The Biological Effects of Novel Synthetic Retinoids

M.Sc. Thesis

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ABSTRACT

Naturally occurring Retinoids are known to play a role in many developmental processes in the early embryo as well as being a potent tool for *in vitro* differentiation of stem cells. The exogenous application of these naturally occurring retinoids as well as some of their synthetic analogs has been shown to yield digit duplication phenotypes and facial phenotypes in the model system of chick. It is not however always appreciated that these naturally occurring retinoids are highly susceptible to isomerisation and that these isomers have different biological activity. Due to this EC23 and EC19, synthetic analogs of these retinoids which are not able to be isomerised, have been synthesized. These two compounds until now have not been tested *in vivo*.

We showed that both EC23 and EC19 are able to yield retinoid phenotypes in the model system of chick. We also showed that EC23 is far more toxic than EC19 or ATRA, while being far more able to create retinoid limb phenotypes at lower concentrations. EC19 in this investigation is shown to produce very few retinoid limb phenotypes in comparison, but is able to yield a far higher frequency of retinoid facial phenotypes.

In conclusion this investigation shows the ability of EC23 to emulate known ATRA limb phenotypes at much lower concentrations. EC19 is shown to yield less limb phenotypes possibly due to its similarities in structure to 13-cis RA, EC19 is also shown to yield facial phenotypes more frequently than EC23 or ATRA.

INTRODUCTION

RETINOIDS

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Retinoids an example of which is All-Trans Retinoic Acid (ATRA) are naturally occurring derivatives of Vitamin A (Retinol). They are known to affect development.

All naturally occurring retinoids are made up of three structurally distinct groups; a hydrophobic end, a polyene linker and an acidic group.

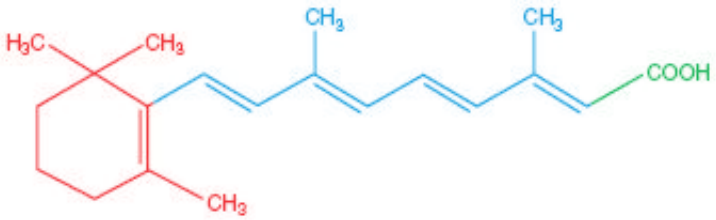


FIGURE 1. MOLECULAR SHAPE OF ALL TRANS RETINOIC ACID. THE AREA IN RED IS THE HYDROPHOBIC HEAD DOMAIN, THE AREA IN BLUE IS THE POLYENE LINKER REGION AND THE AREA IN GREEN IS THE ACIDIC GROUP

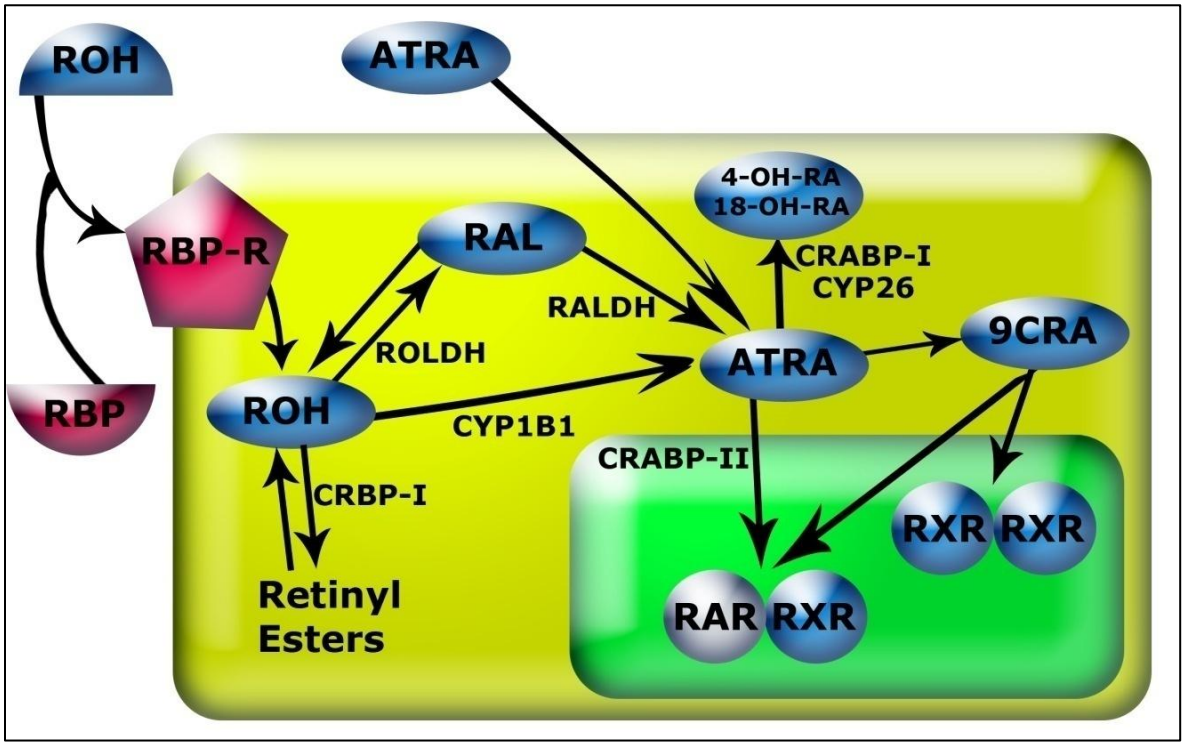


FIGURE 2. OVERVIEW OF THE RETINOID PATHWAY INTO A CELL, ITS METABOLISM AND ITS AFFECT ON SIGNALLING

The following introduction will look in detail at various aspects of retinoid metabolism and signalling. As can be seen in Figure 2. this is a complex system. This study will look at how some of the synthetic retinoids discussed below, and those used in this investigation, fit into this system and importantly how they elicit their own phenotype.

RETINOID UPTAKE AND STORAGE

Vitamin A must be obtained by diet, as it cannot be synthesized by any animal species. It is usually obtained in the form of pre-formed Vitamin A, (usually retinyl esters, a store of ingested Vitamin A) from eating animal products. It is also available from provitamin A carotenoids (such as β-carotenoid) from plants.

Once either of these two compounds is digested, it is generally re-esterified, via enzymatic action. This is aided by Cellular Retinol Binding Protein-II (CRBP-II) which is generally involved in the conversion of Retinol into retinyl esters for chylomicron export to the liver (Ong 1984). There is also a Cellular Retinol Binding Protein- I (CRBP-I) that seems to be ubiquitously expressed throughout the organism. Its role seems to be related to facilitation of cellular uptake and metabolism of Retinol either in its storage or active forms.

Retinol is generally stored as retinyl palmitate, which is formed via a reversible enzymatic activity. Transport around the body and through the vascular system is achieved using Retinol Binding Proteins (RBP) (Kanai, Raz and Goodman 1968) to which the Retinol is bound. These compounds also assist the compound across the cytoplasmic membrane (Maraini and Gozzoli 1975). These RBP seem to play an important role in the material delivery of the Retinol to the embryo (Sapin, Begue, et al. 1998) (Sapin, Ward, et al. 1997). However, the mechanisms and the physiology of maternal-fetal Vitamin A transfer are not fully understood. It must also be pointed out that it does seem that the pathways responsible for delivery of retinoids from the mother to the embryo are complex and overlapping. These pathways include retinyl esters in lipoprotein particles which can be a significant source of retinoids that can be utilized by the fetus to support embryogenesis (Quadro, et al. 2004).

There is a body of work that has shown that the principle source of retinoids in chicken eggs is Retinol, and this accounts for well over 70% of the retinoids present in this system (Plack 1960). It seems in this system that Retinol is solely transported into the egg by maternal plasma RBP (Vieira and Schneider 1993), and all the Retinol found in this system after the eggs is full formed is bound to plasma RBP (Vieira, Kuchler and Schneider 1995).

Once Retinol has entered the cell cytosol it can either be stored locally for future use (as retinyl esters) or be converted into forms that activate certain cellular biochemical pathways leading to a modulation of gene transcription as explained in more detail below.

METABOLISM OF RETINOIDS

To understand how new synthetic retinoids may affect chick development we must first study the metabolism of the endogenous naturally occurring retinoids, because as will seen the control of the endogenous retinoids is multifaceted and quite complex.

As mentioned before Retinoic Acid (RA) is the principal biologically active form of Retinol (Vitamin A). The production of the active form, RA, in the embryo is initially driven by the intracellular conversion of Retinol to RA via a two step oxidative reaction.

The first step is reversible and takes maternally derived Retinol to Retinal; this step is catalyzed by retinol dehydrogenase (RODHs) or by alcohol dehydrogenases (ADHs). One of these alcohol dehydrogenases, ADH3, is expressed ubiquitously within the mouse embryo, which one can presume produces Retinal. This can be supplied as a substrate to the RALDHs throughout the embryo (Molotkov, et al. 2002) for the next step in the conversion process. Molotkov et al., showed the importance of this by using a ADH3 null mutant mice which hence exhibited reduced RA generation *in vivo* and growth deficiency that could be rescued by Retinol supplementation.

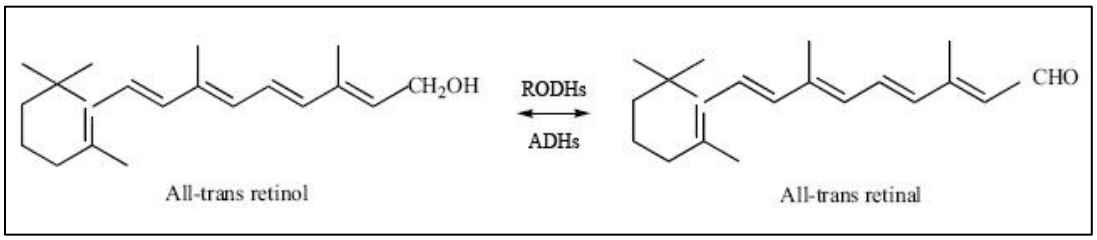


FIGURE 3. THE CONVERSION OF ALL-TRANS RETINOL TO RETINAL BY RETINOL DEHYDROGENASE OR ALCOHOL DEHYDROGENASE.

The second step takes Retinal to all-trans retinoic acid. This step is irreversible and is catalyzed by retinal dehydrogenase (RALDH). There are many RALDHs of which the most important for the embryo are RALDH1, RALDH2 and RALDH3 as reviewed by (Maden, Retinoid signalling in the development of the central nervous system. 2002). Most of the RA-signalling activity in the embryo is attributed to RALDH2 ((Niederreither, et al. 1997) (McCaffery and Dräger 1995) (Blentic, Gale and Maden 2003)).

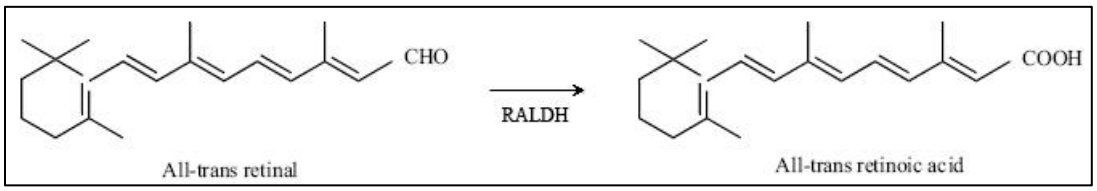


FIGURE 4. THE CONVERSION OF ALL-TRANS RETINAL TO ITS ACTIVE FORM ALL-TRANS RETINOIC ACID BY THE RETINAL DEHYDROGENASES

It must be noted that even with all the areas of RALDH2 activity combined with both RALDH1 and RALDH3 expression the supposed activity is seemingly unable to account for

all of the defects in patterning seen following full Vitamin A deprivation in rat ((White, Shankar, et al. 1998) (White, Highland, et al. 2000)) or quail ((Maden, Gale, et al. 1996) (Gale, Zile and Maden 1999)).

Thus, it has been hypothesized that since these three enzymes presumably cannot alone account for all of the observed RA-patterning processes there must also be at least one other pathway. This RALDH independent pathway has been shown to allow ATRA to be created from All Trans Retinal or All-Trans Retinol. The enzyme responsible for this is CYP1B1, which has been shown to be able to catalyze the conversion of All-Trans Retinol to All-Trans Retinal ((Choudhary, et al. 2004) (Chen, Howald and Juchau 2000) (Zhang, Dunbar and Kaminsky 2000)) and subsequently to ATRA (Chambers, et al. 2007). The work by Chamber et al., utilized microsomes prepared from a baculovirus expression system containing the *in vitro* translated hCyp1B1 cDNA. 35 μM Retinol as a substrate, and retinoids were subsequently extracted with n-butanol and methanol. The Retinal and ATRA generated was then confirmed by HPLC.

CYP1B1 is a member of the cytochrome p450 family of mono-oxygenases. This family includes CYP26s which is discussed below in the context of the catabolism of ATRA. CYP1B1 on the other hand cannot degrade ATRA.

However it may well be that the two paths, both RALDH dependent and RALDH independent, are not separate but work in unison in some situations, for example where the expression of Cyp1B1 and RALDHs overlap. Somites which express Raldh2 and the mid-hindbrain boundary which expresses Raldh3 are two possible examples. In these areas Chambers et al., (Chambers, et al. 2007) proposed the concept in keeping with their results that CYP1B1 may supplement the supply of retinal to the RALDHs as well as making RA.

Chambers et al., then also went on to present the idea that their results would be consistent with, but did not prove, the concept that CYP1B1 may be the sole supplier of RA (Chambers, et al. 2007) in areas that do not express any other known RALDHs (A list of some of the areas this may include is: hindbrain paraxial mesoderm, limb buds and pharyngeal pouches).

The findings discussed above become even more interesting when one takes into account the fact that, while enzymes that can conduct the conversion of All-Trans Retinol to Retinal are ubiquitously expressed, the enzymes that generate RA both in the RALDH pathway (RALDH1-3) and the CYP1B1 pathway (CYP1B1) have a tissue-specific profile. This once again possibly hints at the complexity of retinoid signalling which is discussed later.

These findings also disprove the old concept that RA signalling was controlled through the rate limiting of the Retinal to Retinoic Acid step. Rather the control of the signalling is due to the differential and spatial expression of other factors.

ATRA concentration limiting seems to be also carried out by catabolism (Blaner and Olson 1994). In the 1980's it was demonstrated that there was a sequential conversion of RA to 4-hydroxy- and 4-oxo-RA by hamster liver microsomes (Roberts, Lamb and Sporn 1980).

The biochemical properties of the oxidative process inferred the possible involvement of a cytochrome P450-dependent pathway in RA metabolism (Roberts, Lamb and Sporn 1980) as well as by the ability of known cytochrome P450 inhibitors to inhibit RA catabolism *in vitro* and *in vivo* ((Muindi, Young and Warrell 1994) (Kang, et al. 1996) (Schwartz, et al. 1995)).

The findings above lead to a further clarification of the nature of RA catabolism by the cloning of a novel gene, CYP26, which encoded a cytochrome P450-related hydroxylase ((White, et al. 1996) (Ray, et al. 1997) (Fujii, et al. 1997) (White, et al. 1997)) called CYP26 ((White, et al. 1996) (White, et al. 1997)) , also occasionally referred to as P450RA (Fujii, et al. 1997) so called due to its RA inducibility. It was originally cloned from regenerating, RA-treated zebra fish fin (White, et al. 1997), murine embryonic stem cells ((White, et al. 1996) (Ray, et al. 1997) (Fujii, et al. 1997)) and a human cDNA library (White, et al. 1996).

