.

The release profile of RA depends on several factors of the formulation, including the size, composition, initial concentration of RA loaded and its degradation profile. Studies have reported the sustained release of RA for more than 1 month by encapsulating it into biodegradable microspheres and tuning the release rate by adjusting polymer composition in the formulation[62], by the encapsulation in polyion complex micelles[63] and adjusting polymer composition or drug content, or by the encapsulation in liposomes[50]. In general, formulations with high RA loading showed a slower release profile of the drug[52]. Because of the hydrophobicity of RA, the occurrence of a burst release in most formulations is negligible[45,48,52,64].

Recent developments to improve the delivery of RA have led to the design of formulations that can be controlled remotely (temporally and spatially) by an external stimulus such as light, ultrasound or magnetic forces[65]. These stimuli-responsive biomaterials are suitable to control RA kinetics delivery. In that sense, light-activatable nanoparticles containing RA that disassemble in minutes after activation by a blue laser at 405 nm have been prepared[25,26,61]. Importantly, the formulations presented higher activity than formulations in which RA was released by passive diffusion (not light-triggered), because they rapidly saturated nuclear receptors.

3.2- Capacity of RA-containing formulations to overcome biological barriers

The cellular uptake of some RA-containing nanoparticles has been demonstrated to take below 12 h[45] by clathrin-mediated endocytosis and macropinocytosis[25]. Formulations that escaped the endolysosomal compartment accumulated in the cytoplasm in less than 24 hours[25,46]. It is possible that RA released in the cell cytoplasm binds to transport proteins such as cellular retinoid-binding protein II (CRABP-II) and/or fatty acid-binding protein 5 (FABP5), followed by its transportation to the cell nucleus[66]. The intracellular concentration of formulations containing RA was dependent on the initial formulation loading, type of formulation, and type of cell[25,46]. Uptake between 25 and 80 pg of nanoparticles containing RA *per* cell has been described[25].

The capacity of RA-containing formulations to cross biological barriers such as the blood-brain barrier (BBB) (relevance for the treatment of brain cancer and neurodegenerative disorders) is a topic largely unexplored. In most cases, the formulations have been administered by stereotaxic and not intravenous administration[67]. Experimental data in humans indicate that ATRA administered orally is not able to accumulate in the cerebrospinal fluid[68]. Clinical trials such as NCT00528437 and others are now investigating the pharmacokinetics of 13-*cis* RA and its accumulation in the cerebrospinal fluid.

3.3. Pre-clinical and clinical applications of RA-containing formulations

Formulations containing RA have been used both in pre-clinical and clinical trials (**Figure 3**). Most pre-clinical tests were performed in mice in the context of regenerative medicine[46,61,67,69] and cancer applications[22,25,57]. *In vitro* tests showed that ATRA-containing liposomes[70] or ATRA-containing polymeric nanoparticles[25,71] were 100-1000 times more active than soluble ATRA in cultured tumor cells[25,70] or neural stem cells[71]. *In vivo* tests showed that rats[50] or human patients[72] treated with ATRA-containing liposomes by intravenous administration had no decrease in plasma ATRA levels while animals/humans treated with oral formulation of ATRA (non-liposomal) had a significant decrease in plasma ATRA levels[42,50]. The results indicate that the hepatic metabolism of ATRA encapsulated in liposomes was inferior to the one observed in ATRA administered orally. Both formulations were safe in human trials. The clinical application of RA-containing formulations can be divided into 2 groups: (i) topical and (ii) oral administration. For topical administration, seven RA-containing formulations have reached the market for the treatment of skin-related diseases (**Table 4**). Here, current progresses are concentrated in reducing the toxicity of RA (e.g. by the use of synthetic retinoids[32]), and exploring the combination of RA with other drugs (see **Table 4**). For oral administration, there are four formulations containing RA that have reached the market, particularly for the treatment of skin diseases (three of them) and cancer (only one)

(**Table 4**). Others were tested in clinical trials but did not reach the market. For example, a liposomal-based formulation of RA (monotherapy) was successfully tested in patients with facial acne[73] and refractory hematological malignancies in a phase II clinical trial[72]. In the last application, the remission results (67% clinical remission) were lower than a combinatorial therapy (77% clinical remission) based on oral administration of ATRA plus idarubicin and thus the formulation was not further evaluated. Unfortunately, the liposomal formulation of RA was not tested in combination with other drugs, as it is now being investigated with non-lipossomal RA formulations (**Table 1**), and this deserves further investigation in the near future.

4. RA delivery systems for Regenerative Medicine

4.1. Embryonic stem cells

One of the initial applications of RA for Regenerative Medicine was as a differentiation agent during embryogenesis. The spatiotemporal release of RA by polymeric microparticles incorporated within embryonic bodies, derived from human embryonic stem cells, was reported to induce cell differentiation and tissue formation resembling the phenotype and structure of early human embryos[7]. Initial studies have used RA as a potent regulator of neural differentiation[74]. RA downregulates expressions of geminin and zinc finger protein Zic2, SoxB1 (Sox-1, Sox-2, Sox-3) and Notch-1, which maintain neural progenitor cell proliferation. By halting proliferation, RA shifts signaling towards differentiation. Several platforms have been used for RA delivery alone or in combination with other agents. For example, RA-containing electrospun fibrous meshes and scaffolds reportedly have an improved effect on stem cell dynamics. Electrospun poly(ε-caprolactone) (PCL) fibers were loaded with ATRA, which led to extremely high local concentrations of this agent and to differentiation of murine embryonic cells, within the multilayered scaffolds[75]. Besides the neurogenic potential of RA, recent studies have highlighted the critical role of RA in the derivation of embryonic hematopoietic cells[9], lymphoid organs[13] and Langerhans cells that reside specifically in the epidermis[16]. Particularly, *ex vivo* activation of RA signaling in hemogenic endothelium, a small subpopulation of endothelial cells that can differentiate into hematopoietic cells, increased its transition into a pool of hematopoietic stem cells. Conversely, RA pathway shutdown terminated this process[9]. Additionally, fetal type 3 innate lymphoid cells (ILC) are modulated by RA signaling *in utero* and therefore depend on appropriate maternal dietary intake of retinoids throughout pregnancy. This period also determines the ability of ILC progenitors to differentiate into mature lymphoid tissue-inducing cells[13]. It is possible that some of the RA delivery systems developed so far may be used for embryonic immune and hematopoietic stem cell development studies.

4.2. Adult stem cells

RA is also an important regulator of adult stem cells. For example, ATRA antagonizes stress-induced activation of dormant hematopoietic stem cells by restricting protein translation and oxidative stress[8]. When mice were fed a vitamin A-free diet to deplete the RA reservoir, animals suffered, among other effects, functional impairment of hematopoietic stem cells and their numbers were unable to recover even after injection with an immunostimulant[8]. In addition, RA-based formulations have been used as inflammatory modulators of stem cells. For example, human mesenchymal stem cells exposed to ATRA-loaded solid lipid nanoparticles significantly reduced IL-6 and IL-8 expression[76]. ATRA-containing nanoparticles have been also developed to deliver RA into neural stem cell (NSC) niches [45]. The nanoparticles had a higher effect on neuronal differentiation than solubilized RA both *in vitro* and *in vivo*[45,67]. The effect was mediated by an increase in transcription of the pro-neurogenic genes Ngn1 and Mash1[45,67]. The formulation was then modified to remotely disassemble and release RA with spatial and temporal resolution, triggered by exposure to blue light[61]. A single short pulse of light prompted β-catenin-dependent neuronal differentiation and RARα upregulation. The combined action of blue light and RA enhanced endogenous neurogenesis.

