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13-*cis* Retinoic acid and isomerisation in paediatric oncology—is changing shape the key to success?

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Abstract

Retinoic acid isomers have been used with some success as chemotherapeutic agents, most recently with 13-*cis* retinoic acid showing impressive clinical efficacy in the paediatric malignancy neuroblastoma. The aim of this commentary is to review the evidence that 13-*cis* retinoic acid is a pro-drug, and consider the implications of retinoid metabolism and isomerisation for the further development of retinoic acid for cancer therapy. The low binding affinity of 13-*cis* retinoic acid for retinoic acid receptors, low activity in gene expression assays and the accumulation of the all-*trans* isomer in cells treated with 13-*cis* retinoic acid, coupled with the more-favourable pharmacokinetic profile of 13-*cis* retinoic acid compared to other isomers, suggest that intracellular isomerisation to all-*trans* retinoic acid is the key process underlying the biological activity of 13-*cis* retinoic acid. Intracellular metabolism of all-*trans* retinoic acid by a positive auto-regulatory loop may result in clinical resistance to retinoic acid. Agents that block or reduce the metabolism of all-*trans* retinoic acid are therefore attractive targets for drug development. Devising strategies to deliver 13-*cis* retinoic acid to tumour cells and facilitate the intracellular isomerisation of 13-*cis* retinoic acid, while limiting metabolism of all-*trans* retinoic acid, may have a major impact on the efficacy of 13-*cis* retinoic acid in paediatric oncology.

Keywords: Retinoic acid; Isomerisation; Pro-drug; Metabolism; Neuroblastoma; Paediatric

1. Introduction

Retinoic acid (RA), the main biologically-active derivative of Vitamin A or retinol, regulates cell growth and differentiation and can reverse malignant cell growth in vitro and in vivo [1]. RA exists in several stereoisomeric forms: predominantly all-*trans* retinoic acid (ATRA) and 13-*cis* retinoic acid (13*cis*RA), but also as less-stable isomers such as 9-*cis* retinoic acid (9*cis*RA) (Fig. 1). These are all readily oxidised and/or isomerised in the presence of light or excessive heat. ATRA is the most thermodynamically stable and is the major isomer at equilibrium, but all RA isomers readily isomerise in biological systems [2].

Despite their relative instability, RA isomers each have specific clinical utilities and exhibit contrasting drug pharmacokinetics and toxicities when administered to patients. 13*cis*RA has more-favourable pharmacokinetic properties than ATRA or 9*cis*RA, with higher peak plasma concentrations and a significantly longer half-life, resulting in greater exposure of the body to 13*cis*RA compared to other RA isomers [3].

1.1. Retinoic acid in cancer therapy

The use of retinoids in cancer treatment has progressed significantly over the past 20 years, with ATRA and 13*cis*RA now being established for the treatment of acute promyelocytic leukaemia (APL), prostate cancer and neuroblastoma. As a result of good clinical responses in these malignancies, retinoids are being investigated for their therapeutic potential in an increasing range of malignant diseases.

Abbreviations: AHR, aryl hydrocarbon receptor; APL, acute promyelocytic leukaemia; ATRA, all-*trans* retinoic acid; CEPT, cholesteryl ester transfer protein; CRABP, cellular retinoic acid binding protein; GST, glutathione S-transferase; IGFBP-3, insulin-like growth factor binding protein-3; LRAT, lecithin-retinol acyltransferase; M6P, mannose-6-phosphate; MTD, maximum tolerated dose; PGP, P-glycoprotein; RAG, retinoyl β-glucuronide; 13*cis*RA, 13-*cis* retinoic acid; 9*cis*RA, 9-*cis* retinoic acid; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor

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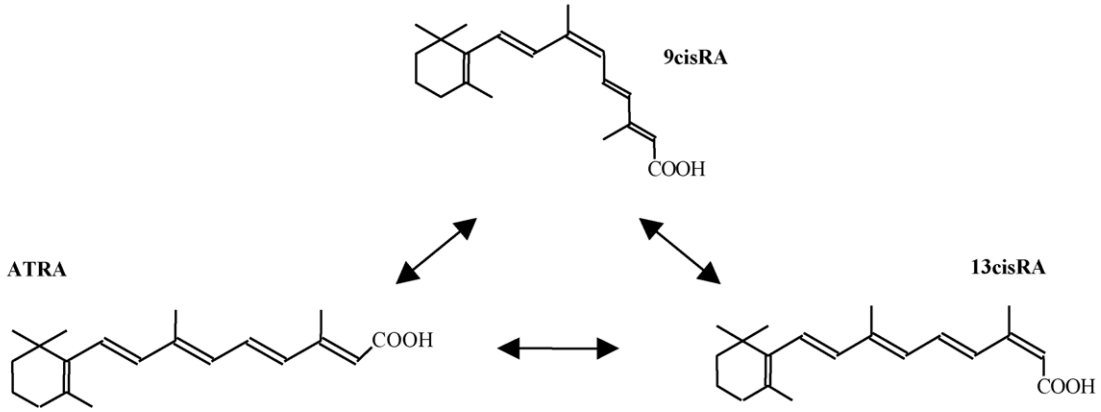

Fig. 1. Structure of the three main retinoic acid isomers.

The toxicities of RA isomers are associated with hypervitaminosis, such as hepatotoxicity, headaches, nausea, vomiting, abdominal pain, and mucocutaneous dryness. For ATRA, administered orally twice daily for 28 days in the treatment of APL, dose limiting toxicities, including pseudotumor cerebri, have limited the maximum tolerated dose (MTD) to 60 mg/m^2. Similar MTDs have been obtained with ATRA or 9*cis*RA in paediatric patients and, in all these studies, low peak plasma drug levels (within the range 0.1–1 μM), a wide degree of interpatient variability, and increased rate of catabolism of the drug with increasing time of treatment are observed with both ATRA and 9*cis*RA. In contrast, the pharmacokinetic properties of 13*cis*RA in neuroblastoma patients are more favourable, with 3-fold higher MTDs facilitating higher peak plasma levels (5–10 μM), and a significantly longer half-life (reviewed in [3]).

1.2. 13cisRA in neuroblastoma therapy

Early phase II trials in the US suggested that low dose, continuous use of 13*cis*RA failed to provide a clinical benefit in neuroblastoma patients with recurrent disease, and this was confirmed by a subsequent European randomized study of 13*cis*RA administered after myeloablative therapy in high-risk neuroblastoma patients. However, when 13*cis*RA was administered to a similar patient population as a high-dose, intermittent regimen, a significant improvement in event-free survival was observed in a US Children's Cancer Group (CCG) phase III randomized trial. In this trial, the plasma concentrations of 13*cis*RA achieved were within the range which results in inhibition of neuroblastoma cell growth in vitro and this may be the most likely factor explaining the good clinical efficacy compared to earlier trials [3]. In addition, an intermittent dosing regimen, with patients receiving 2 weeks of 13*cis*RA followed by a 2-week rest period during each course of treatment, may maintain therapeutic concentrations by limiting the induction of oxidative metabolism of retinoic acid (Veal et al., unpublished data).

These results for neuroblastoma illustrate that designing an optimal dosing regimen using information from in vitro studies, pre-clinical models, pharmacokinetic studies and clinical trials, is critical for obtaining maximum benefit from RA therapy. Ideally, this process of optimising RA delivery should be applied to all malignant diseases which may be responsive to RA. Indeed it may be of interest to examine the effectiveness of high-dose, intermittent 13*cis*RA for the treatment of tumour types where low-dose, continuous therapy lacked efficacy or where ATRA is the isomer currently used clinically.

Our understanding of how RA works as a signalling molecule at a molecular level suggests that ATRA is the main biologically-active form. Since RA isomerises readily, the likelihood that 13*cis*RA acts as a pro-drug for ATRA is, therefore, an important issue. The favourable pharmacokinetics of 13*cis*RA may play a key role in allowing it to act as a reservoir for continuing isomerisation to ATRA.