There are two main cytochrome P450 enzymes that seem to carry out the work of metabolizing ATRA, these are CYP26A1 and CYP26B1. They convert ATRA to 4-oxo-RA, 4-OH-RA, 18-OH-RA and 5,8-epoxy-RA ((Fujii, et al. 1997) (White, et al. 1996) (J. A. White 2000))

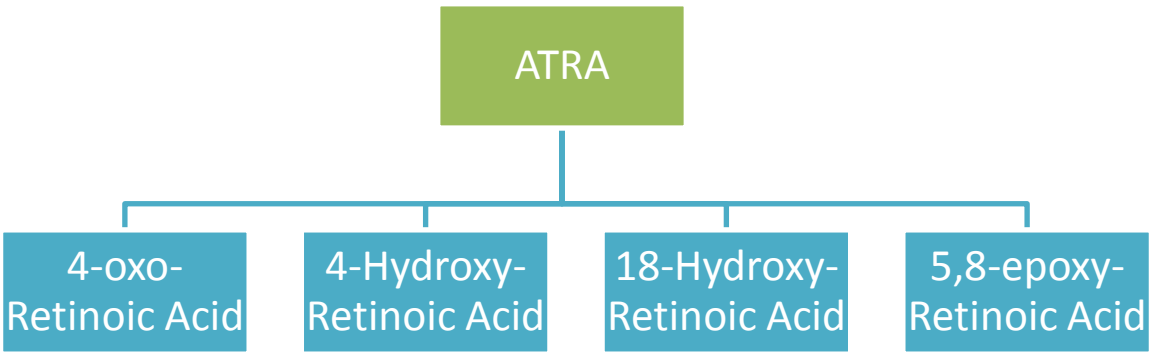


FIGURE 5. FIGURE TO SHOW THE PRODUCTS OF THE METABOLISM OF ATRA BY CYP26S.

The products are excreted and were thought to be inactive. However it has more recently been possibly shown that some of these products may be biologically active, and possibly even have the ability to induce neural differentiation (Sonneveld, et al. 1999). In this investigation it was proposed that it was not ATRA that caused this differentiation but rather CYP26s catalyzing the formation of metabolites (4-Hydroxy- and 18-hydroxy-RA were implicated directly in this investigation) that possibly could yield this effect.

This investigation utilized P19 Embryonal Carcinoma (EC) cell lines stably expressing hCYP26 (human CYP26) which were shown to undergo extensive and rapid neuronal differentiation in monolayer at low concentrations of RA. At these low concentrations of RA the authors believed that endogenous CYP26s would not be induced, as discussed in more detail below. In keeping with this concept, under these conditions their control P19 cells were shown to only differentiate into endoderm-like cells. Sonneveld et al., believed that the

isolation and characterization of these metabolites (4-hydroxy-RA, 18-hydroxy-RA and 4-oxo-RA were all directly identified) in these hCYP26 cells in their investigation helped to give weight to their hypothesis. In addition they also went on to comment on the fact that this is in keeping with data showing CYP26s are highly expressed in the developing CNS, such as hindbrain ectoderm, cephalic mesenchyme (including migrating neural crest cells), and caudal neural plate ((Fujii, et al. 1997) (Trofimova-Griffin and Juchau 1998) (De Roos, et al. 1999) (Iulianella, et al. 1999)).

However, it should be noted that these products of CYP catabolism are probably not used by the embryo. This was shown by the lethal phenotype of the Cyp26a1 $^{-/-}$ mutant mouse. This seems to mimic the effects of excess RA administration, but can be rescued by the heterozygous disruption of Raldh2, which would act to reduce the amount of RA that is synthesized in the embryo (K. E. Niederreither 2002). Although no ATRA is being metabolized by Cyp26A1, and thus metabolites are being produced, normal patterning is still being achieved.

The regulation of the CYP26s promoter region is thought to be at least partly controlled by the RAR- and RXR- families. These have been implicated in regulating CYP26 gene expression in F9 cells (Abu-Abed 1998). When CYP26 cDNA is transfected into cell lines ((White, et al. 1996) (Fujii, et al. 1997) (Abu-Abed 1998)) the net result is the oxidation of exogenous ATRA to products identified as 4-hydroxy- and 4-oxo retinoids. However it is worth noting that CYP26s seemed to be unable to metabolize the precursors of ATRA; Retinol or Retinal (Fujii, et al. 1997). This was assessed *in vitro* using 293T cells which were transfected with a CYP26 expression vector. Microsome fractions were prepared from transfected and untransfected cells. These microsome fractions were then incubated with various retinoids and the reaction products were analyzed by HPLC. This is interesting to note as the results showed that CYP26s could metabolize ATRA as well as 9-cis RA and 13-cis RA.

These transfected cells exhibited reduced sensitivity to RA-induced differentiation and reporter gene trans-activation. This possibly implies that RA is inactivated by the action of CYP26s (Fujii, et al. 1997).

It seems that RAR-α, which is discussed in more detail below is thought to play a large part in the control of the CYP26s promoter region and thus in the control of the concentration of the CYP26s. This was shown by Ozpolat et al., to be the case due to the receptor specific antagonist of RAR- α totally inhibited ATRA-induced expression of CYP26A1 (Ozpolat, Mehta and Lopez-Berstein 2005). Thus indicating that RAR-α plays a major role in CYP26A1 expression, or at least in HepG2 cells which this investigation used.

CYP26 mRNA as mentioned before has been shown to be expressed in embryonic stem cells and during development ((Ray, et al. 1997) (White, et al. 1997) (De Roos, et al. 1999)). It has been shown that in mice treated with a high dose (100 mg/kg, i.p.) of All-Trans RA, CYP26s expression was induced in liver, but not brain (Ray, et al. 1997), though in this study RA metabolism was not studied. Therefore, for some time it was still unclear whether

CYP26s were involved in RA metabolism under physiological conditions and at the time no studies of its potential regulation by Vitamin A nutritional status had been conducted.

In 2000 Yamamoto, Zolfaghari and Ross designed a study to clarify whether the metabolism of RA and the expression of CYP26s were regulated in the liver of intact animals under the following conditions; acute, nontoxic RA treatment and chronic dietary exposure to different levels of Vitamin A.

It was found that there was a reduction in the expression of CYP26s and RA metabolism during Vitamin A deficiency. The results also show there was an up-regulation of CYP26s by acute treatment with RA, and that it was modulated by chronic dietary exposure to Vitamin A. It is also worth noting that in the chronic dietary model, liver Retinol concentration was strongly correlated to CYP26s expression. Thus these results may imply a role of CYP26s in normal retinoid metabolism, possibly especially so when dietary Vitamin A intake and hepatic Retinol reserves are elevated (Yamamoto, Zolfaghari and Ross 2000).

These results perhaps also highlight another aspect of importance, which is the ability of CYP26s to be up or down regulated in response to the amount of an active retinoid present. This ability to be up or down regulated in a dosage dependent manner has also been seen in the up regulation of CYP26A1 expression in intestinal, endothelial, liver and APL cells (Ozpolat, Mehta and Lopez-Berstein 2005). This was shown to lead to a faster metabolism of ATRA and thus a possible role in rapid clearance of ATRA after continuous oral administration in APL. This ability is interesting to consider in the context of the synthetic retinoids discussed below that seem refractory to these methods of metabolism.

This concept, that the level of CYP26s is induced by the concentration of ATRA, is also shown to be occurring in some embryos. Dobbs-McAuliffe et al., work describes this phenomenon in zebra fish embryos (Dobbs-McAuliffe, Zhao and Linney 2004).

It would also, at this the junction be worth mentioning the cellular Retinoic Acid binding proteins (CRABP) family. These are found within some cells and come in two forms CRABP-I and CRABP-II.

CRABP-I is involved in the storage of free ATRA and also thus performs a role in inhibiting the biological activity of the metabolite when stored (Dong, et al. 1999). It has been found that, in general, cells that contain CRABP-I do not respond to ATRA.

However the role of CRABP-II is very much less well understood. It has however been hypothesized that it is involved in the transport of ATRA to its receptors. Therefore it may well be that it plays a role in the sensitizing of cells to ATRA (Budhu, Gillilan and Noy 2001). Once again as seen with other molecules involved in the metabolism (and later on in this introduction the cell signalling that retinoids produce) the specific expression of these important retinoid regulators in different tissues indicates their complex nature of action and possible involvement in many biological processes.

RETINOIDS AFFECT ON SIGNALLING; THE RETINOIC ACID RECEPTORS

RA has been shown to function as the ligand for two families of ligand-activated nuclear retinoid receptors, RAR and RXR, which regulate the transcription of a large number of genes. Although there have been advances in understanding retinoid receptor biology, the regulation of these molecules is still not well understood ((Chambon 1996) (Giguère 1994) (Mangelsdorf, et al. 1995)).

Two of the isomers of RA, All-Trans RA and 9-cis RA, are thought to act through different receptors. It is not known whether they are produced by separate enzymatic pathways, from All-Trans Retinol and 9-cis Retinol, respectively , or whether they can be interconverted by isomerization.

The metabolism of All-Trans Retinoic Acid *in vivo* generates many metabolites with varying retinoid activity ((Haque and Anreola 1998) (Stanley, Michael and Edgar 1998)). Transcription can be altered when these products interact with and activate retinoid nuclear receptors (Mangelsdorf, et al. 1993).

Studies of steroid and thyroid hormone action were the first to create hypotheses of a molecular action between Retinoic Acid and RARs (Evans 1988). The proposed theory suggested that Retinol entered the cell then via enzymatic action was converted to ATRA. After this the ATRA was transported to the nucleus by CRABP-II.

Once in the nucleus the ATRA would bind to Retinoic Acid Receptors (RARs) with high affinity via a ligand binding domain. There would then be a conformation change to the receptor-ligand complex brought about by this interaction. The net effect of these steps would be to enable the binding of a specific Retinoic Acid Response Element (RARE) to a target gene via the DNA-binding domain.

The first nuclear retinoid receptor (now known to be RAR-α1) was independently discovered by two groups in 1987, ((Giguere, et al. 1987) (Petkovich, et al. 1987)). As mentioned above, there are two retinoid receptor families, RAR and RXR (retinoid X receptors (Mangelsdorf, et al. 1990), and each family can be split into three sub-groups α, β, and γ members (De Luca 1991). The RARs can also be further sub-divided into multiple receptor isoforms (i.e. RAR-β1, RAR-β2 and RAR-β3). These molecules are located in the nuclear envelope. The heterogeneity of these molecules is due to each group being encoded by a separate gene that has multiple promoters; thus encoding each subtype. The products are also able to undergo differential splicing which may explain the different isoforms.

It is worth noting that during mouse development the different receptor isoforms are known to have unique distributions. However it has been shown that by knocking out one specific isoform at a time that there is a large amount of redundancy between members of each receptor subtype. Mice homozygous for the null mutation of either RARα1, RARβ2, or RARγ2 were viable and did not seem to display any phenotypic abnormalities (Kastner, Mark and Chambon 1995).

It has been shown that RARs preferentially bind to All-Trans Retinoic acid, with RXRs binding to a stereoisomer, 9-cis Retinoic Acid more readily ((Allegretto, et al. 1993) (Levin, et al. 1992)). These receptors frequently exist as RAR/RXR heterodimers. While RARs must always bind to DNA as heterodimers, they are only able to pair with an RXR. RXR on the other hand may act as homodimers or heterodimers, pairing up with themselves, RARs or other receptor compounds ((S. Kliewer, et al. 1992) (Chen, et al. 1998) (Kersten, Gronemeyer and Noy 1997) (Laudet 1997)). As with RAR there are also three sub groups within the RXR these are RXRα, RXRβ and RXRγ (S. A. Kliewer, et al. 1994). There are also several isoforms.

It is also worth noting that the expression of these RAR and RXR subtypes and isoforms varies greatly throughout the different tissues of an organism. Perhaps this, more clearly than anything else, indicates the possible complex and varied role that retinoids play in adult and embryonic cell systems.

Retinoid Phenotypes in the model system of Chick

Retinoids are known to affect development of many systems in the chick embryo. However within the confines of this investigation we will focus on just two areas in which it is known to elicit an effect in chick.

Digit Duplication

The chick forelimb has often been recognized as a useful model system in development due to its relatively simple pattern with three morphologically distinct digits. It is also relatively accessible to experimental manipulation.

The ability of All-Trans Retinoic Acid (RA) to create duplicate digit patterns that are strikingly similar to those obtained with grafting cells from the ZPA to the same position has been recognized for some time. This has generally been achieved using ion exchange beads that allow the controlled release of All-Trans Retinoic Acid (RA) where they are implanted in the anterior margin of the developing bud ((C. J. Tickle 1985.) (Tickle, Alberts, et al. 1982) (Summerbell 1983)).

These investigations also went on to show that there was concentration sensitivity in this system, showing a dose dependent relationship between the amount of RA applied and the extremity of the phenotype observed. The most mild of these different and progressive outcomes is the formation of an extra digit 2, on top of the normal 234 patterning of the chick forelimb. If the concentration of the retinoid is increased one may then see an embryo with an extra digit 3 and 2, once again on top of the normal chick limb digit pattering of the 234. Finally if higher levels of retinoids are used then a full mirror digit duplication pattern may be seen, thus there will be six digits in total with a new digit 4, 3 and 2 on top of the normal 234 limb pattern.

It is worth mentioning that the tissue concentration of RA required to cause such duplications is in the physiologically significant nanomolar range. In these experiments the local release of RA was postulated to result in an exponentially shaped concentration gradient along the anteroposterior axis (C. J. Tickle 1985.).

More recently a growth-morphogen model of chick wing anteroposterior patterning has been proposed. (Towers et al., 2008) In this model both growth and morphogen gradient are thought to be necessary to normal chick wing development.

This is a twofold process where firstly Shh from the ZPA promotes sufficient AP growth of the mesenchymal field for the three digits to form. Secondly during this time the Shh gradient forms along the anteroposterior axis creating three AP positional values and thus identities for each of the digits. high levels of Shh being digit 4, intermediate levels being digit 3 and low levels being digit 2. These positional values are thought to be remembered.

The concepts of this model can be see in action in the two experiments described below.

Shh signalling can be reduced experimentally using Cyclopamine treatment, with this treatment there is a decrease in Shh-dependent anteroposterior expansion to the extent that only two digits form (Scherz et al., 2007). The reduced Shh signalling specifies only the lower Shh concentration dependent digit 2 and 3.

This link between growth and the morphogen can also be seen in states of reduced proliferation and growth. TSA treatment can be used to irreversibly inhibit AP expansion of the limb bud during digits specification (Towers et al., 2008). In the most severely affected wings, only one digit can forms which is digit 4. This is thought to be because the duration of Shh signalling is not affected, so the most posterior and highest Shh concentration dependent digit 4 is specified.

Facial Phenotypes

It has long been recognized that chick embryos that are treated with Retinoic Acid will often produce a phenotype with a distinct appearance. This entails truncations of the upper beak while the lower outgrowth remains normal (Tamarin, et al. 1984). In these treated faces the inhibited cartilage differentiation of the upper beak is seemingly absent from its normal position leaving just a series of tubercles derived from maxillary primordial, frontonasal mass and lateral nasal processes.

During all of this the paired mandibular primordial that forms the lower beak is unaffected and outgrowth is quite normal. These phenotypes do not require a retinoid soaked bead to be placed next to them; a bead placed in either of the forelimb buds is close enough to the developing face to yield an affect (Tamarin, et al. 1984).

This together with data showing that if a bead is placed in a more posterior position in the developing chick forelimb the facial phenotype is less severe (Tamarin, et al. 1984); lead to the concept that the proximity of the developing face to the implant site affects the extent of

the facial defect. It was also estimated in the same paper that the effective concentration range of Retinoic Acid to which the face is exposed was as much as 10^{-7}M over the 24 hours.

The mechanism for this seems to be heavily linked to the products of two homeobox-containing genes, Msx 1 and Msx 2. These have been implicated in epithelial-mesenchymal interactions.

Msx 1 and Msx 2 (also previously known as Hox-7 and Hox-8) are expressed in overlapping domains at many sites in the embryo, including the developing face and limb (Davidson 1995).

In the developing mouse and chick face, Msx 1 and Msx 2 are expressed in spatially-restricted overlapping domains ((Brown, et al. 1993) (Mina, et al. 1995)). These expression domains in the developing chick face may suggest that they govern the outgrowth of facial primordial by playing a role in epithelial-mesenchymal interactions. In the normal face, high levels of Msx gene transcripts in upper and lower beak primordia correlate with regions of outgrowth (Brown, Robertson and Wedden 1997).