5. RA delivery systems for skin diseases

5.1. RA mode of action

Several RA drugs are available clinically for dermatological treatments, including ATRA and 9-*cis*-RA, among others[77]. For example, 9-*cis*-RA encapsulated in a gel is a FDA-approved topical agent for cutaneous Kaposi´s sarcoma[77]. ATRA also has been tested in clinical trials for treatment of the same disease[78]. This sarcoma is associated with human immunodeficiency virus infection and is characterized by a vascular endothelioma (i.e. tumor of the endothelial cells). ATRA is used clinically for treating photoaging, acne and psoriasis. Indeed, ATRA was approved for acne vulgaris treatment in 1971[2], and since other drugs (called retinoids because they bind to RAR and/or RXR receptors) have been developed. In these clinical applications, the biological effect of ATRA includes: (i) modulation of proliferation and differentiation of skin cells; (ii) anti-inflammatory activity[77]. The existence of several types of receptors and their combinations as heterodimers, as well as ability of ATRA to modulate the activity of multiple kinase signaling pathways independently of the nuclear activation of RAR and RXR receptors, may explain the diversity of ATRA biological actions[36]. ATRA also induces skin angiogenesis and collagen

deposition, increases the mitotic activity of inter- and follicular epithelium, and reduces melanin production[77].

5.2. Type of RA-containing formulations

Conventional formulations containing ATRA require multiple applications to maintain the therapeutic effect. Therefore, several formulations have been developed to improve the long-term effect of ATRA, reducing side effects like desquamation and erythema, skin irritation, and increasing the stability of RA to light[19] (**Figure 4**). In this sense, formulations including phospholipid-based particles (e.g. solid lipid nanoparticles and nanostructured lipidic carriers)[2,79], polymeric nanoparticles[2,27,79,80] or polymers conjugated with RA[53] have been developed to overcome these issues. The carriers presented a particle size between 100 and 400 nm, a range of zeta potential from neutral (liposomes) to negative (polymeric nanoparticles, ethosomes, solid lipid nanoparticles and nanostructured lipidic carriers), high entrapment efficiency (above 65%), high photostability (between 2 and 3-fold higher than commercial tretinoin dissolved in ethanol), moderate to high skin permeation, high skin tolerance and moderate to high anti-psoriatic activity. Some of these formulations are easier to scale up (e.g. solid lipid nanoparticles) than others[79,81]. In addition, some formulations (e.g. ATRA-containing liposomes) improve the local effect of RA in the skin and decrease systemic adsorption[80]. Moreover, ATRA-containing formulations increased significantly the chemical stability of ATRA during normal storage conditions (e.g. stability for 1 year at 25ºC) and after exposure to UV irradiation (2-fold lower photo-degradation)[79,82]. The protective effect of these formulations was linked to their ability to reflect and scatter UV radiation[82]. Interaction of ATRA with a lipophilic amine (stearylamine) decreases ATRA crystallinity, leading to a formulation with less skin irritating properties[79].

5.3. Pre- and clinical applications

RA-containing formulations are in clinical evaluation for the treatment of *acnes vulgaris*, hand eczema and photoaging (**Tables 1 and 3**). An ATRA-loaded liposomal formulation has been tested in a pilot trial[73]. Patients with facial acne treated with the liposomal formulation showed higher lesion improvements than with conventional formulations. The higher efficacy of the ATRA-loaded liposomal formulation was attributed to enhanced penetration of RA across the *stratum corneum*. A commercial tretinoin gel microsphere formulation has reached the market, consisting of microspheres with 10-20 μm in size[77]. A common issue in design of controlled RA release systems for skin applications is limited efficacy in terms of cell targeting (**Figure 4**). Formulations such as

micro- and nanoparticles might have the capacity to target specific cells by specificities in their physicochemical properties or by incorporating in their surface peptides, proteins or aptamers that recognize specific cell receptors. Due to their size, these formulations might accumulate preferentially in some regions of the skin, such as the follicular adduct and thus act in those biological environments. Indeed, follicular targeting is important for treatment of acne, because it increases the therapeutic effect of retinoids, while reducing their potential side effects. Polymeric micelles of diblock methoxy-poly(ethylene glycol)-poly(hexyl-substituted lactic acid) copolymer favor follicular targeted delivery of ATRA[83].

6. RA delivery systems for brain diseases

Several formulations containing RA have been used to treat Alzheimer's disease (AD), Parkinson's disease (PD) and stroke (**Table 1**). Current challenges in the use of RA formulations for brain diseases are ascribed to low accumulation in the brain after intravenous administration (**Figure 5**). Although the formulations having affinity ligands in their surface have increased retention in the brain, only a minor fraction of the injected formulation reaches the brain and crosses the BBB. Cell-mediated delivery (e.g. T cells, monocytes) may be used to overcome this issue: cells can act as transporters of formulations that will be activated by local (e.g. pH) or remote triggers (e.g. light, ultrasound, magnetism)[25,84] once they reach the target site.

6.1. Alzheimer's disease

AD is a neurodegenerative disease caused by accumulation of amyloid-beta peptides, hyperphosphorylated tau filaments and brain vascular changes leading to cerebral amyloid angiopathy[85]. In experimental models of AD, RA: (i) inhibited oxidative damage and mitochondrial dysfunction; (ii) increased ApoE expression and suppressed inflammation; and (iii) improved learning and memory[86,87]. Nanoparticles have been developed to efficiently deliver ATRA and small interfering RNA to promote NSC differentiation in AD[88]. Transplantation of nanoparticle-treated NSCs in AD mice improved cognition and memory.

6.2. Parkinson's disease

PD is characterized by selective degeneration of dopaminergic neurons in the *substantia nigra* and by accumulation of α-synuclein and Lewy bodies (protein inclusions in neurons)[69]. In experimental models of PD, RA protected against neurodegeneration of midbrain dopaminergic neurons in the *substantia nigra*[89,90]. Stereotaxic injection of ATRA-containing nanoparticles in the striatum protected nigral dopaminergic neurons in an *in vivo* PD model[69]. Accordingly, RA-

containing nanoparticles increased expression of Nurr1 and Pitx3, key regulators of dopaminergic neuronal development and maintenance[69,91].

6.3. Stroke

In the case of stroke, changes in the vasculature, or in BBB permeability or function, may cause or enable progression of CNS diseases[92]. RA/RAR signaling is critical for BBB differentiation and integrity[93]. Recent studies have shown protective effects of ATRA-containing nanoparticles in stroke. The formulation enhanced endothelial cell proliferation and tubule network formation and protected against ischemia-induced death in endothelial cell lines and in endothelial progenitor cells isolated from ischemic stroke patients[24]. Moreover, when intravenously injected, RA formulations restored neuronal and vascular functions in a prenatal model of brain ischemia[94].