2. Mechanisms of isomerisation

In experimental use, photoisomerisation of RA before it reaches the target cells and tissues under study may increase variation in biological responses. Many published studies, both in vitro and in vivo, have not taken retinoid isomerisation into account or performed HPLC analysis in order to quantify the amounts of different isomers in the cultures under investigation. Apart from careful handling, photoisomerisation can be reduced using cyclodextrins in the vehicle, and this can also lead to greater clinical efficacy of retinoid treatment, at least with respect to topical application [4]. Within biological systems, the mechanisms responsible for mediating RA isomerisation are poorly characterised. Thiol-containing compounds and proteins catalyze the isomerisation of RA by a thiol-radical mechanism [5], resulting in a mixture of 13*cis*RA, 9*cis*RA, 9,13-di-*cis*RA, and ATRA [6]. This nonstereospecific mechanism may also occur within cells; however, the

degree of isomerisation varies between cell types, and there is evidence for enzymatic mechanisms and some stereospecificity. Marked isomerisation of 13*cis*RA to ATRA occurs in sebocytes, HL-60 and neuroblastoma cells, but to a lesser extent in HaCaT keratinocytes [7,8]. An absence of the 9,13-di-*cis* isomer when HL-60 cells are incubated with 13*cis*RA, 9*cis*RA, or ATRA suggests that isomerisation may be partially under enzymatic control [5]. Glutathione S-transferases (GSTs) can act as isomerases of 13*cis*RA, although it is difficult to discriminate between a specific enzymic reaction and a non-specific reaction catalysed by GST thiol groups [9]. However, the specific conversion of 13*cis*RA to ATRA, high activity compared to other thiol compounds, and rapid heat inactivation of isomerisation, support a role for GST in catalysing retinoid isomerisation. There is also evidence for an embryonic RA isomerase: isomerisation of 13*cis*RA to ATRA and 9*cis*RA in rat conceptal homogenates displays substrate saturation kinetics and inactivation of catalytic activities by heat and urea [10]. Furthermore, ATRA concentrations in mice after treatment with 13*cis*RA were approximately 5% of the 13*cis*RA concentrations detected in plasma, 68% of those found in liver, and 20% of those found in tumour [11]. These data suggest that intracellular enzymatic catalysis of the conversion of 13*cis*RA to ATRA may represent an important activation mechanism for 13*cis*RA in certain tissues.

3. Mechanism of action of retinoic acid isomers

3.1. Receptor-dependent activity

The cellular effects of RA result from uptake and metabolism by target cells. At a molecular level, RA regulates gene expression mainly, although probably not exclusively, via two families of ligand-activated receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (reviewed in [12]). RA isomers exhibit different receptor binding properties, with ATRA itself binding to RARs with high affinity and 9*cis*RA binding with high affinity to both RARs and RXRs. 13*cis*RA itself does not bind well to either class of receptor and has apparent binding affinities 100-fold less than ATRA or 9*cis*RA [13].

However, comparisons of the activity of 13*cis*RA against ATRA in various cell types have shown similar properties with respect to inhibition of cell proliferation after exposure to RA for several days [7,8,14]. This does not correlate with available data on the relative binding affinities of 13*cis*RA with the nuclear receptors, indicating that 13*cis*RA may be acting through pathways independent of the retinoid receptors or via conversion to the more transcriptionally active all-*trans* isomer. 13*cis*RA and ATRA have similar activities in regard to growth inhibition after prolonged periods of time, whereas gene-expression studies in which cells are exposed to RA for only a few hours indicate that 13*cis*RA has much lower activity than ATRA [15]. RAR activation by concentrations of 13*cis*RA greater than 100 nm has been reported to be comparable to that for ATRA over 6 h in cell-based reporter assays [13], suggesting that high concentrations of 13*cis*RA or longer incubation times facilitate the formation of sufficient ATRA to obtain an effect. Time course studies have shown delayed responses to 13*cis*RA compared to ATRA in neuroblastoma, leukaemia and sebocyte cell lines, and in a human neuroblastoma xenograft model [7,8,16]. These observations support the hypothesis that 13*cis*RA functions as a pro-drug, generating ATRA via isomerisation, though do not exclude other mechanisms of action for 13*cis*RA.