As mentioned above retinoid application to developing chick faces reproducibly inhibits upper beak outgrowth but the lower beak is unaffected (Tamarin, et al. 1984). The retinoid treatment seems to be yielding its effect though Msx 1 and Msx 2. This can be hypothesized due to the fact that the aforementioned high level of Msx gene transcription in normal face outgrowth does not occur in the upper beak primordial where transcript is rapidly down-regulated during ATRA administration (Brown, Robertson and Wedden 1997). It does however remain relatively unchanged in lower beak primordial, which is, as mentioned before where the outgrowth is unaffected.

It is worth noting that these decreases in gene expression precede retinoid-induced morphological changes in the upper beak. This suggests, along with the data above, that Msx gene products are involved in mediating the effect of retinoids on facial development (Brown, Robertson and Wedden 1997).

However this may only be representing one aspect of what is happening in this system. More Recently findings have shown that the retinoids seem to be needed for normal patterning of the upper beak. This is shown by a member of the aldehyde dehydrogenase (ALDH) family, ALDH6, being expressed in a restricted region of ventral ectoderm covering the FNP of chick embryos (Schneider, et al. 2001). These nasal placodes have also been shown to express retinaldehyde dehydrogenase-3 (RALDH3).

It is also shown that the main role of endogenous RA signalling occurs earlier than when exogenous RA application has been shown to yield phenotypes. This is hinted by expression of ALDH6 in epithelial cells of the presumptive FNP beginning precisely at stage 10 and ending at stage 12 (Schneider, et al. 2001). This concept is further developed by two retinoid receptors, *RAR*β and *RXR*γ, being found in neural crest mesenchyme ((Schneider, et al. 2001) (Hoover and Glover 1998) (Rowe and Brickell, Expression of the chicken retinoid X receptor-gamma gene in migrating cranial neural crest cells. 1995) (Rowe, Richman and

Brickell 1992)) which subsequently then migrates out of the rostral neural tube and eventually accumulates around the eyes in the FNP.

A good illustration of the need for this early Retinoic Acid signalling comes from treatment with RAR/RXR antagonists. In these experiments chick embryos of stage 10 treated with this RAR/RXR antagonist show no development of the entire frontonasal process. However at stage 18 the same treatment yields no facial phenotype at all, even with a fourfold increase in the antagonist (Schneider, et al. 2001).

It is thought that truncation at this early stage is yielded through a down regulation of FGF8 and Shh (Sonic hedgehog) in the ectoderm of the frontonasal process (Schneider, et al. 2001). FGF8 and Shh, as shown by data from studies in the limb ((Helms, et al. 1996) (Stratford, et al. 1999)) are candidate downstream targets of retinoid signalling.

Up to stage 20 FGF 8 expression is seen across the entire frontonasal mass disappearing by stage 24 in normal patterning. If this drop does not occur the frontonasal process does not continue to develop properly (Firnberg and Neubuser 2002). This is what is shown to happen in cpp (cleft primary palate) a chick mutation that leads to a truncation of the frontonasal process (MacDonald, Abbott and Richman 2003). In cpp there is prolonged Fgf8 expression in the cpp mutant frontonasal mass which may serve to maintain higher levels of retinoid synthesis and/or retinoid responsiveness (MacDonald, Abbott and Richman 2003). This is possibly due to the ability of FGF8 to up regulate Radlh2 and RAR, as shown in the mammalian equivalent of the frontonasal mass, medial nasal prominence mesenchyme, when FGF8 is added to culture medium (Bhasin, et al. 2003). Perhaps this phenocopying by the cpp mutant of exogenous RA application sheds light on a mechanism for the latter.

The Bone Morphogenic Proteins (BMP's) are also thought to play a role in normal beak patterning (Francis-West, Tatla and Brickell 1994). It seems normal patterning and identity of the frontonasal mass is dependent on high RA, low BMP levels for the initial signal that is followed by regulation of transcription factors and a late up regulation of BMPs (as reviewed by (Richman and Lee 2003). This becomes more interesting when it is known that it has been shown in chick forebrain that BMP4 represses Fgf8, and Shh maintains Fgf8 expression (Ohkuboa, Chiangb and Rubenstein 2002).

SYNTHETIC RETINOIC ACID ANALOGS

TTNPB

TTNPB (4-[E-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid) the aromatic retinoid is a selective and highly potent Retinoic Acid analog which binds Retinoic Acid Receptors (RAR) α, β, and γ, which as mentioned before are nuclear transcription factors. Thus TTNPB is able, as natural occurring retinoids do, produce a ligand-activated transcription of genes that possess RAREs.

TTNPB has been shown to be 1000-fold more potent as a teratogen than ATRA in several species and in the inhibition of chondrogenesis in the mouse limb bud cell culture (Pignatello, Kauffman and Levin 1997).

In 1997 Pignatello et al., published a study as to the factors responsible for the potency of TTNPB. These included binding to nuclear Retinoic Acid Receptors (RARs and RXRs), cytosolic binding proteins (CRABPs), and metabolic disposition of TTNPB. In this study TTNPB was shown to bind to RAR α, β, and γ with K_ds in the nanomolar range. It must be noted that these binding affinities are 10-fold less than those of ATRA. Thus although the affinities are high for TTNPB, being no higher than those of ATRA, it seems to indicate that it is unlikely that the binding affinities to nuclear receptors alone can account for the potency of TTNPB. It is worth noting that in this investigation any competitive binding assays and saturation kinetics were achieved using nucleosol or cytosol fractions obtained from COS-1 cells transfected with cDNAs encoding the appropriate nuclear receptor or binding protein.

The next part of the study looked at the binding affinities of TTNPB for the CRABPs. These were once again shown to be significantly lower than those of ATRA. TTNPB was also shown to not compete with 9-cis RA for binding to RXR α, β, or γ.

Finally the metabolic disposition of TTNPB and ATRA was studied. A well characterized model for retinoid teratogenesis the mouse limb bud cell cultures were used to do this. The media of the limb bud cell cultures were treated with either retinoid. The disappearance of ATRA from this media over the 72 hours was significantly faster than that of TTNPB. The uptake experiments however showed that both retinoids reached approximately equal concentrations in the end. The final part of this line of experiment however showed that TTNPB disappeared from the limb bud cell at a significantly slower rate than ATRA. These results collectively possibly indicate that the potency of TTNPB is due to a lower affinity to CRABPs resistance to metabolism and thus prolonged nuclear receptor activation (Pignatello, Kauffman and Levin 1997).

Further investigations have since been conducted into the role of half-life in retinoid toxicity by Pignatello et al., in 2002. A series of experiments was performed to determine to what extent or whether at all, the inhibition of ATRA metabolism by Liarozole increased its toxicity comparable to that of TTNPB. The experiments were conducted using the mouse limb bud system. This was done by collecting forelimb and hindlimb buds which were dissected from embryonic day 12 embryos which were then disassociated to create Mouse embryonic limb bud cell cultures. These cells were exposed to the ATRA, TTNPB and Liarozole by simply adding them to their media.

It is worth briefly mentioning the role that Liarozole is a known to play. It is a known inhibitor of ATRA 4-hydroxylation, a metabolism step of ATRA catalysted by CYP26 as discussed above.

The results were quite conclusive. In the absence of Liarozole, the IC_{50} for inhibition of chondrogenesis by ATRA was 140 nM compared to 0.3 nM for TTNPB. This represents a 467 times difference between the two. However following Liarozole treatment of the limb bud cultures, the ability and potency of ATRA needed to inhibit chondrogenesis was increased by approximately 14 times. An interesting point of note is that although Liarozole markedly

increased the overall toxicity of ATRA in these mouse limb bud cultures, the overall catabolism of ATRA by CYP26 was only reduced by about 10%.

The results from the above investigation seem to clearly indicate that a relatively minor decrease in the metabolism of ATRA is associated with a marked and relatively large scale increase in its toxicity in the mouse limb bud system. This toxicity is thought to be most likely due to the prolonging of the effective half-life of ATRA, especially during a critical period of development. Thus this investigation concluded that the prolonged half-life of TTNPB may be the most significant factor contributing to the extremely high teratogenicity of TTNPB (Pignatello, Kauffman and Levin 2002).

EC23 AND EC19

Naturally occurring retinoids including the aforementioned All-Trans Retinoic Acid are known to degrade readily. This degradation is mostly through photo mediated degradation (isomerisation). In one study by Christie et al., in 2008 it was shown that over a three day period of exposure to ordinary laboratory (fumehood) fluorescent light, which emits in the visible to near-UV range, there was substantial isomerisation/degradation of the ATRA with approximately only 37% remaining unchanged (Christie, et al. 2008). This investigation also found that ATRA not exposed to the light and kept completely dark over the same period of time remained stable. EC23 and EC19 on the other hand were shown to be stable under both conditions (Christie, et al. 2008).

This susceptibility is mainly due to its molecular structure. ATRA along with other much used natural retinoids, 9-cis and 13-cis retinoid acid, function as chromophones thus selectively absorbing light. As mentioned before retinoids are composed of three structurally distinct areas; a hydrophobic end, a polyene linker region and an acid group. It is the polyene linker region that causes this ability to absorb light. This is due to the fact that it is highly conjugated and thus can depend on solvent absorption between 300-400nm. Thus it is particularly susceptible to photo-isomerisation (Murayama, Suzuki and Matsui 1997) and can degrade into a range of different retinoid acid isomers.

Thus it could be that the findings in the past, that the concentration of retinoid levels drop markedly over time in culture (Han, et al. 1995), could be a consequence of both their degradation and metabolism. This could easily lead to inaccuracies in any laboratory based procedure that utilizes naturally occurring retinoids and ATRA in particular, in the presence of light as the total concentration of the retinoid would be impossible to know. Also, and almost more importantly, the composition of which isomers were present would also be unknown. Thus any results obtained would always have to have the caveat that any effects seen could in part be due to the effect of these isomers; as well as the stochastic final concentration of the retinoid.

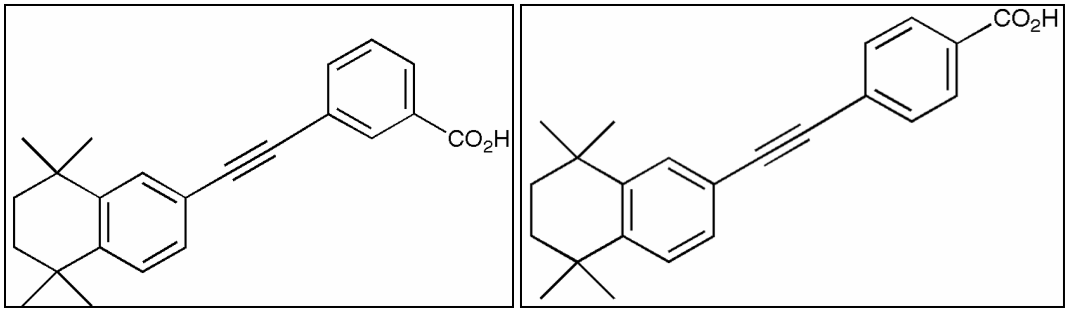


FIGURE 6. THE STRUCTURE OF EC19 (TO THE LEFT) AND EC23 (TO THE RIGHT)

With the point above in mind, the need for and uses of, a photo stable equivalent of ATRA and other retinoids can easily be seen. It was for these reasons that EC23 and EC19 were designed and synthesized. Due to the large differences in molecular shape, because of the different combinations of olefin geometries in natural retinoids polyene containing regions, EC23 and EC19 were synthesized using a stero-controlled synthesis of the polyene building blocks. The difference between the two compounds comes from the acid group substitution either being in the 'para' as in EC23, thus mimicking the molecular shape of ATRA; or 'meta' position as in EC19. From current data EC23 also seems to mimic the effect of ATRA as it is a known potent pan-RAR agonist, whilst being inactive towards RXRs (Gambone, et al. 2002).

EC23 or EC19 have never been tested *in vivo* however there has been testing *in vitro* including work by Maltman et al., (In press) which looked at the novel application of proteomic biomarker profiling technology to stem cell lysates to compare the differentiation effects of ATRA with those of two stable synthetic retinoid analogues, EC19 and EC23.

The investigation used MALDI-TOF MS (matrix-assisted laser desorption ionisation time-of-flight mass spectrometry) protein profiles to support previous findings into the functional relationships between these compounds, when applied to the Embryonal Carcinoma cell line Tera2.SP12. These previous findings include immunological analysis of marker proteins that revealed that the Tera2.sp12 embryonic carcinoma stem cells followed similar differentiation patterns in response to ATRA and EC23 treatment. EC19 on the other hand stimulated a distinctly different pathway of differentiation (Christie, et al. 2008).

This new investigation found that cellular retinoid responsive proteins CRABP-I, CRABP-II, CRBP-1, and the actin filament regulatory protein profilin-1 were up-regulated in response to ATRA and EC23 (Maltman et al. in press).

The up-regulation of CRABPs by ATRA and EC23 suggests that the latter may stimulate bona-fide retinoid responsive pathways in the cell.

However not all these up regulations are uniform, although the MALDI profiles demonstrate that both CRABP-I and II are up-regulated by both compounds. CRABP-I induction was shown to be greater in response to EC23. As mentioned above the natural function of

CRABP-I is to lower ATRA by facilitating its enzymatic breakdown and also as a possible simple sequestering mechanism.

Thus the up regulation of CRABP-I by EC23 could be because it is more biologically active/potent than ATRA at the same concentrations. Alternatively it may well be that it is just more resistant to cellular metabolism. Both of these possible explanations could aid the explanation as to the enhanced neurogenic effect observed in the EC23-treated cell cultures.

The investigation also reported that the level of induced cellular Retinol binding protein I (CRBP-I) seemed to be similar in ATRA and EC23 samples.

As shown above, although the synthetic retinoids EC23 and EC19 have both been tested *in vitro*, their possible effects *in vivo* were up until now untested. Since the effects of naturally occurring retinoids (ATRA, 9-cis RA and 13-cis RA) and synthetic retinoids on the chick limb bud system are quite well documented it was proposed that this system offered a good start to begin to explore the biological effects of these compounds *in vivo*.

CYCLOPAMINE

Cyclopamine (11-deoxojervine) is a naturally occurring chemical that belongs to the group of steroidal jerveratrum alkaloids. It was originally identified and isolated from the corn lily (*Veratrum californicum*). It is a teratogen and exposure has been shown in the past to cause cyclopia (holoprosencephaly).

The ability of Cyclopamine to be such a potent teratogen is because it can inhibit the hedgehog signalling pathway. This seems to be achieved by influencing the balance between the active and inactive forms of the Smoothened protein.

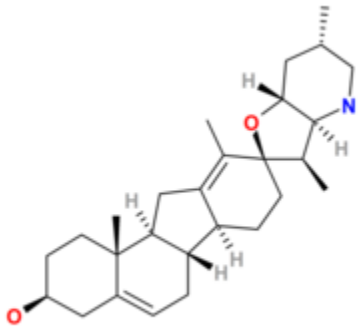


FIGURE 7. STRUCTURE OF CYCLOPAMINE MOLECULE

The compound is currently being investigated as a treatment agent in many forms of cancer including basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma as well for tumors that result from excessive Shh activity, glioblastoma, and as a treatment agent for multiple myeloma.

Due to Cyclopamine's ability to inhibit Shh it has been reported to also yield a limb phenotype. Cyclopamine treatment leads to either fusion of digits 3 and 4, or loss of digit 4 all together (Scherz, et al. 2007). The extent of the phenotype seen is determined by the time at which the embryo is exposed to Cyclopamine, with the most extreme phenotypes being exposed earlier and relatively milder phenotypes being the product of later exposures. It was shown by Scherz et al., (Scherz, et al. 2007) that if the chick embryo was exposed on embryonic day 3 the resulting forelimb was truncated to almost unrecognizable levels leaving no distinct distal element. If the exposure occurred on embryonic day 3.5 then

the limb was normal apart from the absence of digit 4 in the forelimb. Finally if the exposure occurred on embryonic day 4 then there was a partial digit 4 in the forelimb.