7. RA delivery systems for the treatment of cancer

7.1. Blood cancers

7.1.1. RA mode of action

One of the first applications of RA delivery systems was for treatment of blood cancers. The antitumor activity of ATRA was demonstrated in 1980 in acute promyelocytic leukemia (a subtype of acute myeloid leukemia (AML) accounting for 5% of AML cases)[3]. Since then, many clinical trials have evaluated the antitumoral efficacy of the drug alone or in combination with arsenic trioxide or idarubicin[95]. RA promoted terminal differentiation of leukemic cells, while reducing their proliferation. The antitumoral activity of RA (enhanced by cooperation with arsenic trioxide)[11] was due to inhibition and degradation of prolyl isomerase Pin1, which has a critical role coordinating multiple phosphorylation events during oncogenesis[12]. Therefore, RA has the unique property of blocking multiple cancer-driving pathways simultaneously. Unfortunately, the therapeutic efficiency of ATRA-based therapies remains to be demonstrated in patients with AML without acute promyelocytic leukemia.

7.1.2. Type of RA-containing formulations

Physicochemical properties as well as release properties of RA-containing formulations for treatment of blood cancers are summarized in **Table 3**. With exception of some formulations for parenteral administration[64] and for intracellular delivery in *ex vivo* conditions[25,26], most RA-containing formulations have been developed for intravenous delivery. These formulations were

401 based in liposomes[50,72,96], microspheres[62], polymeric micelles[64] and nanoparticles[51,97]. In most cases,
402 ATRA was encapsulated in the formulation[50,72], or chemically conjugated to the carrier[51]. This
403 effort was motivated by the fact that some of the patients treated with ATRA relapsed during the 4-
404 6 weeks treatment of daily oral administration. Follow-up studies indicated that resistance was due
405 to the decrease of ATRA concentration in the plasma, and thus inability of the drug to differentiate
406 leukemic cells[42]. The decrease was attributed to an induction of ATRA catabolism and increased
407 levels of RA-binding protein[20]. Several ATRA delivery systems showed relative success in
408 reducing induction of ATRA catabolism and thus maintaining RA for longer periods in blood
409 plasma. For example, microspheres of poly(L-lactide) showed a nearly constant release rate of
410 ATRA for 5 weeks[62]. In addition, intravenous administration of RA-containing liposomes for 7
411 weeks in rats did not enhance RA catabolism[50]. Maintenance of RA in the plasma reduced the
412 number of relapses[98].
413 Targeted delivery of RA-containing formulations to bone marrow has recently attracted
414 much attention[99]. Bone marrow is the residence of hematopoietic stem cells that give rise to
415 myeloid and lymphoid cell lineages. In leukemia patients, hematopoietic stem cells are the target of
416 genetic mutations and thus aberrant activity. These altered cells are difficult to eliminate by
417 conventional antitumoral agents because drugs do not reach bone marrow at the required
418 concentration, and because the stem cell niche (a physical and functional entity that supports the
419 self-renewal and differentiation of stem cells) changes after chemotherapy, protecting altered
420 cells[100]. RA is a potential treatment, combined with other drugs, for AML[101] and chronic myeloid
421 leukemia[102]. In the last 10 years, a new therapeutic paradigm has emerged based in the targeted
422 delivery of formulations to bone marrow[99]. Targeted delivery can be achieved by surface
423 modification of nanoparticles with bisphosphonate to promote binding to bone[103], by surface
424 modification of liposomes with folate to target the folate receptor, which is highly expressed in
425 AML cells[21,104], by the conjugation of nanoparticles with the surface of hematopoietic stem cells[105],
426 or by surface modification of nanoparticles with antibodies (e.g. CD45.1, CD117)[106,107] or aptamers
427 (E-selectin thioaptamer)[108]. Recently, we have developed a new platform to target bone marrow,
428 based on light-activatable nanoparticle formulations containing ATRA[25,26]. The formulation was
429 highly internalized by leukemia-initiating cells and accumulated in the cell cytoplasm. Once
430 leukemia-initiating cells transfected with light-activatable nanoparticles containing RA were
431 administered intravenously in leukemic mice they tend to home in the bone marrow, in the
432 proximity of other leukemia cells. The irradiation of bone marrow with a blue light induced photo-
433 disassembly of the nanoparticles within the cells and consequently their differentiation. These cells

then secreted extracellular vesicles containing ATRA that interfered with cells supporting the stem cell niche.

7.1.3. Pre- and clinical applications

Most RA-containing formulations are in clinical evaluation for treatment of blood tumors (**Table 1**). Current challenges for translation of RA formulations to the clinic are related to two factors: (i) capacity to incorporate multiple drugs besides RA; (ii) efficiently target hematopoietic cells (**Figure 5**). In most cases, RA-based monotherapies are less efficient in interfering with tumorigenesis and cancer progression than combination therapies[29]. The efficiency of combination therapies is likely ascribed to several factors: (i) a combination of drugs may act at different sites of the anti-proliferative signaling pathway and thus be more effective in the inhibition process; (ii) combination therapies may decrease the probability of cancer resistance; (iii) synergies between the drugs reduces the dose necessary for therapy, as well as their toxicity and treatment time. Currently, several clinical trials are investigating the combination of ATRA with arsenic trioxide, with arsenic trioxide and gemtuzumab ozogamicin (a monoclonal antibody against CD33 conjugated with ozogamicin, a cytotoxic agent), with epigenetic regulators such as tranylcypromine, with decitabine, cytarabine and granulocyte-stimulating factor, among others, in the context of AML (**Table 1**). Unfortunately, most RA formulations tested so far for blood cancers have not explored simultaneous controlled release of RA and other agents. Thus, further investigation is needed to address this issue. It should be noted that a liposomal formulation having 2 drugs (non-RA drugs) has been approved recently for AML[109]. Another important challenge in clinical translation of RA formulations is targeting. Pre-clinical tests have addressed different leukemia cell targets, not yet explored in clinical trials. The recent re-approval of gemtuzumab ozogamicin[109] may inspire new approaches for RA formulation targeting.

7.2. RA delivery systems for the treatment of solid tumors

7.2.1. RA mode of action

One of the first applications of RA formulations for treatment of solid tumors was in mild/moderate intraepithelial cervical neoplasia[4,5]. ATRA was released by a collagen sponge with a cervical cap at the tumor site by insertion in the cervix. A dose of ~0.4% of RA was selected for a phase II trial. Fifty percent of the patients showed total regression of the disease[4,5]. Systemic and cervical side effects were mild and vaginal side effects were moderate and tolerable. Unfortunately, the formulation did not reach the market, likely because it was not sufficient to reverse or suppress

more advanced dysplasia with acceptable local side effects in a phase III clinical trial[110]. The antitumoral activity of RAs is linked to their differentiation and cell growth arrest properties[111]. RA has been tested in several clinical trials and the results showed that RA alone did not present significant antitumoral activity against breast cancer[112]; however, when RA was combined with tamoxifen or paclitaxel it showed moderate antitumoral activity[113,114].