3.2. Receptor-independent activity

While we view the main biological and cellular effects of RA as mediated by ATRA at the level of RARs, leaving aside the possible functions of 9*cis*RA, this may be an oversimplification. For example, 13*cis*RA is more effective than ATRA as an inhibitor of acetate incorporation into lipids in cultured sebocytes and can also function as a modulator of enzymes such as retinol dehydrogenase RoDH-4, 9-*cis* retinol dehydrogenase, class 4 alcohol dehyrogenase, and lecithin-retinol acyltransferase (LRAT) [17]. Therefore, 13*cis*RA may have the ability to inhibit retinol oxidation and retinol esterification without isomerisation to ATRA. In other enzyme systems, 13*cis*RA is a potent activator of human cholesteryl ester transfer protein (CEPT) in plasma, an enzyme of lipid metabolism [18], and is reported to be a stereospecific suicide inhibitor of thioredoxin reductase, an enzyme critical for the redox regulation of protein function and signalling [19]. These specific properties of 13*cis*RA may explain teratogenic properties which cannot be accounted for by isomerisation to ATRA [2].

ATRA may also have effects independent of nuclear receptors. It has recently been discovered that ATRA is a ligand for the mannose-6-phosphate (M6P)/insulin-like growth factor-II receptor, and is capable of altering the intracellular distribution of this receptor and M6P-containing lysosomal enzymes [20]. As this cell-surface receptor plays a role in the regulation of cell growth and may function as a tumour suppressor, this may be a novel RA-response pathway. As yet, there is no evidence to suggest that 13*cis*RA is also a ligand for this receptor.

4. Stereospecificity of cellular uptake

The biological activity of RA will also be determined by rate of uptake into target cells. Entry into cells may occur via passive diffusion through the plasma membrane, but the fact that uptake of RA varies between cell lines suggests that other mechanisms may be involved. Studies in APL

cells have implicated a role for P-glycoprotein (PGP), a multidrug resistance transporter. The PGP inhibitor, verapamil, restores the ability of ATRA to induce differentiation in ATRA-resistant APL cells expressing multidrug-resistance-1 gene transcripts [21].

Evidence also suggests that ATRA is preferentially taken up by cells compared to 13*cis*RA. In neuroblastoma cells, intracellular concentrations of RA are approximately 10-fold higher after incubation with ATRA for 6 h compared to incubation with 13*cis*RA [8]. Furthermore, in pregnant mice dosed with 13*cis*RA, transfer to the placenta and mouse embryo is very efficient for ATRA compared to 13*cis*RA, suggesting selective uptake as well as *cis*/*trans* isomerisation [2].

Cellular uptake of RA can be regulated by the presence of albumin, which acts as a carrier for hydrophobic molecules in vivo [22]. Albumin does not appear to bind either ATRA or 13*cis*RA preferentially, as its presence restricts the uptake of both isomers. Blocking albumin/RA interactions with a fatty acid source may enhance the bioavailability of 13*cis*RA and improve its clinical activity.

5. Retinoic acid metabolism

5.1. Constitutive metabolism

Although RA metabolism plays a central role in retinoid signalling pathways, the enzymes involved have only recently been identified. Oxidative catabolism is the first step and is mediated by cytochrome P450 enzymes (CYP), mainly located in the liver. ATRA is metabolised by CYP3A7, CYP1A1, and CYP2C8, and to a lesser extent by CYP1A2, CYP2C9, and CYP3A enzymes, whereas 13*cis*RA is a substrate for CYP3A4, CYP3A7 and CYP2C8 [23]. The contribution of these P450 enzymes to retinoic acid metabolism in vivo is not known but may be relatively minor due to their high K_m values in vitro (in the micromolar range). 13*cis*RA can also undergo hydroperoxide-dependent co-oxidation by prostaglandin H synthase [24]. In addition, there is evidence that the aryl hydrocarbon receptor (AHR) signalling pathway plays a role in hepatic retinoid metabolism in the mouse, via CYP2C39 [25], although a link to retinoid metabolism in human cells remains uncertain. RA and oxidised RA can undergo further metabolism by UDP-glucuronosyltransferases to form glucuronide derivatives, such as all-*trans* retinoyl β-glucuronide (RAG), which also exhibits good biological activity resulting from hydrolysis to ATRA (reviewed in [26]).