AIMS OF THE INVESTIGATION

The major aims of this investigation are set out below:

1. To assess the ability of EC23 and EC19 to emulate known ATRA limb phenotypes. Assessing both the frequency at which these phenotypes are yielded and also the concentrations needed to do so.

2. To assess the ability of EC23 and EC19 to emulate known ATRA facial phenotypes. Assessing both the frequency at which these phenotypes are yielded and also the concentrations needed to do so.

3. To analyse if EC23 and EC19 show different patterns of limb and facial phenotype yield when compared to ATRA and between these two novel synthetic retinoids, and to try and understand why this may be the case if so.

4. To see if a retinol equivalent of EC23 (EC23-OL) could be converted into the active form like Retinol which is known to be converted into the biologically active form, ATRA. This would possibly show how closely EC23 mimics ATRA.

METHODOLOGY

IN OVO OPERATIONS

EGG PREPARATION

The chicken eggs used in the investigation were supplied from a commercial source, PD Hook Hatcheries, Dalton, North Yorkshire, and were from white leghorn chickens (flock age 35 – 45 weeks). Fertilized eggs waiting to be placed into the incubator were stored at 10°C for up to a week to prevent any development. Eggs were collected approximately once a week from the commercial source. This was found to allow consistent development between eggs.

Prior to incubation the eggs were removed from the chiller and wiped with tissue paper soaked in 75% ethanol. This was done to reduce the risk of contamination later in the procedure from *campylobacter sp.* and other sources.

The eggs were then punched at the rounded end of the egg and 2ml of albumen was removed using a 10ml syringe and a 19G needle angled downwards to avoid damage to the yolk and embryo, thus creating a working space for the operation to be conducted. The eggs were then placed back on the tray on their side ready for incubation.

Incubation post operation was conducted at 37°C in humidified conditions in a bench top incubator. Before the eggs were operated on they were incubated for approximately 96 hours in a 37°C fixed temperature growth room, allowing the embryos to reach approximately development stage 20 (Hamburger-Hamilton staging - Hamburger-Hamilton, 1951).

RETINOID SOURCING

The retinoids used in this experiment where sourced from a variety of different locations as explained below.

ATRA was obtained from a commercial source (SIGMA) while EC23 and EC19 were synthesized by High Force Research Limited.

The Retinol equivalent of EC23 was synthesized by Josef Gluyas of the Durham University Chemistry Department and was assessed to be pure by NMR (Nuclear Magnetic Resonance). The sample was assessed by producing a nuclear magnetic resonance spectra which allows the chemists involved to distinguish atoms within a molecule. This showed the spectra expected without any other peaks that would have indicated there were other compounds were also present.

ION EXCHANGE BEAD PREPARATION AND LOADING

AG1-X2 ion exchange beads (mesh size 200-400, for an effective size between 50 and 150 μm) were obtained from a commercial source (BioRad) and first converted from the chloride to the formate derivative. This was achieved using the protocol as advised by the manufacturer which is described below. These beads were used to load EC23, EC19 and ATRA.

They were rederivatized to formate form by inserting them into 50ml centrifuge tubes and rinsing with 2 bead volumes of 1M NaOH, then rinsed with 4 bead volumes of De-ionised water. This converted the bead from the resin-Cl to the resin-OH derivative. The beads were then washed in 2 bead volumes of formic acid and finally the beads had 2 washes in 4 bead volume deionized water. The beads were then dried on filter paper. This completed the change with the beads as they were changed from resin-OH to resin-formate. During this procedure the ion exchange beads change colour from white to a light tan.

Amberlite beads, which were used to load the Retinol equivalent of EC23 (EC23-OL), required no preparation before their level of hydration was standardized as described below.

The beads were stored in this dried stage until 24 hours before an operation. At this point they were transferred to a 5 Molar NaCl solution to standardize hydration. Twenty beads, 30 minutes before the operation were then transferred into 100μl of the appropriate concentration solution of the specified compound (EC23 or EC23 rentinol)in DMSO and constantly agitated for 30 minutes. After this the beads were removed and washed in Tydrode's saline immediately before being transferred into the *in ovo* system.

Tydrode's saline was made fresh from 10 times stock made using the following recipe (500ml stock)

40g NaCl
1g KCl
0.25g $NaH_2PO_4 \cdot 2H_2O$

0.05g of both sodium bicarbonate and glucose were added to 50ml 1x stock immediately prior to use. The 10x stock was diluted in 18MOhm reverse osmosis purified water.

0.5ml of a 100x antibiotic and antimycotic solution was added to reduce the risk of infection.

OPERATIONS

The eggs were then windowed by carefully removing a small amount of the shell at the top approximately 2cm by 2cm using a pair of forceps.

After this procedure some Tyrode's saline solution was dripped into the open egg to stop any unnecessary desiccation of the material inside. Using a Tungsten needle the vitteline membrane was removed, taking care not to damage the embryo while doing so. Any other

membranes occluding the limb bud were also removed taking care not to puncture any of the vasculature of the embryo.

An incision was made parallel to the AER (Apical Ectodermal Ridge) on the anterior side of the limb bud and the ectoderm was pulled back to create a pocket into which the retinoid soaked bead was implanted with the help of sharpened watch makers tweezers. Tyrode's saline with antibiotic and antimycotic was then added to the egg (approx 1ml) to stop any desiccation of the embryo.

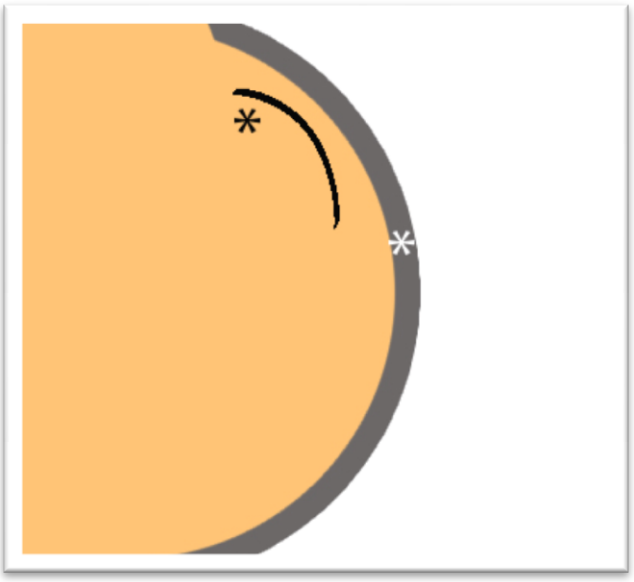


FIGURE 8. LIMB BUD, WHITE ASTRIX SHOWS THE AER AND THE BLACK ASTERIX NEXT TO THE BLACK LINE DENOTES THE AREA IN WHICH THE INCISION IS MADE TO CREATE THE POCKET FOR THE BEAD.

The egg was then taped up and allowed to incubate for a further 7 days (until H&H stage 35 is achieved).

CYCLOPAMINE PREPARATION AND ADMINISTRATION

Cyclopamine in crystallized form was obtained from a commercial source (SIGMA). 1mg was dissolved in 1ml of 45% HCB in sterile PBS and the solution was then stirred for 1-2 hours at 65°C to allow the cyclopamine to load, as described by previous investigations (Incardona, et al. 1998).

It was administered at embryonic day 3 by first windowing the egg and ripping the vitaline membrane as mentioned above. Measures to avoid desiccation of the embryo, as described above were also utilized. If present other membranes around the area in which the limb bud will form were also cleared. 5µl of the solution described above was then administered to this area using a 2-10µl micropipette. The egg was then resealed using the aforementioned technique and returned to the incubator.

If further retinoid bead implanting was to take place, this would happen the following day following the protocol as described above.

Recovery, Staining and Imagining

After the embryo had reached approximately H&H stage 35 it was removed from incubation and the tape was removed. Using forceps the membranes over the embryo were removed and the embryo was sacrificed by severing the neck and spinal column. Initial scoring was then conducted. If a phenotype could be recognized, or there was any level of uncertainty as to whether there was a phenotype present, the embryo was removed from the egg and exposed to a cartilage staining preparation.

The method for this cartilage staining in whole mount was modified from Simons and Van Horn (Simons and Van Horn 1971).

First the embryos were fixed and stained in the following:

96% ethanol – 80ml
Acetic acid – 20ml
Alcian blue 8GX – 15mg

The embryos were immersed in this solution for 5 days. After this was completed the embryo was dehydrated in ethanol over five days with three changes of 100% ethanol.

The embryo was then photographed at this stage.

After the entire embryo had been checked for any further abnormalities the torso was then isolated and was placed into 0.5% aqueous solution of KOH to start the clearing process. The specimen was left in this solution until all the cartilaginous elements were visible. The solution was then changed to the following:

Deionised water 79ml
Glycerol 20ml
KOH 1g

The specimens were then left in this solution for approximately 48 hours or until it was deemed that it would not clear any more. At this point the specimens were transferred into progressively more concentrated glycerol solutions with 1% KOH (50%, 80%, 100% glycerol) leaving them for about 3 days in each solution. They were then stored in 100% glycerol.

After the embryo was cleared the specimen was re-photographed and the limbs, once the shoulder girdle had been checked for abnormalities, were transferred in to storage plates.

All embryos were photographed using a JVC Digital Camera KY-F1030 mounted on a Zeiss stemi SV 11 though a 10x eye piece. Embryos were generally photographed with the objective magnification at 1-1.2x yielding a final magnification of 10-12x.

BEAD LOADING ASSAYS

A series of UV spectrophotometer assays were conducted to assess the ability of the beads to take up the retinoids used in this investigation.

THE NANODROP

It is believed that this is the first time that the NanoDrop ND-1000 from Thermo Scientific Limited, has been used in a bead loading assay. The NanoDrop is a full-spectrum UV/Vis spectrophotometer that can make readings on samples of approximately 2µl. Below are some of the advantages, limitations and necessary adaptations needed to facilitate its use.

The NanoDrop allowed samples around 2µl to be used and assessed to see their UV and visible absorption profile. One great advantage of being able to work on these small qualities is that it allowed very little of the retinoid to be wasted. This is an important factor for the Retinol equivalent of EC23 due to its very limited supply.

However due to the nature of the machine the solvent volatility was an issue of concern. DMSO proved to be fine to use but methanol proved impossible with the sample evaporating faster than the reading could be made.

Initial investigation revealed that the NanoDrop seemed to be far less tolerant of small particles, which were believed to be associated with the ion exchange beads used. These small particles often caused an overall increase in absorbance across the whole spectrum. It is for this reason that the samples were centrifuged as described below.

STANDARD CURVES

Standards curves were generated by starting with 20µl of a 0.01mg/ml solution in DMSO of the compound and diluting it one to one in DMSO for each of five steps. Thus producing the following series of concentrations 0.01, 0.005, 0.0025, 0.00125, 0.000625, 0.0003125mg/ml. These six samples were then run though a NanoDrop ND-1000 Spectrophotometer. These results along with two repeats were used to create the standard curve.

LOADING ASSAYS

200µl of a 0.01mg/ml solution in DMSO of the appropriate compound was made up. Forty of the appropriate beads where then put into the solution. Then immediately 10µl of the solution was removed taking care to leave the beads at the bottom. The sample was then left to agitate till the next appropriate sampling point. This sampling continued to occur at 0, 10, 20, 60, 120, 240 minutes. All of these samples were then spun at 14,000rpm for two minutes to reduce problems with fine particles associated with the beads. They were then all sampled by the NanoDrop. This procedure was then repeated twice again to create a triplicate of results.

RESULTS

The overall aim of the investigation was to compare the ability of two newly developed novel photo stable synthetic retinoids to affect development *in vivo*. To this end a series of experiments was designed and performed to test their biological activity in a system known to be affected by retinoids, chick. The two systems in chick that were the focus of the experiments were the chick limb bud and facial phenotypes.

In the past AG1-X2 ion exchange beads were used as a delivery system for ATRA. These beads were soaked in a range of different concentrations of their respective retinoids and then implanted into the anterior of the chick limb bud. After further incubation the embryos were inspected for known retinoid digit duplication phenotypes and facial phenotypes.

BEAD LOADING ASSAYS

Initial investigations looked at whether the novel compounds could be delivered to embryos using the same technique.

Therefore a method to study whether novel retinoids would load onto beads was needed. A convenient way to do this in the investigation was to use UV/visible absorbance spectra of compounds in a DMSO solution. However it was first necessary to establish that a compound gives a spectrum that can be used in this way. Thus the first step was to investigate whether the compounds gave a consistent spectrum.

Figure 9. shows the UV/visible absorbance spectra of all four retinoids used in this investigation. EC23 had a broad peak of absorbance between 305 and 325 while EC19 and EC23-OL gave two peaked spectra at 288 and 307; 291 and 309 respectively. ATRA only had a single absorbance peak at 362.

Figure 9. also shows that EC19, EC23 and EC23-OL do not absorb in the visible spectrum at all, unlike ATRA.

From this data we can conclude that all the retinoids used yield UV/visible absorbance spectra that would allow them to be used in this bead loading assay. It also showed the lack of absorbance of EC19, EC23 and EC23-OL in the visible spectrum. This is probably related to their photo stability, a factor for which they were originally developed.

STANDARD CURVES

The investigation then went on to see if the characteristic spectra could be used to estimate concentrations in solution. This was investigated by testing if it was possible to obtain a linear correlation of absorbance with concentration.

A standard curve was created for each of the four retinoids. Triplicates of dilution series were performed for EC23, EC19, ATRA and EC23-OL. Figure 10. and Figure 11. show that

there is a direct and linear relationship between absorbance and concentration over the range shown.

Thus it is possible to measure the concentration of the novel retinoids in solution using their UV/Viable light spectra.

LOADING ASSAYS

Past investigations have shown that AG1-X2 ion exchange beads are able to load ATRA in a DMSO solution (Eichele, Tickle and Alberts 1984). However it was unknown whether the novel retinoids EC23, EC19 and EC23-OL are able to be loaded in a similar manner and to what degree if they can be loaded.

The NanoDrop provided a useful tool to do this bead loading assay, however due to its nature it did cause a few problems that had to be addressed. The first problem was the selection of the solvent to be used. Due to the extremely small volume sampled by the machine (2µl) evaporation was a problem to be considered. DMSO does not seem to be sufficiently volatile to cause a problem.

Preliminary investigation showed that when readings were taken on the NanoDrop of a solution to which the AG1-X2 beads were added a rapid increase in absorbance was seen globally over all wave lengths. Random peaks were also seen.

It was assumed that this was due to small particulate matter associated with the beads. By centrifuging the samples prior to using the NanoDrop this issue seemed to be resolved.

The loading assay used 200µl of a 0.01mg/ml retinoid in DMSO solution (thus a total of 0.002mg of the appropriate retinoid) and 40 beads, the assay ran for 240 minutes as described in the methodology. Triplicates of the bead loading assay were performed for EC23, EC19, ATRA and EC23-OL.

The EC23 samples as can be seen in Figure 12. yielded a 36% drop in absorbance over the 240 minutes. The kinetics of this loading seems quite simple, with a relatively constant drop in absorbance slowing slightly as time proceeded.

The EC19 samples as can be seen in Figure 13. yielded a 37% drop in absorbance over the 240 minutes. The kinetics of this loading seems quite simple, with a relatively constant drop in absorbance slowing slightly as time proceeded.

The ATRA samples as can be seen in Figure 14. yielded a 10% drop in absorbance over the 240 minutes. The kinetics of this loading seems quite simple, with a relatively constant, however small scale, drop in absorbance slowing slightly as time proceeded.

The EC23-OL samples as can be seen in Figure 15. yielded a 5% drop in absorbance over the 240 minutes. The kinetics of this loading as seen in Figure 15. seem somewhat complex with a fast and large initial drop in absorbance which then increases again before commencing a gradual drop.