7.2.2. Type of RA-containing formulations

In the last years, significant effort has been made by the scientific community to develop formulations able to release RA and other neoplastic drugs. For example, nanoparticles containing both ATRA and paclitaxel (to inhibit cell division because the cell cannot disrupt the polymerized tubulin for cell division) had superior efficacy than formulations containing only paclitaxel[54]. In addition, pH-sensitive nanoparticles have been designed to release all-*trans* retinal and doxorubicin in weakly acidic tumors (pH 6.5) or in acidic intracellular environments such as endosomes/lysosomes (pH 4.5-5.5)[115]. All-*trans* retinal was chemically conjugated to the nanoparticle polymer by hydrazone bonds that were labile under acidic conditions. Compared to free drugs, the nanoparticle formulation increased the accumulation of the all-*trans*-retinal and doxorubicin at the tumor site and induced higher levels of cell senescence and anti-tumoral activity. Progress also has been made in targeting cancer-initiating cells, which are resistant to chemotherapy and associated with tumor recurrence. ATRA liposomes and nanoparticles encapsulating simultaneously ATRA and doxorubicin have been used successfully to arrest proliferation of breast cancer-initiating cells and to differentiate them[116,117]. In a combinatorial approach, RA differentiated cancer-initiating cells, whereas antineoplastic agents, such as doxorubicin, killed non-cancer initiating cells.

7.2.3. Pre- and clinical applications

The results of several clinical trials indicate that 13-*cis* RA, alone or in combination, seems to be effective at different levels against malignant gliomas[118], and cancer occurring in the central and peripheral nervous system[119]. Moreover, ATRA inhibits proliferation of glioblastoma cells *in vitro* and *in vivo*, while promoting their differentiation[120,121]. With high concentrations, ATRA may induce cell apoptosis[122]. RA-coated solid lipid nanoparticles showed higher *in vitro* toxicity relatively to glioblastoma cells than the parent drug[123]. In addition, polymeric micelles composed by methoxy (mPEG)-grafted chitosan and encapsulating ATRA was more effective at inhibiting glioblastoma cell line migration *in vitro* than the free agent[63].

Immune system plays an important role in modulating tumor progression; however, limited infiltration of immune cells in the tumor site, as well as existence of immunosuppressive agents, hamper their biological role. Therefore, a combination of chemotherapy with immunotherapy might be a better strategy to fight tumor biology. Indeed, immunotherapy may increase sensitivity of cancer cells to chemotherapy and thus reduce its side effects[124]. Recently, a chemo-immunotherapy approach for melanoma has been developed based on biodegradable hollow mesoporous nanoparticles containing three drugs: doxorubicin, ATRA, and interleukin (IL)-2[23]. IL-2 is a T cell growth factor and thus can facilitate proliferation and activation of T cells. Animals treated with formulations containing the three agents were the ones with the most effective tumor growth inhibition and decreased metastasis. ATRA was effective in differentiating myeloid-derived suppressor cells, and in synergy with doxorubicin, contributed to increase the number of dendritic cells at the tumor site; IL-2 facilitated the proliferation of $CD8^+$ T cells at the tumor site to promote effective tumor killing.

8. RA delivery systems for immune diseases

8.1. RA mode of action

Inflammation is essential for the regulation of tissue homeostasis and barrier integrity (e.g. BBB, mucosal barrier). However, when dysregulated, it contributes to the pathophysiology of many diseases. In fact, retinoids have been described as potent anti-inflammatory and therefore protective in pathologies such as chronic obstructive pulmonary disease[76], rheumatoid arthritis[125], psoriasis[126] and inflammatory bowel disease[127]. At cellular levels, RA inhibited IL-6-driven induction of proinflammatory Th_{17} cells and promoted the differentiation of T_{reg} cells, which are important to suppress excessive immune responses[128].

The role of RA in the gastrointestinal tract is particularly relevant since it is produced and metabolized in the intestine, where it regulates the differentiation and function of diverse immune cells, and supports mucosal barrier immunity[129]. Indeed, ATRA regulates the activity of $CD161^+$-T_{reg} cells that support wound repair in intestinal mucosa[14]. These C-type lectin CD161 regulatory T cells were found to induce cytokines that promoted epithelial barrier healing in the gut. Accordingly, several studies have focused on the impact of vitamin A intake (i.e. RA obtained from diet), both during development and in the adult. Accordingly, the levels of retinoids obtained from the maternal diet increase the size of secondary lymphoid organs and the efficacy of adult immune responses[13], while the depletion of vitamin A, in the adult, led to a decrease of Th1, Th17, and ILC3 responses (a synonym of lowered immunity to bacterial infection), an effect which is counteracted through time[130].

8.2. Type of RA-containing formulations

Several RA formulations have been developed to target immune cells and induce an immunomodulatory response, such as solid lipid nanoparticles[76], polymeric nanoparticles[59], nanostructured lipid carriers[127], among others. RA formulations with an average diameter of 130 to 250 nm[59,76] and an entrapment efficiency of ATRA between 2.3 and 310 μg[127] *per* mg of nanoparticle have been developed. Macrophage phagocytosis was influenced largely by the physicochemical properties of nanoparticles. For example, uptake is higher in nanoparticles with high negative or positive surface charges[131]. In general, RA formulations showed sustained intracellular RA delivery for a few days[59,76]. RA formulations are taken up by macrophages and induce anti-inflammatory responses by suppressing NF-kB signaling and increasing bone morphogenetic protein 2 signaling (pivotal for bone and cartilage development), as well as by enhancing production of anti-inflammatory cytokines (e.g. IL-10)[59,127]. In addition, RA formulations have the capacity to enhance differentiation of naïve T cells to regulatory T cells[127]. The advantages of RA-containing nanoparticles *versus* free RA were demonstrated in inhibiting expression of IL-6 and IL-8 in alveolar epithelial cells[76], but were not demonstrated in induction of immune cell differentiation.

8.3. Pre- and clinical applications

Clinical trials using free RA in the context of immune thrombocytopenia (severe bleeding disorder) and sclerosing cholangitis (inflammation and scarring of the bile ducts) are active and will evaluate the role of RA as immunotherapy (**Table 1**). Current challenges for using RA formulations for immune diseases are related to the *in vivo* demonstration of RA effects. This requires use of formulations able to target specific cells, particularly immune cells and their surface receptors (**Figure 5**). There are classes of molecules, including antibodies, carbohydrates, peptides, aptamers that can be attached to RA formulations to target more specific immune cell populations.

9. Future outlook

The advantages of RA formulations compared to free RA have been demonstrated in various biomedical applications. These are related to: (i) increased bioavailability; (ii) decreased toxicity and skin irritation; (iii) increased *in vivo* half-life; (iv) increased RA photostability; (v) increased cell targeting and capacity to cross biological barriers. Advantages of RA formulations have been demonstrated in preclinical (in all applications described in this review) and clinical trials (cancer

and skin applications)[73,132]. In the case of cancer, the high efficacy of RA formulations has been attributed to a longer RA half-life. In fact, recent studies indicate that the antitumoral activity of RA might be enhanced with formulations that extend the *in vivo* half-life of RA[12].