Extensive metabolism has been observed during a 14 day course of 13*cis*RA treatment in neuroblastoma patients, with plasma concentrations of the 13*cis*-4-oxo metabolite being greater than those of 13*cis*RA by day 14 in the majority of patients studied (Veal et al., unpublished data), (Fig. 2). The greater stability of 13*cis*-4-oxoRA suggests that this retinoid may play a role in the toxicity observed with 13*cis*RA treatment, perhaps via isomerisation to the more active all-*trans* metabolite.

5.2. Induced metabolism of RA in target tissues

An adaptive hypercatabolic response to ATRA therapy in leukaemia patients, with ATRA concentrations reduced

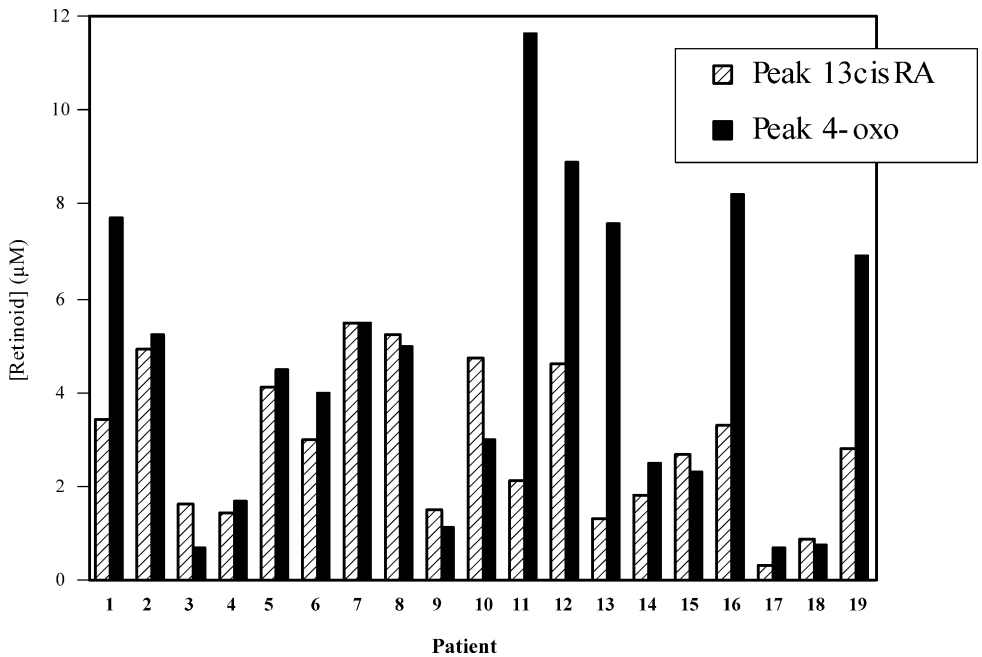

Fig. 2. Peak plasma concentrations of 13*cis*RA and 13*cis*-4-oxoRA observed on day 14 of treatment in patients with high-risk neuroblastoma. Patients were treated with an oral dose of 80 mg/m^{2}13*cis*RA.

by up to 80%, indicate that RA metabolism is highly inducible by RA itself (reviewed in [27]). This is extensively supported by in vitro studies [28]. Although physiological lipid hydroperoxides, generated by the arachidonic acid–lipoxygenase system, may be involved in the self-induced oxidative catabolism of RA [29], the use of non-specific P450 inhibitors such as ketoconazole and liarozole suggest that increased CYP activity may be primarily responsible (reviewed in [30]). In many extrahepatic tissues and cell lines, RA pretreatment induces ATRA metabolism [31,32]. A novel cytochrome P450 was cloned which catalysed the 4-hydroxylation of ATRA [33], and this was subsequently termed CYP26A1, after the identification of the related enzymes CYP26B1 and CYP26C1 [34,35]. ATRA is the preferred substrate for CYP26B1 and CYP26C1, although CYP26C1 can recognise and metabolise 9*cis*RA. 13*cis*RA can compete with ATRA for metabolism by the CYP26 family but only at concentrations greater than 10 μM. Induction of CYP26 expression in response to increasing RA constitutes a developmentally-important negative feedback loop controlling RA concentrations within cells: CYP26A1-null mice die during mid-late gestation and have severe abnormalities similar to those induced by teratogenic doses of ATRA [36].