As the solutions used are of a known starting concentration, and as shown in the standard curves their absorption is directly related to their concentration, it is possible to calculate from the percentage decreases how much retinoid was loaded. As forty beads were used in each loading, it is also possible to calculate the loading per bead. This data is presented in Table 1. EC23 loaded $1.65x10^{-5}$mg per bead, EC19 loaded $1.8x10^{-5}$mg per bead, ATRA loaded $4x10^{-6}$mg per bead and EC23-OL loaded $1x10^{-6}$mg per bead.

As can be seen from the results outlined above EC23 and EC19 seem to load to a similar degree. ATRA however only loads to a much smaller degree $4x10^{-6}$mg/bead. This shows that there is approximately a four times reduction in the possible effective delivery of ATRA when compared to EC23 and EC19, assuming they unload off the bead in a similar fashion.

This low loading of ATRA however is no lower than has been previously found (Eichele, Tickle and Alberts 1984), in which it can be calculated that they loaded $1.6x10^{-6}$mg/bead over the same period of time. The discrepancy between the $4x10^{-6}$mg/bead found in this investigation and the $1.6x10^{-6}$mg/bead of the Eichele et al., 1984 investigation is most likely due to the number of beads used per 200µl of retinoid solution. This investigation used 40 beads while the Eichele et al., 1984 investigation used 600 beads and thus perhaps their beads were not individually loaded to such a degree.

The loading of EC23-OL in this experiment was the lowest of any of the retinoids. However it may well be that this was due to how the Amberlite beads load as they are a size exclusion bead not a ion exchange bead like the AG1-X2. They may load the solution as a whole with DMSO and retinoid in roughly the same proportions as found in the solution. If this were the case the bead would still load retinoid but would appear to be having no effect on the concentration of retinoid in the solution.

In summary both EC23 and EC19 load to a similar degree. ATRA loads to a much smaller degree but the amount it does load is in line with previous experiments. EC23-OL only loads a very small amount which may be due to the nature of the bead used to load it. Thus, given the possibility of the Amberlite beads loading solvent and retinoid at equal rates, a better assay may be to look for release from a loaded bead rather than loss of retinoid from solution.

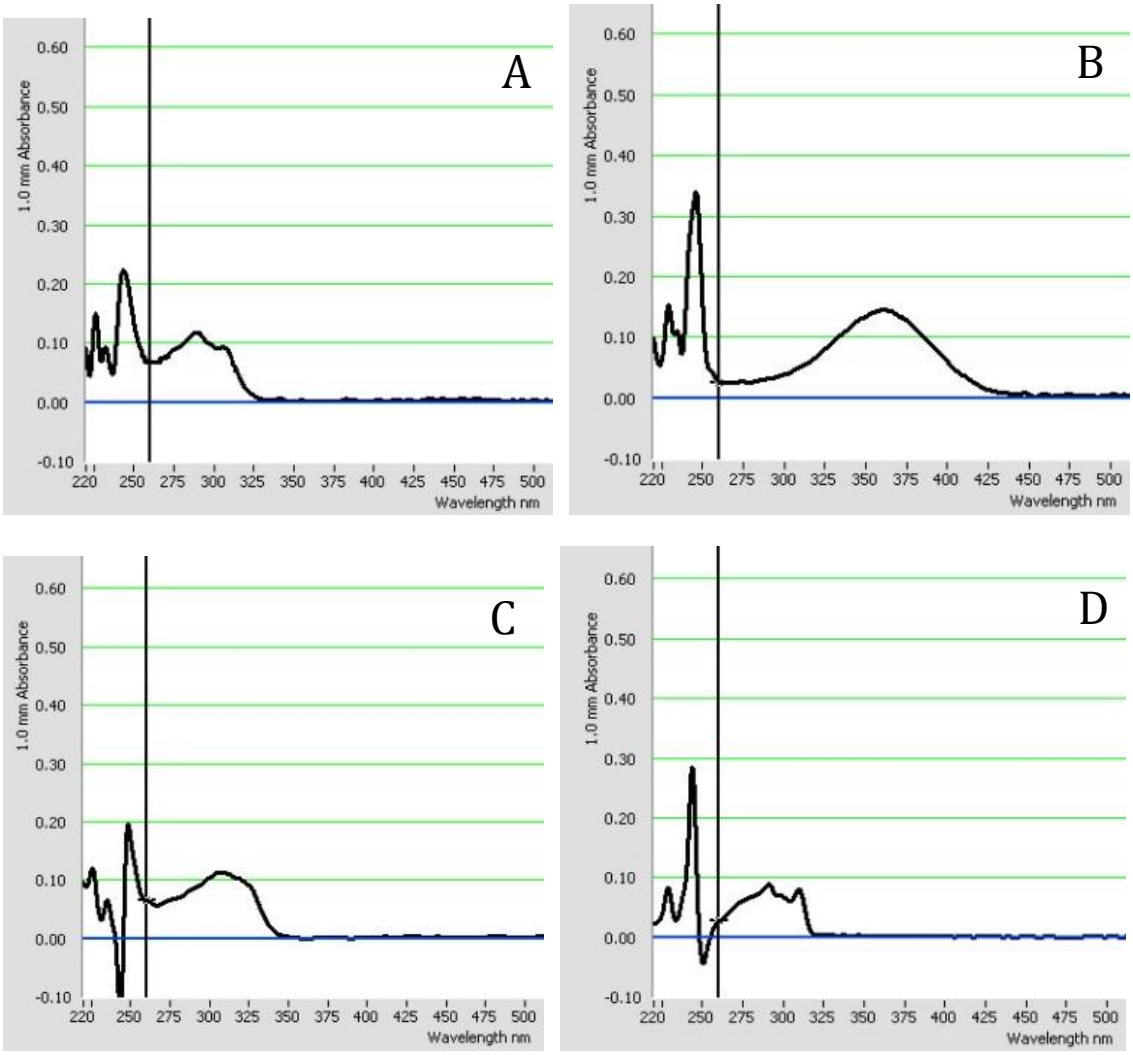


FIGURE 9. UV AND VIABLE LIGHT ABSORPTION SPECTRUMS OF A 0.01MG/ML IN DMSO SOLUTIONS OF EC19 (PLATE A), ATRA (PLATE B), EC23 (PLATE C) AND EC23-OL (PLATE D). THE AREA TO THE RIGHT OF THE BLACK LINE IN ALL THE PLOTS IS DUE TO THE ABSORPTION PROFILE OF THE SOLVENT USED, DMSO. THIS SEEMS TO CHANGE NOTICEABLY FROM ONE READING TO ANOTHER BUT DID NOT AFFECT THE RETINOID SPECTRUMS.

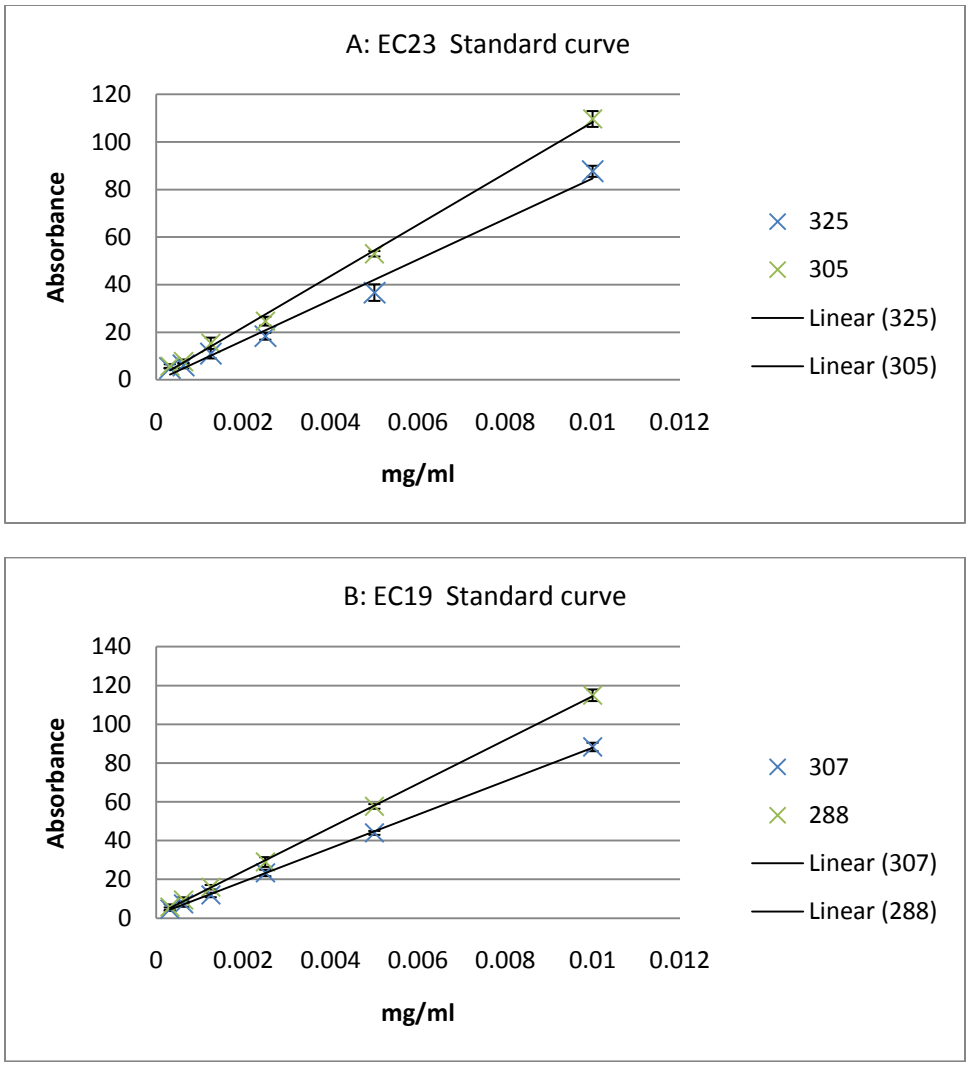
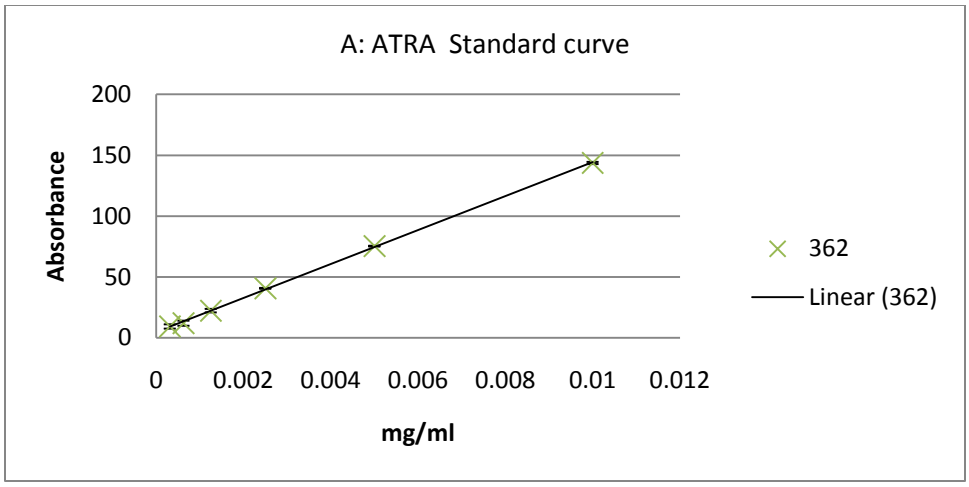


FIGURE 10. RETINOID STANDARD CURVES. PLATE A, EC23; PLATE B, EC19 (ERROR BARS SHOW STANDARD ERROR OF THE MEAN)



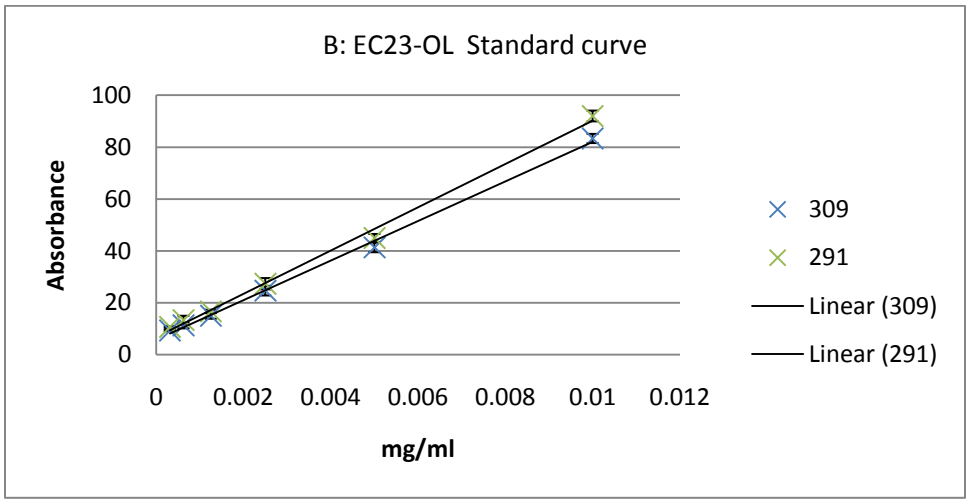


FIGURE 11. RETINOID STANDARD CURVES. PLATE A, ATRA; PLATE B, EC23-OL (ERROR BARS SHOW STANDARD ERROR OF THE MEAN)

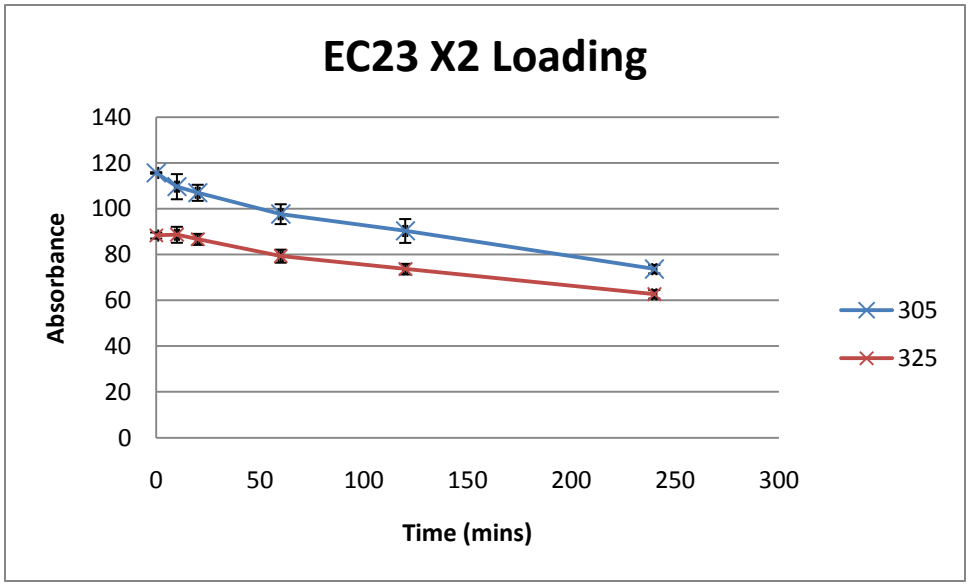


FIGURE 12. EC23 X2 BEAD LOADING ASSAY (ERROR BARS SHOW STANDARD ERROR OF THE MEAN)