RA formulations have some limitations that preclude immediate translation into the clinic. ATRA, 9-*cis*-RA and 13-*cis*-RA belong to the first generation of retinoids. They are being replaced in some applications by third-generation retinoids[133]. Although many formulations have been tested in preclinical models, clinical translation of the formulations is relatively low. For example, although nanoparticles delivered systemically have been tested in many animal tests, clinical translation is relatively poor because of delivery (e.g. limited efficacy in terms of cell targeting, limited capacity to cross biological barriers), technical (e.g. scale-up) and regulatory aspects (e.g. study design and approval challenges). Use of antibodies, peptides, aptamers immobilized in the surface of nanoparticles to target specific cellular receptors may accelerate clinical translation of RA formulations. Indeed, the recent FDA approval of gemtozumab ozogamicin (anti-CD33 antibody conjugated with a cytotoxic agent)[109] or daratumumab (anti-CD38 antibody)[134] might accelerate clinical testing and approval of other formulations conjugated with antibodies. In addition, nanoparticles/microparticles used in pre-clinical studies have been prepared in small batches. Scale-up of these formulations is challenging, as they must comply with regulatory guidelines, in terms of narrow size distribution, precise chemical composition and drug loading[135]. In the last years, progress has been made in producing more controlled formulations with size and composition levels compatible with microfluidic systems[136]. It is expected that these technological advances might accelerate clinical translation of some RA formulations.

Despite progress in the last 50 years, many issues remain to be addressed. For example, further preclinical and clinical studies are necessary to evaluate the mechanism of RA formulations biodistribution, phamarcokinetics, clearance and toxicology. This requires development of theranostic RA formulations that may be tracked *in vivo* by luminescence, magnetic resonance imaging or positron emission tomography. Theranostic RA formulations are particularly relevant if they are injected intravenously. Moreover, further formulations should be developed with capacity to release multiple drugs in combination with RA. Pathologies discussed in this review (neurologic diseases, cancer, immune diseases) are multifactorial and display complex signaling pathways and symptoms. Although some progress has been made in the last 5 years regarding formulations with capacity to release RA in combination with other agents [55,115], further effort is needed to better control *in vivo* the half-life of each drug to match clinical dosage programs. Another area that deserves further investigation is development of stimuli-responsive RA formulations. These systems have ability to undergo physical and/or chemical changes in response to endogenous

biological or external triggers[65]. The concept here is that the systems retain the drug and release it after a specific trigger, enhancing its therapeutic efficacy and minimizing systemic toxicity. These systems may be used for the targeted delivery of RA in hematopoietic[25] or neurogenic niches[61]. Development of these systems requires development of linkers susceptible to pH or enzymes present in the targeted cell/tissue. For example, several RA-polymer conjugates have been developed to release RA by hydrolytic cleavage of ester bonds[51,53], by enzymatic cleavage of amide bonds by proteases present in the targeted tissue[28,65-67] or by reducing environments[57] such as the cell cytoplasm. In addition, linkers may be cleaved by an external trigger, such as light with the consequent release of RA[25,26].

RA is a very deeply conserved as a pathway and evolutionarily ancient. The overlap and pleiotropic effects of RA is one of the key reasons for targeted delivery. It is expected that the knowledge gathers in the development of retinoid formulations inspire others in the development of formulations based in different drugs.

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Table 1- RA formulations tested in past and ongoing clinical trials.

Drug	Indication	Clinical trial
ATRA-containing liposomes	Acne	Phase I/II (NA) [73]
ATRA-containing collagen sponge	Mild/moderate intraepithelial cervical neoplasia	Phase II (NA) [4,5]
ATRA + pembrolizumab	Advanced melanoma	Phase I/Ib (NCT03200847)
ATRA + ipilimumab	Advanced melanoma	Phase II (NCT02403778)
13-***cis*** RA + cabozantinib	Solid tumors	Phase I (NCT03611595)
13-***cis*** RA + temozolomide + thiotepa + carboplatin	Brain tumor	Phase II (NCT00528437)
13-***cis*** RA + 3F8/GM-CSF	Neuroblastoma	Phase II (NCT01183429)
13-***cis*** RA + 3F8/GM-CSF	Neuroblastoma	Phase II (NCT01183897)
13-***cis*** RA	Neuroblatoma	Phase I/II (NCT03291080)
13-***cis*** RA + dinutuximab + lenalidomide	Neuroblastoma	Phase I (NCT01711554)
13-***cis*** RA + several drugs	Neuroblastoma	NA (NCT01526603)
ATRA	Cholangitis, sclerosing	Phase II (NCT03359174)
ATRA + arsenic trioxide	APL	Phase II (NCT01404949)
ATRA + arsenic trioxide	APL	Phase III (NCT02339740)
ATRA + arsenic trioxide + gemtuzumab ozogamicin	APL	Phase II (NCT01409161)
ATRA + idarubicin	APL	NA (NCT01064557)
ATRA + arsenic trioxide + Realgar-Indigo naturalis formula	APL	Phase III (NCT02899169)
ATRA + several drugs	APL	Phase IV (NCT02200978)
ATRA + several drugs	APL	Phase III (NCT02688140)
ATRA + several drugs	APL	Phase III (NCT00482833)
ATRA	Acne vulgaris	Phase IV (NCT02620813)
ATRA	Multiple myeloma	Phase I/II (NCT02751255)
ATRA + rituximab	Immune thrombocytopenia	Phase II (NCT03304288)
ATRA + 5-azacitidine + lupron	Prostate cancer	Phase II (NCT03572387)
ATRA	Olfactory loss	NA (NCT03574701)
ATRA + tranylcypromine + cytarabine	AML	Phase I/II (NCT02717884)
ATRA + tranylcypromine	AML	Phase I (NCT02273102)
ATRA + gemtuzumab ozogamicin	AML	Phase III (NCT00893399)
ATRA +decitabine + cytarabine + G-CSF	AML	Phase II (NCT03356080)
ATRA + arsenic trioxide + cytarabine	AML	Phase I/II (NCT03031249)
ATRA + pioglitozone + azacitidine	AML	Phase II (NCT02942758)
ATRA + gemcitabine + Nab-paclitaxel	Pancreatic cancer	Phase I (NCT03307148)
ATRA + INCB059872+ azacitidine + nivolumab	Advanced malignancies	Phase I/II (NCT02712905)
13-cis RA + vorinostat + temozolamide	Glioblastoma	Phase I/II (NCT00555399)

Retinol + bakuchiol	Photoaging; wrinkles	Phase I/II (NCT03112863)
9-*cis* RA + cyclosporine A	Hand eczema	Phase III (NCT03026946)

644 APL= Acute promyelocytic leukemia; ATRA= all-*trans* retinoic acid; GM-CSF= Granulocyte-macrophage colony-
645 stimulating factor; NA= Not available.
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653 **Table 2-** Synthetic retinoids in terminated or active (**in bold**) clinical trials and approved for commercialization.