The relationship between RA sensitivity and RA catabolism is complex. Some RA-sensitive cell lines show a high metabolic activity, while RA-resistant cells do not appear to metabolise RA significantly [8,37]. Conversely, other cell lines that are fast metabolisers of RA are relatively resistant to RA [38]. Although some oxidised metabolites exhibit significant biological activity in some cell lines [13,39], the ability of CYP26 to perform multiple hydroxylations of the β-ionone ring to form increasingly inactive and water-soluble products suggests that the ATRA metabolites have no further significance as signalling molecules [40]. The overall effect of RA turnover may differ between cell types, and may not be directly linked to the expression of the RARs or other intracellular molecules that mediate RA response.

5.3. Cellular retinoic acid binding proteins

In addition to the nuclear receptors, another class of proteins that bind to ATRA with high affinity are the cellular retinoic acid binding proteins (CRABP I and II), members of a family of highly conserved intracellular lipid-binding proteins. Induction of both CRABP I and II in response to RA occurs in a number of cell lines. Although their exact function remains to be established, CRABPs may be involved in the cellular transport and metabolism of ATRA. ATRA bound to CRABP I is a more favourable substrate for metabolism compared to 'free' ATRA, indicating a direct role for CRABP I in ATRA metabolism, and CRABP I overexpression in F9 and HNSCC cells reduces cellular response to RA. On this basis, CRABP I appears an effective mediator of retinoid degradation, though the extent of its involvement in ATRA metabolism in vivo is questionable [41].

Recent data indicate a role for CRABP II as a co-activator of RAR-mediated transcription by facilitating the formation of the ATRA–RAR complex [42]. 13*cis*RA does not bind well to either CRABP I or II, with K_d values 10–80-fold greater than those for ATRA [43]. CRABP levels should, therefore, have relatively little impact on the biological effect of 13*cis*RA in the absence of isomerisation to ATRA.

6. Resistance to retinoic acid

Resistance to RA represents a significant drawback to its clinical utility but its molecular basis is not fully understood. Many cell lines selected for resistance to ATRA, including neuroblastoma and APL cells, are also cross-resistant to 13*cis*RA [44,45], implying they share a similar mechanism of action and subsequently similar mechanisms of resistance. Down-regulation of MYCN is thought to precede RA-induced differentiation and growth inhibition, and aberrant expression of total myc (MYCN and /or *c-myc*) is associated with RA resistance [45]. Therefore, agents that work synergistically to down-regulate MYCN expression, such as the cytokine interferon-γ, or small-molecule antagonists designed to abolish function, may lead to the development of novel combination therapies [46,47]. It is unknown whether variation in RAR expression contributes significantly to RA-resistance in vivo, although a positive correlation between RARβ expression in primary neuroblastoma tumour samples and patient prognosis has been reported [48]. The involvement of other intracellular signalling molecules, such as the CRABPs, in relation to the in vivo response to retinoid treatment in neuroblastoma is not known. The emergence of effector molecules such as BAG-1 and insulin-like growth factor binding protein-3 (IGFBP-3), which inhibit the binding of RARs to retinoic acid response elements (RAREs) and block the formation of RAR/RXR heterodimers, respectively, suggest further potential mechanisms of RA resistance [49,50]. If 13*cis*RA is acting as a pro-drug for ATRA, then these potential mechanisms of resistance may also apply to 13*cis*RA.