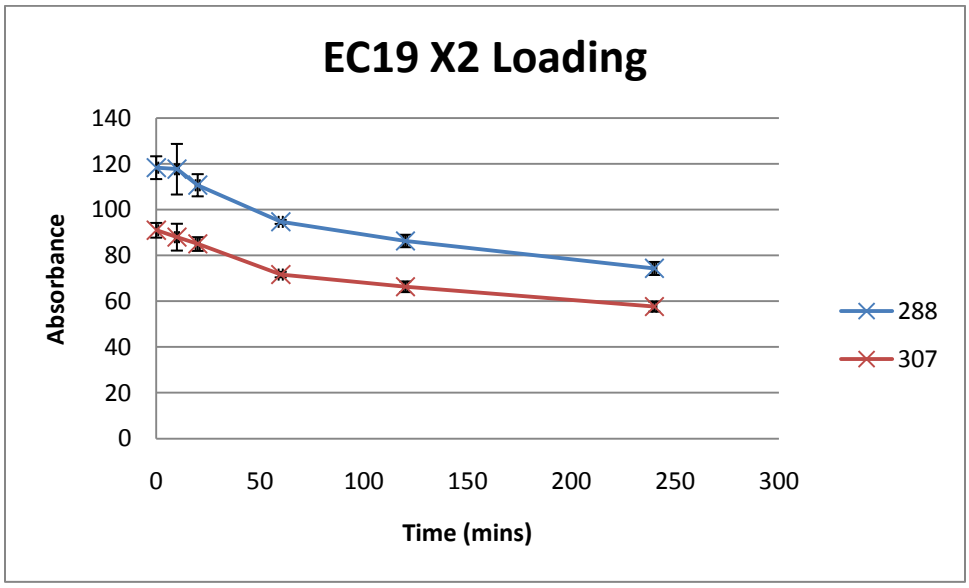


FIGURE 13. EC19 X2 BEAD LOADING ASSAY (ERROR BARS SHOW STANDARD ERROR OF THE MEAN)

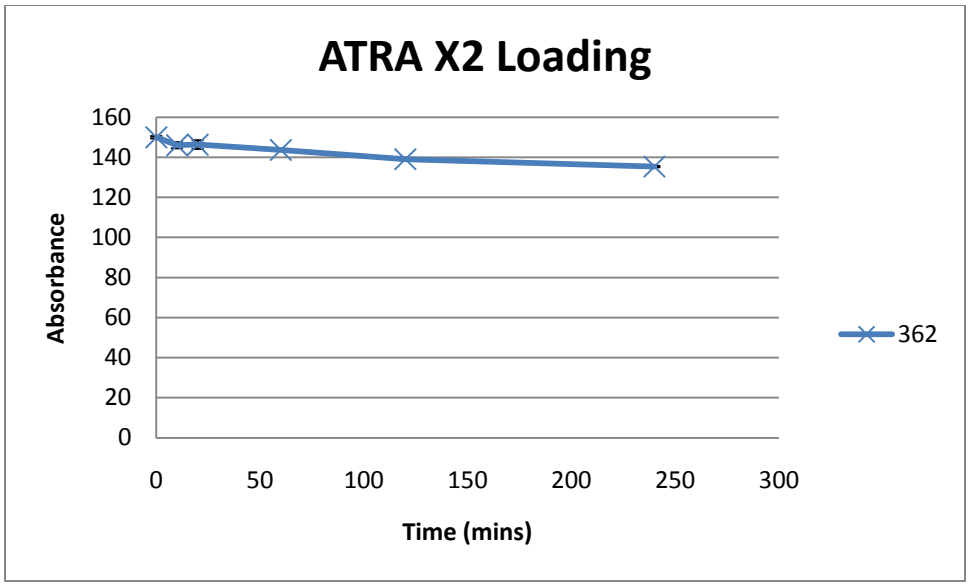


FIGURE 14. ATRA X2 BEAD LOADING ASSAY (ERROR BARS SHOW STANDARD ERROR OF THE MEAN)

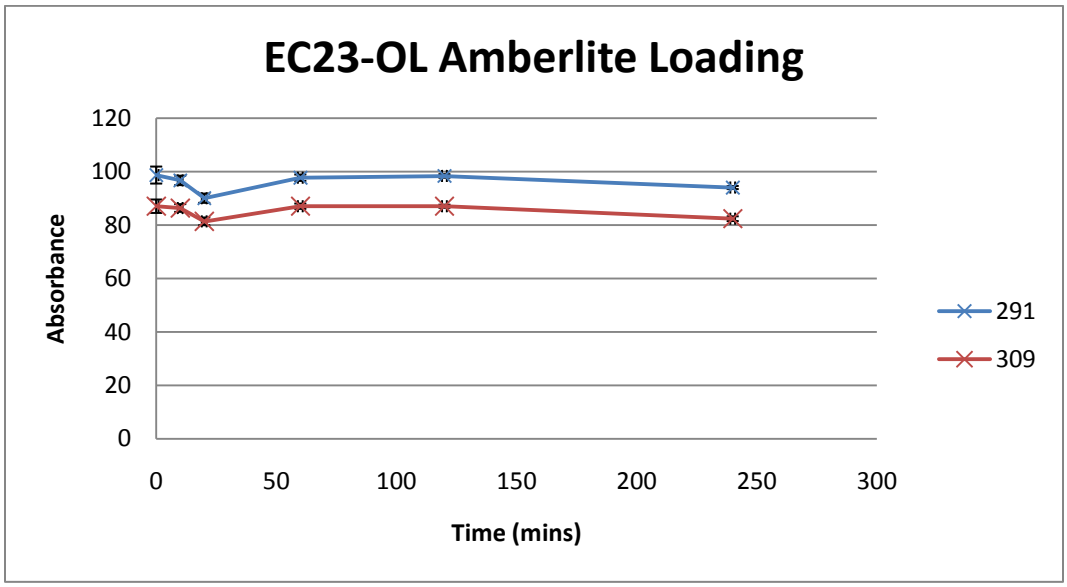


FIGURE 15. EC23-OL AMBERLITE BEAD LOADING ASSAY (ERROR BARS SHOW STANDARD ERROR OF THE MEAN)

Compound	Amount loaded over the course of 240 minutes (mg)
EC23	$1.65x10^{-5}$
EC19	$1.8x10^{-5}$
ATRA	$4x10^{-6}$
EC23-OL	$1x10^{-6}$

TABLE 1. TABLE TO SHOW THE TOTAL AMOUNT OF RETINOID LOADED OVER THE COURSE OF THE 240 MINUTES OF THE BEAD LOADING ASSAY

IN OVO OPERATIONS

EC23, EC19 AND ATRA

Past investigations have shown that ATRA is able to effect the development of chick embryos when applied locally using ion exchange beads.

The investigation has already shown that it is possible to load these synthetic retinoids onto the ion exchange bead AG1-X2, though it does assume that retinoids used were released in a way similar to that described by Eichele, Tickle and Alberts (Eichele, Tickle and Alberts 1984).

The investigation then proceeded to assess the activity of these retinoids in a biological system. The investigation carried out the assessment of these retinoids using chick embryos treated with different concentrations of the retinoids.

The toxicity of these three different retinoids was assessed first.

From Table 2. it can be seen that the dose required for toxicity between the three different retinoids varies greatly.

The level of toxicity of EC23 seems to be much greater with no embryos surviving to stage 35 at a concentration of 0.1mg/ml. From the results, EC19 and ATRA seem to exhibit a much lower and also a more similar level of toxicity with 3mg/ml and 10mg/ml for EC19, and ATRA was shown to cause no decrease in embryo viability to stage 35. Due to time limitations on this investigation it was not possible to take EC19 to 10mg/ml or either of the two retinoids beyond this concentration to find their toxic concentration.

In context of the bead loading data discussed earlier, EC19 and EC23 loaded to similar amounts, however ATRA loaded approximately four times less onto the AG1-X2 beads used. This perhaps goes some way to explain why EC23 had a higher level of toxicity than ATRA. However even at 10mg/ml ATRA was shown to still allow high levels of embryo viability up to stage 35, unlike EC23 which showed complete toxicity at 0.1mg/ml. It appeared that there was more to the toxicity of EC23 than its concentration. This was perhaps further strengthened by EC19 loading more than EC23, while EC19 appeared to have a much lower level of toxicity.

Having established the different toxicity levels of the retinoids the investigation proceeded to assess the differences in their biological effects.

Figure 16. shows that EC23 yielded a retinoid response similar to that of an ATRA concentration one hundred times higher; images of these phenotypes can be seen in Figure 17. At 0.001mg/ml EC23 was the only retinoid in this investigation that was able to yield a retinoid like limb phenotype, as can be seen by the 2234 digit pattern in Figure 17. plate A; the 32234 digit pattern shown in Figure 17. plate E and the 432234 or full digit duplication

seen in plate G. Even at 0.01mg/ml it was the only retinoid that was able to achieve a range of limb phenotypes (see Figure 17. Plate B for a 2234 digit pattern, Plate C and D for a 22234 digit pattern, Plate F for a 32234 digit pattern and Plate H for a 432234 digit pattern). The only phenotype seen in ATRA at this level was the simplest one (2234 digit patterning as seen in Figure 18. Plate A) and this was only seen in less than 5% of the specimens.

ATRA can be seen, from the data above, to cause a retinoid like limb phenotype (as seen in Figure 18.) response like those reported by Tickle et al., (Tickle, Crawley and Farrar 1989) starting at 0.01mg/ml. At this level however only the mildest limb phenotype was seen, the 2234 (as seen in Figure 18. Plate A) and in less than 5% of results. Its effects are significantly clearer between 0.1mg/ml and 10mg/ml (as can be seen in Figure 16. with examples of these phenotypes seen in Figure 18. Plate B which shows a 2234 digit pattern from a 3mg/ml treatment, and Plate C that shows a 32234 digit duplication from a 1mg/ml treatment).

The frequency of 5% of phenotypes seen in this investigation was much lower than has been seen in previous studies which have shown a 60% yield of phenotypes when treated with AG1-X2 bead soaked in a 0.01mg/ml solution of ATRA in DMSO under similar conditions (Tickle, Crawley and Farrar 1989).

EC19 started to show a retinoid like limb phenotype (as seen in Figure 19.) between 0.1mg/ml and 3mg/ml (examples of which are shown by the 2234 digit pattern seen in Figure 19. Plate A and B for the 0.1mg/ml and 3mg/ml treatments respectively). However it must be noted that at 1mg/ml no retinoid like limb phenotypes were seen. This may be due to the small sample size. The limb phenotypes seen at 0.1mg/ml and 3mg/ml were once again only the mildest form the 2234.

During these assays for biological activity, the frequency of facial phenotypes was also noted.

The difference in the frequency of the three different retinoids to generate facial phenotypes is marked as seen in Figure 16. although as mentioned above, EC19 seemed to have a lower frequency of limb phenotypes. However EC19 did seem to yield facial phenotypes at a far higher frequency than the other two retinoids see Figure 16. Plate B, C and D. These were not as fully truncated as seen with the EC23 sample (see Figure 20. to compare the extremity of the phenotypes seen between the different retinoids) although they seemed to still be easily discernable from the normal phenotype.

In summary the toxicity of the retinoids varied greatly with EC23 being much more toxic than the other two retinoids tested, even in light of the reduced loading of ATRA. EC23 however was shown to also create the retinoid phenotype of digital duplication in the forelimbs and at a concentration one hundred times lower than ATRA. Even with the knowledge that ATRA loads four times less onto the AG1-X2 beads used to deliver the retinoids, the difference in concentration needed to elicit a phenotype is still impressive.

EC19 was also able to yield the simplest of the retinoid digit duplication phenotypes but needed a far higher concentration to do so and its frequency was also much reduced. It did however yield a much higher frequency of facial phenotypes than the other two retinoids across the entire range tested.

EC23 Retinol Equivalent

Retinol is known to be converted into the biologically active form, ATRA. To see how closely EC23 mimics ATRA the Retinol equivalent of EC23, EC23-OL was used. The Retinol equivalent of EC23 was delivered using the technique described above in the methodology.

A total of 34 EC23-OL bead implantation operations were performed with a 41% survival rate through to approximately stage 35 (Hamburger and Hamilton 1951).

No phenotypes, retiniod or otherwise, were observed and their development in general seemed to remain normal.

These results of this however must be taken in the context of the bead loading data as explained above. The bead loading showed that very little of the active retinoid seemed to load onto the bead. Thus it is possible that the normal phenotype seen is purely due to no active retinoid being presented to the limb.

However as mentioned above, in the bead loading assays this may well be just a product of the way in which the amberlite beads load. Without conducting further experiments to see what was loaded it is impossible to state whether the retinoid did in fact load to a significant level.

It is interesting to note that even if only $1x10^{-6}$mg of EC23-OL per bead was loaded from a 0.01mg/ml in DMSO solution this is only a log and a half lower than the $1.65x10^{-5}$mg per bead loaded for EC23. Thus since phenotypes are seen in EC23 treatments as low as 0.001mg/ml and if all of the EC23-OL was converted to the active EC23 form then we would still expect to see some level of phenotypes from these experiments. This possibly indicates that EC23-OL cannot be recognized by the endogenous Retinol to Retinoic Acid enzymes.

Cyclompamine

Cyclopamine is a known Shh inhibitor. Shh is thought to play a role in the signalling of endogenous retinoids in the limb. Cyclopamine treatment has been shown to effect limb development (Scherz, et al. 2007). A combined treatment of Cyclopamine and EC23 was thus used to see if this phenotype could be rescued.

Control experiments

It was deemed a prerequisite for this to work to demonstrate a cyclopamine only phenotype. Thus a series of control experiments were first run to see if this investigation could yield phenotypes like those described by Scherz et al., (Scherz, et al. 2007).

A total of 14 cyclopamine control experiments were set up as described in the methodology. Embryonic day 3, embryos were treated 5µl of a cyclopamine and HCB solution in PBS with a 36 percent survival rate through to approximately stage 35 (Hamburger and Hamilton 1951). No phenotypes were seen in contrast to previous investigations (Scherz, et al. 2007).

Higher volumes of Cyclopamine were also administered, both 10µl and 20µl. However this lead to a 0% survival rate beyond stage 29 in all 8 operations conducted.

0.1mg/ml EC23

A total of 9 cyclopamine pre treated EC23 bead implantation operations were performed with a 0% survival rate through to approximately stage 35 (Hamburger and Hamilton 1951).

0.01mg/ml Ec23

A total of 17 cyclopamine pre treated EC23 bead implantation operations were performed with a 41% survival rate through to approximately stage 35 (Hamburger and Hamilton 1951). 43% of those that survived showed 2234 duplications. No other phenotypes were witnessed.

As can be seen from these results above no Cyclopamine only phenotypes were seen (as described by Scherz et al., (Scherz, et al. 2007). No other phenotypes were also witnessed bar those that we believe are due to the sole effect of EC23.

A: EC23

Concentration (mg/ml in DMSO)	Number of operations conducted	Percentage (number) of embryos surviving up to stage 35
0.0001	32	75 (24)
0.001	70	36 (25)
0.01	57	53 (30)
0.1	31	0 (0)

B: EC19

Concentration (mg/ml in DMSO)	Number of operations conducted	Percentage (number) of embryos surviving up to stage 35
0.01	26	23 (6)
0.1	19	47 (9)
1	25	80 (20)
3	31	68 (21)

C: ATRA

Concentration (mg/ml in DMSO)	Number of operations conducted	Percentage (number) of embryos surviving up to stage 35
0.01	135	31 (42)
0.1	47	30 (14)
1	24	38 (9)
3	27	26 (7)
10	22	50 (11)

TABLE 2. TABLES TO SHOW THE NUMBER OF BEAD IMPLANTATION OPERATIONS PERFORMED FOR EACH CONCENTRATION AND RETINOID, ALSO DISPLAYS SURVIVAL RATE THROUGH TO APPROXIMATELY STAGE 35 (HAMBURGER AND HAMILTON 1951). PLATE A, EC23 DATA; PLATE B, EC19, PLATE C, ATRA.