Drug	Receptor activity	Indication	Clinical trial
Tamibarotene (or Am80)	RARα agonist	Crohn's disease	Phase II (NCT00417391)
		APL	Phase II (NCT00520208)
		Advanced non-small cell lung cancer	Phase I (NCT01337154)
		AML or myelodysplastic syndrome	**Phase II (NCT02807558)**
Palovarotene	RARγ agonist	Eye dry disease	**Phase I (CTP300302)**
		Fibrodysplasia ossificans progressive	**Phase III (NCT03312634)**
		Multiple osteochondromas	**Phase II (NCT03442985)**
Trifarotene (cream)	RARγ agonist	Moderate facial and truncal acne vulgaris	**Phase III (NCT03915860)**
		Autosomal recessive ichthyosis with lamellar scale	**Phase II (NCT03738800)**
		Early cutaneous T-cell lymphoma	Phase I (NCT01804335)
Bexarotene (capsules)	Pan-agonist	Refractory cutaneous T-cell lymphoma	Approved by FDA since 1999
Tazarotene (gel or cream)	Pan-agonist	Hand-foot-skin reactions	**Phase II (NCT04071756)**
		Facial acne vulgaris	Approved by FDA since 1997
		Plaque psoriasis	Approved by FDA since 1997
Adapalene (solution, cream and lotion)	Pan-agonist	Acne	Approved by FDA since 1996
AGN194204	RXR agonist	Prostate cancer	Phase II (NCT01540071)
UAB30 / 9-cis-UAB30	RXR agonist	Non-melanoma skin cancer	**Phase I/II (NCT03327064)**
Fenretinide (oral powder and intravenous liquid emulsion)	Atypical retinoid	Peripheral T-cell lymphoma	**Phase II (NCT02495415)**
		Solid tumor (relapsed malignancies)	**Phase I (NCT01553071)**
		High risk cancer	Phase III (NCT01479192)
		Prevention of bladder cancer	Phase III (NCT00004154)
		Cervical neoplasia	Phase III (NCT00003075)
		Schizophrenia	Phase III (NCT00534898)
		Breast cancer	**Phase III (NCT01357772)**

654 APL= Acute promyelocytic leukemia; AML=Acute myeloid leukemia; FDA=Food Drug Administration.

Table 3- Examples of formulations for the controlled release of RA in context of cancer, brain diseases, skin diseases, immune diseases, stem cell differentiation, among others.

Type	Diameter	Loading (µg/mg of carrier)	Delivery	Applications	Ref.
NPs	≃ 500 nm	≃ 76	Up to 3 h in the presence of trypsin	Pharmaceutical	[97]
NPs	< 200 nm	154	Release up to 10 days	Cancer	[52]
NPs	170-230 nm	20	80% cumulative drug release in 4 days	Immune diseases	[59]
NPs	170-185 nm	275	Sustained drug release observed over 72 h	Cancer	[57]
NPs	≃ 200 nm	86	17% cumulative RA release for 21 days	Stem cells and brain diseases	[24,45,67]
NPs	160 nm	150	50% release after 10 min irradiation	Cancer and brain diseases	[25,61]
NPs	≃ 100 nm	68-87	5 - 100% release of RA in 5 days	Stem cells and brain diseases	[46]
NPs	≃ 214 nm	30	38% cumulative release of RA in 48 h	Cancer	[123]
MPs	≃ 5.6 µm	80	Pseudo-zero order release for 5 weeks	Pharmaceutical	[62]
MPs	≃ 8 µm	3	Release up to 10 days	Stem cells	[7]
MPs	190 µm	57	Less than 3 h	Cancer	[137]
Mic	100-500 nm	40 - 130	NA	Pharmaceutical	[49]
Mic	100-400 nm	2.6	Parenteral administration; < 5% in 3 days	Cancer	[64]
Mic	50-200 nm	80% (w/w)	1-month delivery	Cancer	[48]
Lip	NA	NA	4.4 µg/mL blood *per* day (in humans)	Cancer	[50,72,98]
EFM	0.95-1.89 µm	5	80% cumulative RA release in 3.5 months	Stem cells	[138]
EFM	<5 µm	≃ 10	≃ 9.0 µM for 1 h	Stem cells	[75]
Scaffold	NA	≃ 20	Release over 8-28 h	Regenerative medicine	[139]
Scaffold	0.1-0.85 µm fibers	≃ 0.3 - 7	Sustained release of RA for up to 1 week	Stem cells	[140]

EFM= Electrospun fibrous mesh; h= hour; Lip= Liposome; Mic= Micelles; MPs= Microparticles; NA= Not available; NPs= Nanoparticles; min= minute.

Table 4- Marketed RA formulations.

Name/Company	Composition	Indication	Year approved	Ref.
9-*cis* RA (brand name: Panretin)/Eisai Inc.	Topical formulation; 0.05% or 0.1% gel containing alitretinoin	Cutaneous Kaposi´s sarcoma; Treatment of recalcitrant chronic hand dermatitis	2000	[141,142]
13-*cis* RA (brand name: Accutane)/Roche	Topical formulation; 0.05% and 0.1% cream	Photoaging and acne	1982	[142]
ATRA	Topical formulation; 0.05% cream	Photoaging and acne	1971	[2]
Gel microsphere formulation containing ATRA/Advanced Polymer Systems	Topical formulation; macroporous beads, 10-25 μm in diameter	Acne	1997	[77,143]
9-*cis* RA	Topical formulation; 0.1% gel	AIDS-related Kaposi´s sarcoma	1999	[77]
Retinol	Topical formulation; 0.5-5% lotion, cream	Cosmetic	NA	[77]
Retinaldehyde	Topical formulation: 0.01, 0.015, 0.1% cream	Cosmetic	NA	[77]
ATRA (brand name: Vesanoid)/Roche	Oral formulation of Tretinoin	Acute myeloid leukemia, in particular, acute promyelocytic leukemia	2000	[134]
Acitretin	Oral formulation	Psoriasis, disorders of keratinization	1997	[144]
13-*cis* RA	Oral formulation	Severe acne/related disorder	1982	[77]
Retinol	Oral formulation	Prevent/treat hypovitaminosis A	NA	[77]

ATRA= all-*trans* retinoic acid; NA= Not available

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Captions

Figure 1- Milestones in RA formulations research.

Figure 2- Retinoid chemical structures (a) and RA signaling pathway (b). Blood-circulating retinol is internalized through membrane transporter and receptor *stimulated by retinoic acid 6* (STRA6) and converted into all-*trans* RA, which binds to cellular retinoic-acid-binding protein type 2 for signaling in the nucleus. RA triggers gene transcription by binding to RA receptors (RAR) and to the retinoid X receptor (RXR). In the presence of the ligand, RAR and RXR heterodimerize on retinoic acid-response element (RARE) sequences located in promoter regions inducing the transcription of target genes. Of note, RXR may also homodimerize and trigger gene transcription (not depicted in the illustration). RA signaling may also occur via activation of receptors associated with lipid rafts located on the cell surface, which trigger transcriptional activation of target genes by histone and receptor phosphorylation in the cell nucleus. CRBP= cellular retinol-binding protein 1; MAPK= mitogen activated protein kinases; P = phosphorylation; RAL = retinaldehyde; RALDH= retinaldehyde dehydrogenase; RBP4 = retinol-binding protein 4; RDH = retinol dehydrogenase.

Figure 3- Main outcomes of RA-based therapies in pathological contexts. AD= Alzheimer's disease; PD= Parkinson's disease.

Figure 4- Challenges and advances in topical and systemic administration of RA-containing formulations. Challenges include: (a) crossing endothelial barriers for extravasation of RA-containing formulations into a specific body region; (b) targeting of RA-containing formulations to specific cells; (c) development of formulations that combine RA with other pharmacological agents, able to release each agent with a specific release kinetics. Advances include (a) development of formulations with less toxicity; (b) release of RA with variable release kinetics to achieve variable biological action; (c) action mechanism of RA during development and disease; (d) use of cells to transport RA-containing formulations to specific regions in the body followed by the triggering of the formulations by intrinsic (e.g. temperature, pH) or extrinsic (e.g. ultrasound, light) stimuli.