6.1. Retinoic acid metabolism and resistance

Variation in plasma 13*cis*RA levels after treatment of neuroblastoma patients with 13*cis*RA may be due to a number of factors, such as differences in drug exposure, cellular uptake or metabolism. Studies in both leukaemia and neuroblastoma cells have shown no differences between ATRA-resistant sub-lines and their ATRA-sensitive parental cell lines in terms of RA accumulation [8,51], indicating that differential RA uptake may have only a

minor influence on RA sensitivity. Increased ATRA metabolism in response to ATRA treatment, both in vitro and in vivo, has led to the hypothesis that metabolism plays a major role in the emergence of RA resistance. The principal basis of this idea is the observation that RA upregulates the expression of CYP26 in many cell types. Perhaps the most convincing evidence for the role of metabolism in RA resistance is the impact of inhibitors of RA metabolism: non-specific P450 inhibitors such as ketoconazole, fluconazole, and liarozole increase ATRA effectiveness both in vitro and in vivo [28]. The most promising of these, liarozole, suppresses ATRA metabolism in vitro and in vivo, and exerts ATRA-mimetic effects by enhancing endogenous plasma levels of ATRA. However, one of the limitations to the use of liarozole is its lack of specificity (reviewed in [52]).

The importance of drug specificity and timing in obtaining a clinical response were recently demonstrated in a phase I clinical trial evaluating a combination of ATRA and ketoconazole in adults with solid tumours. This study did not show enhanced plasma levels of ATRA or tumour response [53]. However, ATRA in combination with liarozole and liposomal-ATRA can increase, or prevent the decline of, plasma ATRA concentrations in patients without increased toxicity. This implies that there is a benefit for reducing ATRA metabolism in humans [54–56]. Novel CYP26 inhibitors are now emerging, such as R116010, a compound shown to have a 100-fold increased potency against RA metabolism compared to that of liarozole in intact human T47D breast cancer cells. R116010 also has improved specificity, suggesting it is less likely to produce adverse side-effects [31]. In addition, there may be a genetic basis for the interpatient variability in plasma concentrations of 13*cis*-4-oxoRA and 13*cis*RA after treatment with 13*cis*RA, and pharmacogenetic studies are needed to assess the potential association between expression levels or mutations in CYP26 (or other relevant CYPs) with rates of metabolism in vivo.

7. Future of RA in paediatric oncology

Clearly, there is strong evidence that 13*cis*RA acts primarily as a prodrug for ATRA. Since 13*cis*RA has favourable pharmacokinetic properties, devising strategies to target 13*cis*RA to tumour cells, facilitate intracellular isomerisation to ATRA and limit metabolism of ATRA may have a major impact on the efficacy of 13*cis*RA in paediatric oncology. Targeting 13*cis*RA to tumour cells is also only part of the story, and methods to enable longer term administration with less toxicity also need to be developed. There is considerable scope for improving the way in which 13*cis*RA is delivered to the patient: small children are often unable to swallow 13*cis*RA in capsule form and lower drug exposures may result when the drug is extracted from the capsules and administered with food (Veal et al., unpublished data). Therefore, improvements in drug formulation may increase the clinical benefit of 13*cis*RA still further. A liposome-encapsulated form of ATRA has shown some success in the treatment of APL and may provide a more reliable dosage of ATRA for patients unable to swallow or absorb medications [55]. The route of delivery is important and enhanced efficacy may be achieved by minimising exposure of the drug to major organs of RA metabolism such as the liver. However, the effects on isomerisation, metabolism and activity of 13*cis*RA delivered in liposomal or nano-particulate formulations need to be investigated.

With respect to minimising the metabolism of ATRA within target cells, the CYP26 inhibitor R116010 has recently been shown to increase the intracellular levels of ATRA following incubation of neuroblastoma cells with 13*cis*RA, leading to a subsequent increase in the effectiveness of 13*cis*RA [57]. The use of ATRA metabolism inhibitors in combination with 13*cis*RA has not yet been assessed in pre-clinical animal models of neuroblastoma that we are aware of, but there is considerable potential for CYP26 inhibitors to become part of 13*cis*RA treatment and these are attractive targets for future drug development.

In summary, delivering 13*cis*RA to target tissues and then facilitating its conversion to ATRA in an environment where ATRA metabolism is minimised may be the key to the future development of RA as a therapy for paediatric cancer.

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