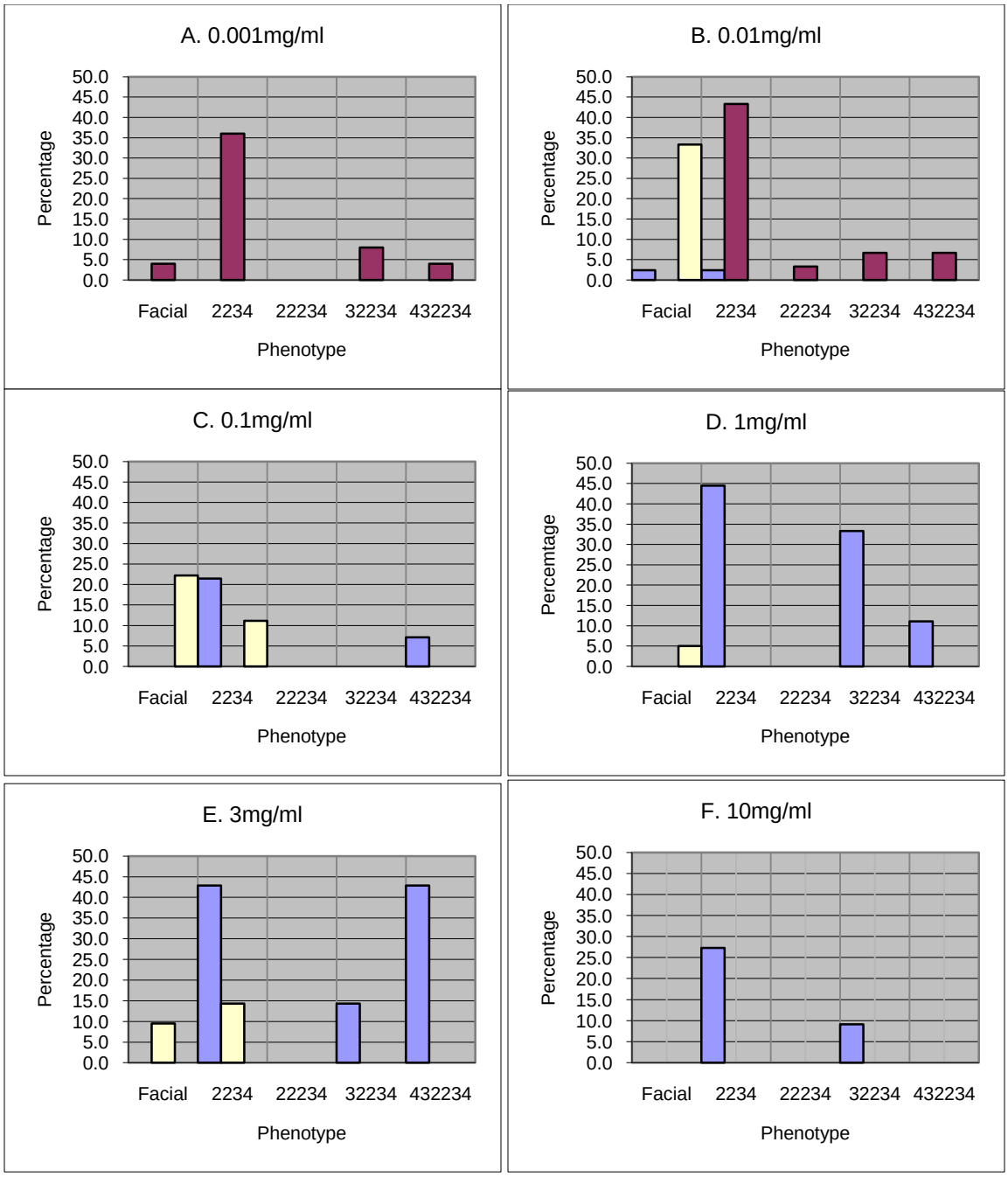


FIGURE 16. GRAPHS TO SHOW THE RELATIVE ABUNDANCE OF DIFFERENT PHENOTYPES SEEN AT EACH OF THE DIFFERENT CONCENTRATIONS AND WITH EACH OF THE DIFFERENT RETINOIDS. THE Y AXIS PERCENTAGE REFERS TO THE PERCENTAGE OF SPECIMENS SHOWING THIS PHENOTYPE AGAINST ALL THOSE THAT SURVIVED TO STAGE 35 (HAMBURGER AND HAMILTON 1951). KEY CAN BE SEEN TO THE RIGHT. PLATE A, 0.001MG/ML; PLATE B, 0.01MG/ML; PLATE C 0.1MG/ML; PLATE D, 1MG/ML; PLATE E, 3MG/ML; PLATE F, 10MG/ML.

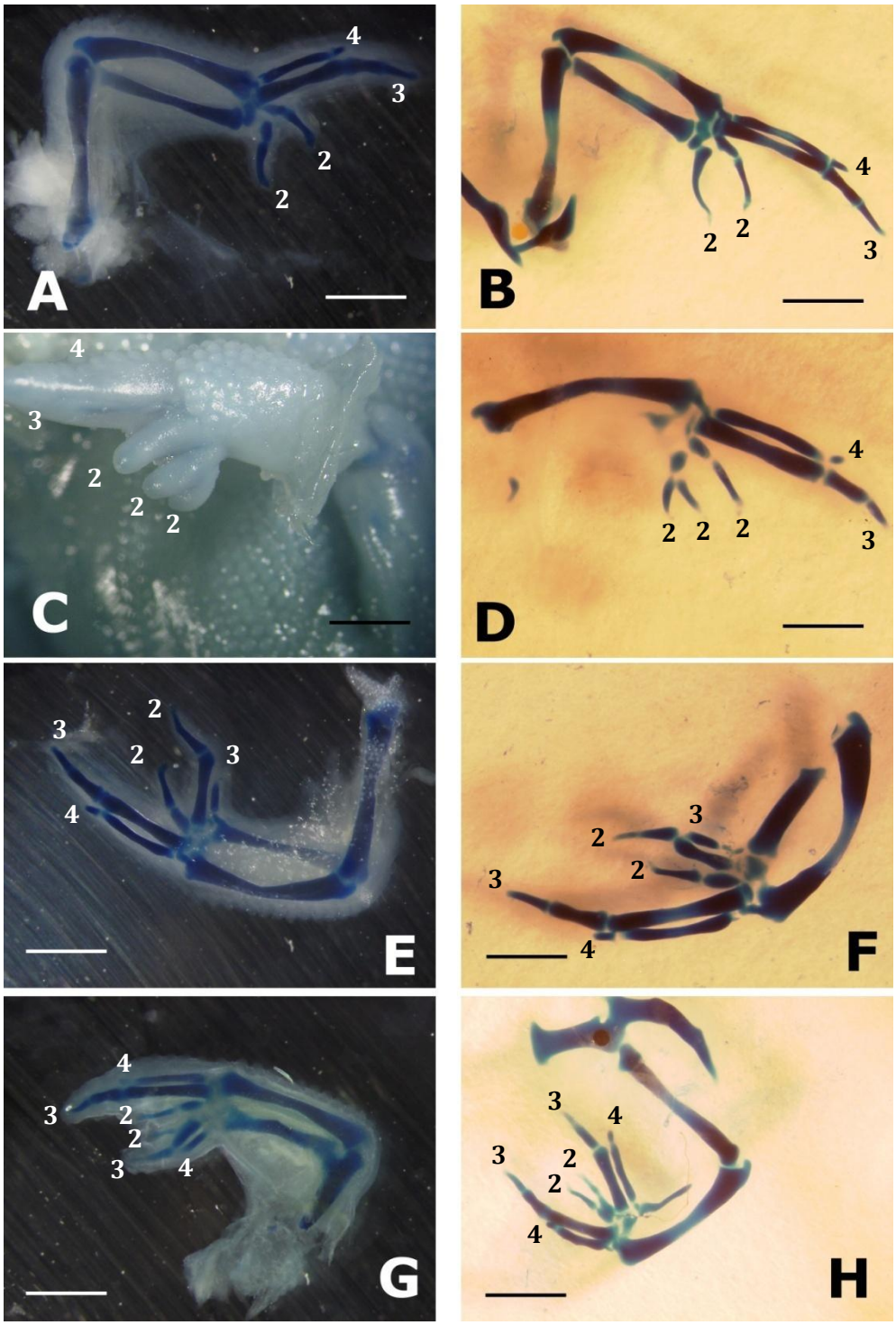


FIGURE 17. FIGURE TO SHOW THE RANGE OF PHENOTYPES SEEN DURING EC23 TREATMENT. PLATE A AND B SHOW EMBRYOS EXHIBITING 2234 PATTERNING, PLATE A SHOWS THIS DEMONSTRATED IN A SPECIMEN EXPOSED TO 0.001 MG/ML, WHILE PLATE B SHOWS THIS WHEN EXPOSED TO 0.01MG/ML. PLATE C (UNCLEARED SPECIMEN) AND D (CLEARED SPECIMEN) SHOW THE 22234 PATERNING SEEN AT AN EC23 CONCENTRATION OF 0.01MG/ML. PLATE E AND F SHOW 32234 PATTERNING AT 0.001MG/ML AND 0.01MG/ML RESPECTIVELY. PLATE G AND H SHOW 432234 PATTERNING AT 0.001MG/ML AND 0.01MG/ML RESPECTIVELY. ALL SCALE BARS EQUAL 2MM.

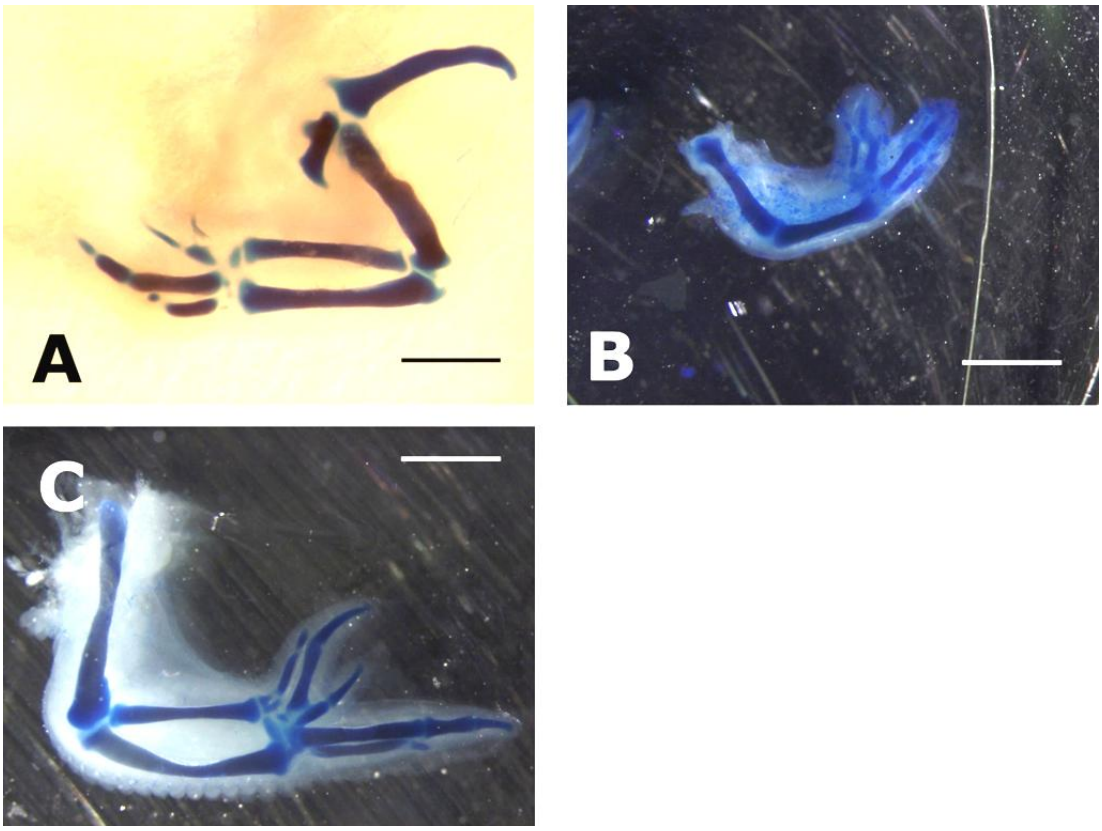


FIGURE 18. FIGURE TO SHOW THE RANGE OF PHENOTYPES SEEN DURING ATRA TREATMENT. PLATE A AND B SHOW 2234 PATTERNING IN 0.01MG/ML AND 3MG/ML RESPECTIVELY. NOTE THAT IN PLATE B BOTH RADIUS AND ULNA ARE PRESENT HOWEVER THE NATURE OF THE ANGLE USED DOES NOT CLEARLY SHOW THIS. PLATE C SHOWS 32234 PATTERNING AT 1MG/ML. DUE TO PROBLEMS WITH THE STAINING PROCEDURE USED SOME OF THE SPECIMENS WERE UNABLE TO BE CLEARED TO ALLOW THEIR PHENOTYPES TO BE PHOTGRAPHED. SCALE BARS EQUAL 2MM.

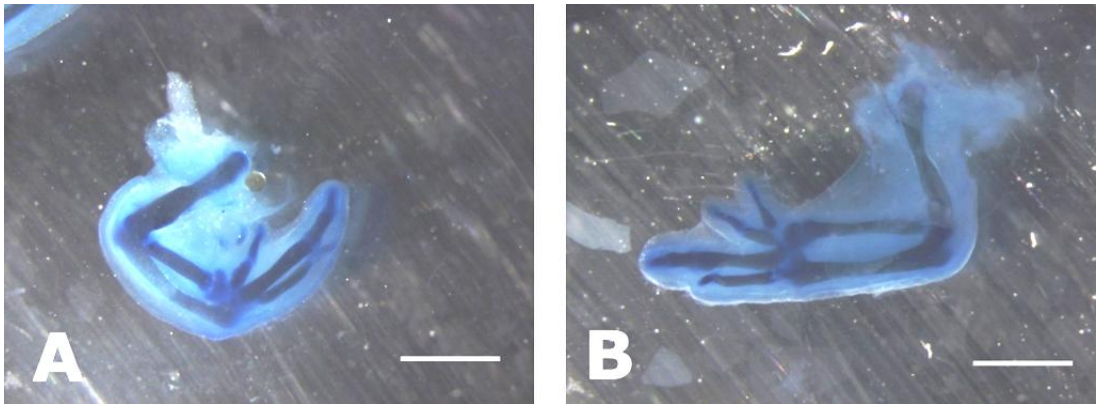


FIGURE 19. FIGURE TO SHOW THE RANGE OF PHENOTYPES SEEN DURING EC19 TREATMENT. PLATE A AND B SHOW 2234 PATTERNING AT 0.1MG/ML AND 3MG/ML RESPECTIVELY. THE DIGIT DUPLICATION IN THE 0.1MG/ML IN THIS IMAGE IS SLIGHTLY OCCLUDED BY SOME TISSUE THAT DID NOT CLEAR AS WELL AS SOME OF THE OTHER SPECIMENS. SCALE BARS EQUAL 2MM.