Figure 1

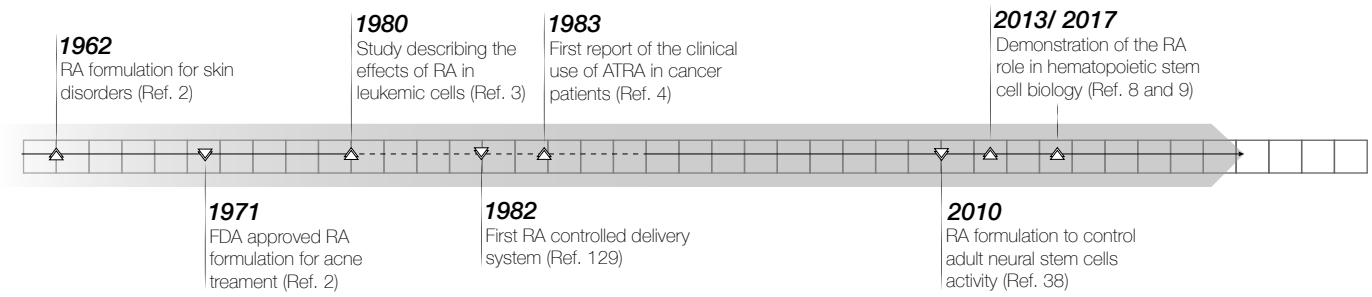
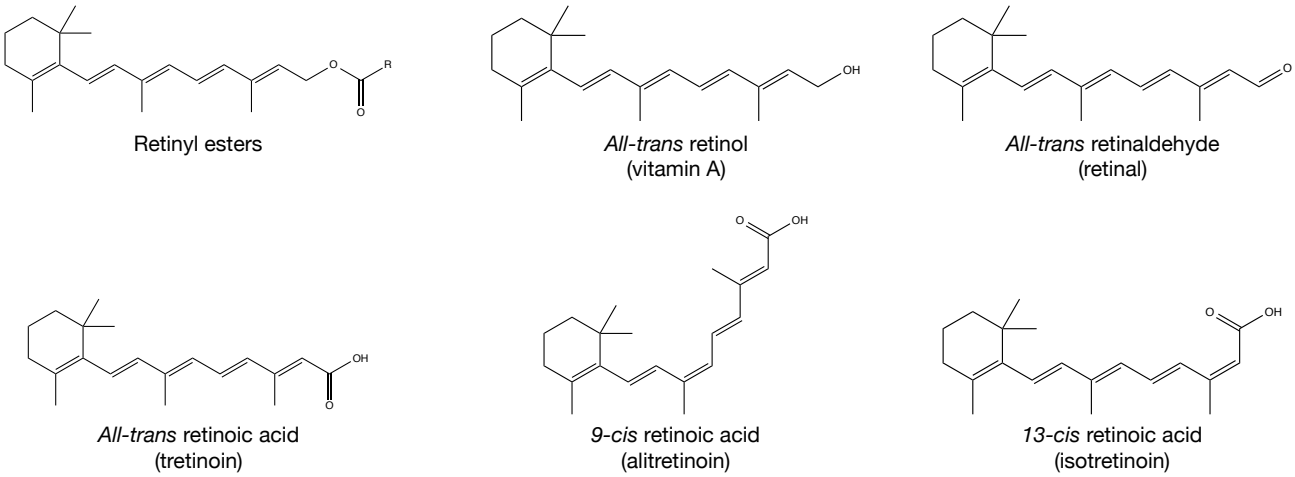

1962
RA formulation for skin disorders (Ref. 2)
1971
FDA approved RA formulation for acne treament (Ref. 2)
1980
Study describing the effects of RA in leukemic cells (Ref. 3)
1982
First RA controlled delivery system (Ref. 129)
1983
First report of the clinical use of ATRA in cancer patients (Ref. 4)
2010
RA formulation to control adult neural stem cells activity (Ref. 38)
2013/ 2017
Demonstration of the RA role in hematopoietic stem cell biology (Ref. 8 and 9)

Figure 2

a


Retinyl esters
All-trans retinol
(vitamin A)
All-trans retinaldehyde
(retinal)
All-trans retinoic acid
(tretinoin)
9-cis retinoic acid
(alitretinoin)
13-cis retinoic acid
(isotretinoin)