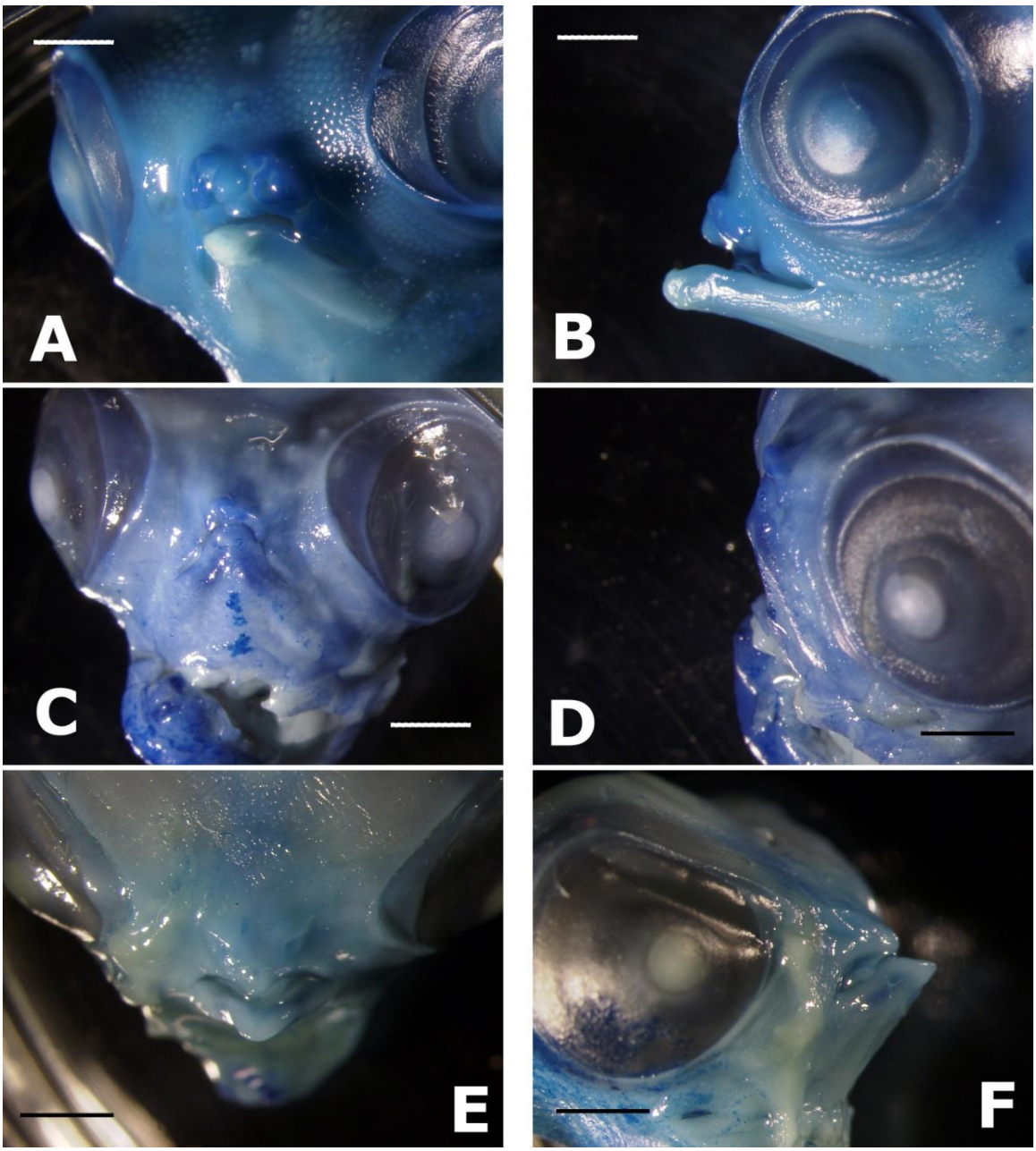


FIGURE 20. FIGURE TO SHOW THE RANGE OF FACIAL PHENOTYPES SEEN DURING TREATMENT WITH THE DIFFERENT RETINOIDS. PLATE A AND B SHOW A FACIAL PHENOTYPE FROM EC23 AT 0.01MG/ML. PLATE C AND D SHOW A FACIAL PHENOTYPE SEEN IN ATRA AT 10MG/ML. IN PLATE C AND D THE STAINING OF THIS SPECIMEN CAUSED THE LOWER BEAK TO PRESS AGAINST THE REST OF THE FACE, HOWEVER WHEN THE EMBRYO WAS ORGINALLY RECOVERED THE LOWER BEAK POINTED OUTWARDS MUCH LIKE IN PLATE A AND B. PLATE E AND F SHOW A FACIAL PHENOTYPE SEEN IN EC19 AT 0.1MG/ML. IT IS WORTH NOTING THAT THE SLIGHTLY FLATTENED PROFILE OF THE UPPER HEAD IN PLATE F IS DUE TO HOW THE EMBRYO WAS RESTING IN THE STAINING DISH. AS CAN BE SEEN IN PLATE E AND F THE PHENOTYPE YIELDED BY EC19, ALTHOUGH MORE PREVALENT, DOES SEEM TO BE LESS EXTREME THAN THOSE SEEN IN EC19 AND ATRA (PLATES A, B, C AND D). SCALE BARS EQUAL 2MM.

Discussion

Differential toxicity between the different retinoids used has been shown in this investigation. EC23 was by far the most toxic of the retinoids tested in this experiment, with no embryo surviving to stage 35 at or above 0.1mg/ml. The level at which EC19 and ATRA became as toxic was not found in this investigation despite going up to 3mg/ml for EC19 and 10mg/ml for ATRA.

Differences in dose response for limb phenotypes were also noted in this investigation. EC23 was shown to yield limb phenotypes at an approximately 100 times lower effective dose than that of ATRA. EC19 was only able to yield the most simple of the digit duplication phenotypes the 2234, at low frequency and only at its higher concentrations.

Dose response for facial phenotypes differed between the retinoids. Facial phenotypes were seen in all three retinoids but only at extremely low frequencies in ATRA and EC23. EC19 however was able to yield these phenotypes at a far higher frequency and over a range of concentrations. However the phenotypes seen in EC19 generally seemed less severe than those seen in ATRA and EC23.

EC23, EC19 AND ATRA

As can be seen in Figure 16. and mentioned above EC23 seemed to yield a retinoid response similar to that of an ATRA concentration one hundred times higher. The level of toxicity of EC23 also seems much greater. It can however be postulated that both of these factors are actually facets of the same feature. Both of these effects may be due the molecular shape of EC23 which possibly makes it more refractory to catabolism by the same endogenous means as the natural occurring retinoids.

This was also postulated to explain the toxicity of TTNPB ((Pignatello, Kauffman and Levin 1997) (Pignatello, Kauffman and Levin 2002)); a compound with which EC23 and EC19 share many similarities (see Figure 21).

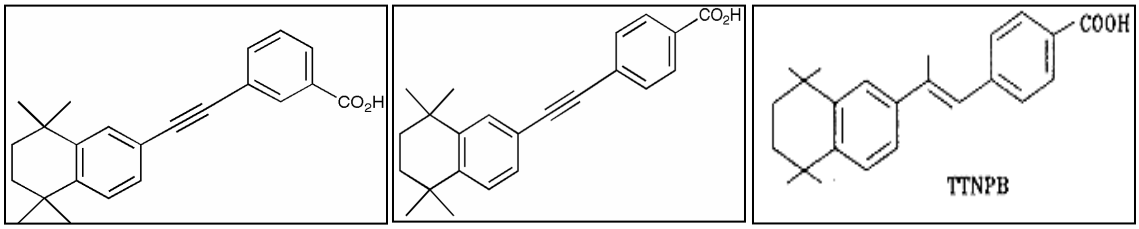


FIGURE 21. THREE SYNTHETIC RETINOIDS, FROM LEFT TO RIGHT EC19, EC23 AND TTNPB

The results from Pignatello et al., 2002 seem to clearly indicate that a relatively minor decrease in the metabolism of ATRA, achieved by the experimental inhibition of ATRA catabolism, is associated with a marked and relatively large scale increase in its toxicity. As

mentioned before this toxicity is thought to be due to the prolonging of the effective half-life of ATRA especially during a critical period of development.

The findings from this investigation seem to indicate that the prolonged half-life of TTNPB, due to it being more refractory to catabolism than ATRA, may well be the most significant factor contributing to the extremely high teratogenicity of TTNPB (Pignatello, Kauffman and Levin 2002).

This concept of an effectively prolonged half-life has already been postulated by work done with EC23 *in vitro* (Maltman et al. in press). As mentioned above, CRABP-I (which has the natural function of lowering ATRA concentration by facilitating its enzymatic breakdown and also as a possible simple sequestering mechanism) induction was shown to be greater in response to EC23 than ATRA. Thus the up regulation of CRABP-I by EC23 could be because it is more biologically active/potent than ATRA at the same concentrations as Maltman et al., postulates, possibly because EC23 was able to bind nuclear receptors more effectively.

Alternatively it may yield this effect as it is possibly present much longer than the faster catabolized ATRA. This may be due to EC23 being a poor substrate for the CYP26's. This would be more in keeping with idea suggested by Pignatello et al., (Pignatello, Kauffman and Levin 2002).

However this raises an interesting point as presumably due to their similar structures EC19 is equally as refractory to catabolism as EC23. Thus why are the effects of EC19 so limited in comparison?

From this pattern in the data one could presume that EC19 may have a low affinity for CRABP-II and thus cannot be as easily transported into the nucleus. The same effect may be yielded if EC19 has a low affinity for the RBP as this would limit its movement into the cell.

However even if neither of the above scenarios were true EC19 may still yield an effect as its more stable structure may mean that it is a poor substrate for CRABP-I and CYP26. CRABP-I and CYP26 as mentioned in the introduction are thought to be involved in the metabolism of ATRA to more polar molecules. Thus if EC19 was a poor substrate for these two catalysts then even at small levels and low affinities it would be able to yield some effect.

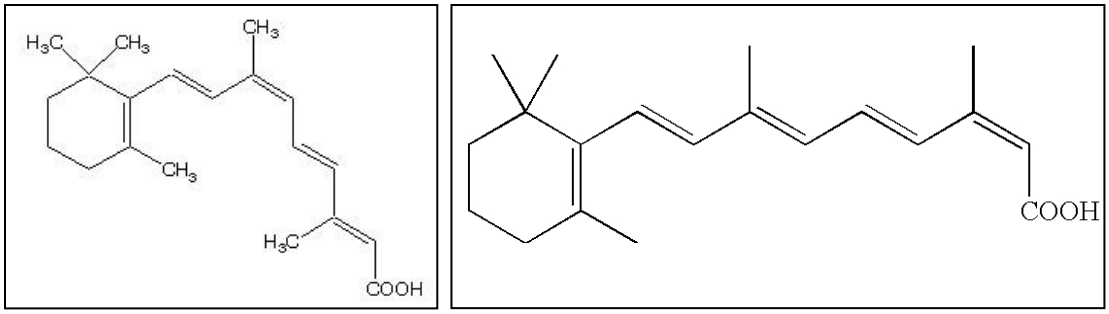


FIGURE 22. FIGURE TO SHOW THE STRUCTURES OF 9-CIS (LEFT) AND 13-CIS RETINOIC ACID(RIGHT).

However the difference in the effect seen between EC19 and EC23 may be due to the fact that EC19 emulates the shape of the cis retinoids (9-cis and 13-cis as seen in Figure 22.) more than the All-Trans ATRA which EC23 was designed to emulate in shape. Investigations in the past have found 9-cis Retinoic Acid to be 25 times more efficient at inducing digit duplication phenotypes than ATRA. Some have even gone so far as to state that it is possible, in the chick limb bud system, that the actual active species is 9-cis Retinoic Acid and All-Trans Retinoic Acid serves as a precursor to it (Thaller, Hofmann and Eichele 1993).

But if this were the case our data would contradict the idea that EC23 acts in a similar way to ATRA, as there is no way that EC23 could be converted to a 9-cis equivalent by the enzymes that use ATRA as a substrate. Thus we would presume that EC23 is most likely active as EC23, which suggests that it can bind to the same receptors as whatever natural metabolite it is emulating. Thus perhaps 9-cis RA and EC23 can bind to the same receptor, but ATRA cannot before it is converted.

EC19 however is more similar around carbon 13 to the second cis isomer mentioned, 13-cis RA. It should be noted that, although the angle of the terminal carboxylic acid group is similar it is attached to a benzene ring not a polyene. Thus perhaps this is too simple a similarity and the shape of the whole molecule might be more important

13-cis RA has also been shown to yield retinoid like phenotypes, however it has shown to be far less potent with about a 10 fold decrease in its ability when compared to ATRA in the mouse limb bud system (Kwasigroch, Bullen and Quillen 1991).

The effect of EC19 in terms of its general ability to elicit facial phenotypes more than limb phenotypes may also be related to the differential effects observed *in vitro* by Christie et al., in 2008. In this investigation EC23 was shown to induce neural differentiation in a similar manner to ATRA. However the cultured human pluripotent stem cells exposed to EC19 seemed to produce very few neurons and large numbers of epithelial-like cells (Christie, et al. 2008).

Thus the example of the decrease in effect of the naturally occurring 13-cis RA together with the differential effects of EC19 when compare to EC23 *in vitro* seem to give some further

weight to a differential phenotype *in vivo* as we have observed. However the mechanism for this is not clear.

We find that ATRA yields retinoid phenotypes like those previously reported (Tickle, Crawley and Farrar 1989) starting at 0.01mg/ml. However at this level only the mildest limb phenotype is seen, the 2234, and at much less than 5% of the time. Its affects occur at higher frequency between 0.1mg/ml and 10mg/ml.

Past investigations have found that limb phenotypes are yielded at 0.01mg/ml (Tickle, Crawley and Farrar 1989) however the percentage of embryos with phenotypes was much higher (approximately 60% in the paper referenced). The reasons for this are unclear, with one of the few explanations we can consider being that the ATRA used in these experiments may have had a higher 9-cis RA concentration to start with, which as discussed above is thought to be a more active retinoid in the chick limb bud system.

However the above comparisons between the retinoids tested must also be looked at in the light of the results obtained regarding their relative loadings onto the AG1-X2 beads used. As mentioned above the loading for both EC23 and EC19 was very similar, with a 36% and 37% drop in absorbance respectively. ATRA however was much lower only achieving a 10% drop in absorbance during loading. With this in mind we must examine our claim that EC23 seems to be able to yield limb phenotypes at a concentration 100 fold lower than ATRA. Due to the fact that ATRA is loading only one fourth the amount of active compound this figure may be closer to 25 times, however this assumes that all the retinoids would unload to the same degree.

It is also worth considering at this stage the EC23-OL experiments conducted. If as suggested there was a loading of $1x10^{-6}$mg of EC23-OL per bead from the 0.01mg/ml in DMSO solution this is only a log and a half lower than the $1.65x10^{-5}$mg per bead loaded for EC23. Thus since phenotypes are seen in EC23 treatments as low as 0.001mg/ml and if all of the EC23-OL was converted to the active EC23 form, then it would be expected some level of phenotypes from these experiments would be seen. This possibly indicates that EC23-OL cannot be recognized by the endogenous Retinol to Retinoic Acid enzymes.

FUTURE DIRECTION AND FURTHER THOUGHTS

As discussed above the EC23 Retinol equivalent (EC23-OL) seems to yield no biological affect at a concentration EC23 is known to have a biological effect. However due to the time frame of this investigation it was not explored further. Thus if time and resources allowed (only a very small quantity of EC23-OL was available for use in this investigation) it would have been beneficial to explore higher concentrations of the compound. It also may have been useful to have looked into other delivery mechanisms for this retinoid.

It would have also been beneficial to run bead assays in which the unloading of the retinoids, under roughly the same conditions to which they would have been exposed to *in vivo,* was quantified. The reason for this is, although EC23 and EC19 were loaded to a great degree, it may be that they could not be unloaded to the same degree. Thus the effective delivery may in fact have been smaller. Bead stripping assays would also be useful to see if, when forced, all the retinoids in their original state could be released from the beads. This would be interesting as currently it is uncertain if any of the retinoids used have been changed or broken down by being loaded onto these beads.

If time had allowed it may have been worthwhile investigating what the upper toxic limit of ATRA and EC19 would have been. However due to the nature of these compounds additional safety procedures may have been warranted at the concentrations of the retinoids in DMSO this would have required. It would be of interest to see if EC19 yielded a larger effect at higher concentrations or if its affect was held back by transport or other means as described above.

It would have been interesting to know the range over which ATRA worked and how much larger this band was than EC23. An investigation may have revealed that the toxicity of EC23 may be more complex than just being caused by its higher potency.

CONCLUSIONS

In conclusion this investigation shows the ability of EC23 to emulate known ATRA limb phenotypes. It has also been shown that EC23 appears to generate this effect even at much lower concentrations.

The effects of EC19 on this system have also been shown and the difference in the phenotypes EC19 is able to achieve. As mentioned above the reduction in the ability of EC19 to yield limb phenotypes may well be due to its similarities in structure to 13-cis RA. However the ability of EC19 to yield facial phenotypes does seem much greater than that of EC23 or ATRA. However the mechanism for this is as yet unknown.

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