b

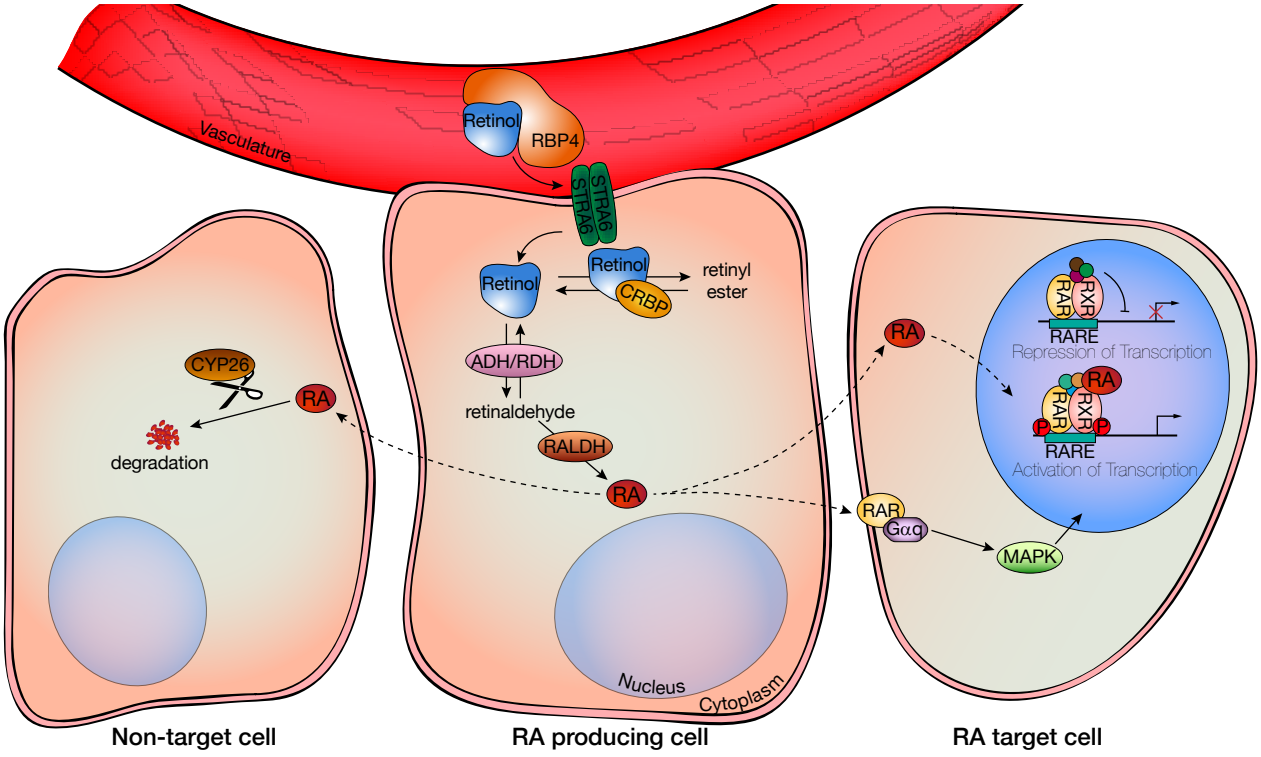
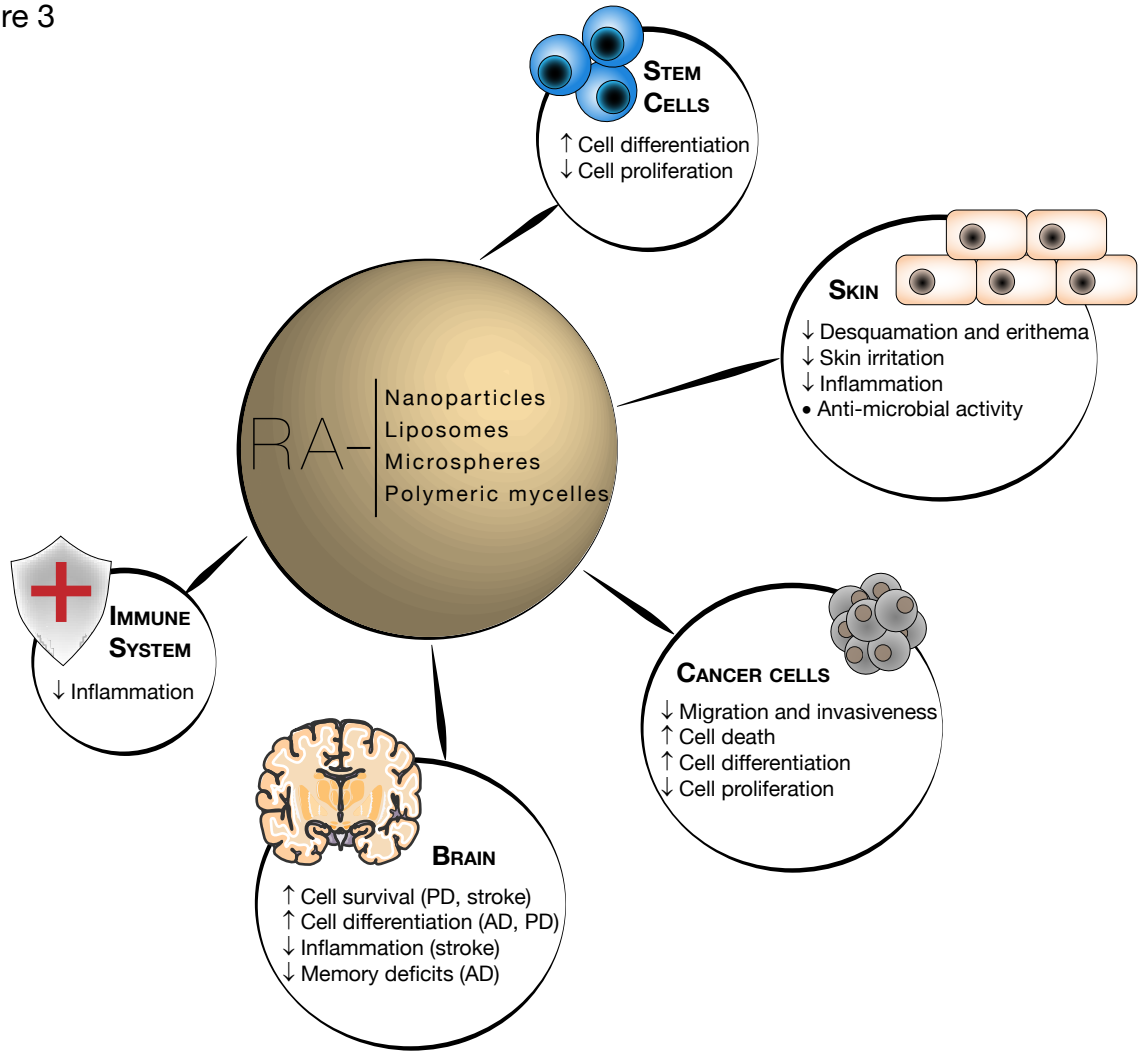
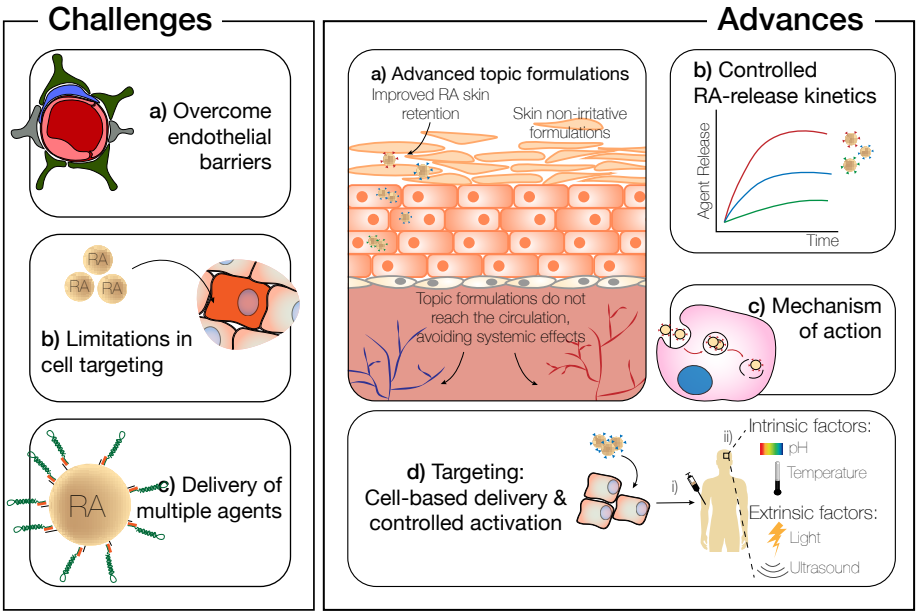

Vasculature
Retinol
RBP4
STRA6
Retinol
Retinol
CRBP
retinyl
ester
ADH/RDH
retinaldehyde
RALDH
RA
CYP26
RA
degradation
RA
RAR
RXR
RARE
Repression of Transcription
RA
RAR
RXR
P
P
RARE
Activation of Transcription
RAR
Gαq
MAPK
Nucleus
Cytoplasm
Non-target cell
RA producing cell
RA target cell

Figure 3


STEM CELLS
↑ Cell differentiation
↓ Cell proliferation
SKIN
↓ Desquamation and erithema
↓ Skin irritation
↓ Inflammation
• Anti-microbial activity
RA–
Nanoparticles
Liposomes
Microspheres
Polymeric mycelles
IMMUNE SYSTEM
↓ Inflammation
CANCER CELLS
↓ Migration and invasiveness
↑ Cell death
↑ Cell differentiation
↓ Cell proliferation
BRAIN
↑ Cell survival (PD, stroke)
↑ Cell differentiation (AD, PD)
↓ Inflammation (stroke)
↓ Memory deficits (AD)


Challenges
a) Overcome endothelial barriers
RA
RA
RA
b) Limitations in cell targeting
RA
c) Delivery of multiple agents
Advances
a) Advanced topic formulations
Improved RA skin retention
Skin non-irritative formulations
Topic formulations do not reach the circulation, avoiding systemic effects
b) Controlled RA-release kinetics
Agent Release
Time
c) Mechanism of action
d) Targeting: Cell-based delivery & controlled activation
i)
ii)
Intrinsic factors:
pH
Temperature
Extrinsic factors:
Light
Ultrasound