CANCER CHEMOPREVENTION BY WATER SOLUBLE ASTAXANTHIN DERIVATIVES

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

BIOMEDICAL SCIENCES
(CELL AND MOLECULAR BIOLOGY)

MAY 2004

By
Laura M. Hix

Thesis Committee:
Tom Humphreys, Co-Chairperson
John Bertram, Co-Chairperson
Pratibha Nerurkar
# TABLE OF CONTENTS

List of Figures

Chapter 1: Introduction

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Compounds

2.1.2. Cell lines and culture conditions

2.2. Methods

2.2.1. Analysis of Cx43 expression

2.2.2. Immunofluorescence

2.2.3. Gap junctional communication assay

2.2.4. MCA-induced neoplastic transformation assay

2.2.5. Statistical analysis

Chapter 3: Results

3.1. Formulation and Solubility Studies

3.2. Molecular Analysis of Cx43

3.3. Cellular Analysis of Cx43

3.4. Induction of Gap Junctional Communication

3.5. Inhibition of MCA-induced Neoplastic Transformation

Chapter 4: Discussion

Appendix

References
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Structures of the disodium salt astaxanthin derivatives</td>
<td>8</td>
</tr>
<tr>
<td>2.</td>
<td>UV/vis absorption spectra of racemic dAST and AST</td>
<td>14</td>
</tr>
<tr>
<td>3.</td>
<td>Western blot demonstrating Cx43 protein expression in 10T1/2 cells treated with two different</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>formulations of racemic dAST</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Western blot demonstrating induction of Cx43 protein expression in 10T1/2 cells treated with</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>dAST derivatives</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Western blot demonstrating induction of Cx43 protein expression in 10T1/2 cells treated with</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>pAST, AST and CTX</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Immunofluorescence demonstrating dAST increases assembly of Cx43</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>immunoreactive junctional plaques</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Micrographs demonstrating dAST enhances functional GJIC in 10T1/2 cells</td>
<td>23</td>
</tr>
<tr>
<td>8.</td>
<td>Dose-dependent effects of pAST and AST on MCA-induced neoplastic transformation</td>
<td>25</td>
</tr>
</tbody>
</table>
CHAPTER 1. INTRODUCTION

It is now well established that the major contributing factor to cancer risk in Western societies is lifestyle rather than genetics. Current epidemiological data suggests that approximately 70% of the estimated 1.37 million cancer cases in 2004 will be linked to preventable lifestyle factors such as tobacco use, alcohol, diet, infections (including sexually transmitted diseases), occupational exposures, pollution, sunlight exposure (UV radiation), and stress (Cancer prevention and early detection, American Cancer Society, 2004); [1]. Despite considerable improvements in the detection and treatment of cancer, it is estimated that 563,700 Americans will die of cancer in the year 2004 (Cancer facts and figures, American Cancer Society, 2004). Cancer chemoprevention, defined as the reduction in cancer incidence through the use of pharmaceuticals, vitamins, minerals and other chemicals, has emerged as a powerful strategy in the fight against cancer. Potential targets for chemoprevention include the general population, subgroups at risk due to lifestyle or other environmental factors, individuals with precancerous lesions, and cancer survivors at risk for secondary tumors.

A growing body of epidemiological evidence has linked increased intake and/or blood levels of several dietary carotenoids, plant pigments found in green, yellow and orange fruits and vegetables, to decreased risk of cancer at many anatomic sites. This activity appears to be independent of the pro-vitamin A activity of these carotenoids [2]. Studies in experimental animal and cell culture models of carcinogenesis have confirmed the cancer chemopreventative activity of many dietary carotenoids. In these systems, activity does not correlate with the pro-vitamin A properties of these compounds, nor does it correlate with their ability to act as lipid-phase antioxidants [3].
In the C3H/10T1/2 mouse embryo fibroblast (10T1/2) cell system developed in the lab of the late Charles Heidelberger, cells undergo neoplastic transformation in response to many chemical and physical carcinogens in a dose-dependent manner [4]. This cell system has been shown to effectively mimic the initiation and transformation events of tumor formation in whole animals, and represents one of the most widely characterized in vitro models for carcinogenesis [5]. Studies conducted in the 10T1/2 cell system revealed that carotenoids active in inhibiting neoplastic transformation upregulated gap junctional intercellular communication (GJIC) in direct relationship to their activity as chemopreventative agents [3].

GJIC permits the transfer of ions and small hydrophilic molecules <1 kiloDalton (kDa) in size by passive diffusion through aqueous channels (connexons) that span the plasma membranes of adjoining cells. Individual connexons are comprised of proteins known as connexins. Communicating cells typically form several thousand connexons that assemble into plaques. A family of approximately 20 connexin members is expressed in mammals. These connexins exhibit developmental- and differentiation-specific expression, allowing the formation of communicating compartments between compatible family members. There is growing evidence that these compartments are vital to normal growth and development.

Interest in gap junctions and carcinogenesis stems from several independent lines of evidence. Following the discovery of junctional communication in cultured normal cells, it was soon discovered that neoplastically-transformed cells were deficient in GJIC. Moreover, inhibition of GJIC was a very early event after activation of the oncogene src [6]. Human and animal tumors of many pathologic types were found to be deficient in
GJIC, either as a consequence of decreased connexin expression or faulty assembly into the plasma membrane. Restoration of junctional communication between transformed cells and growth-inhibited normal cells resulted in growth arrest of the transformed cells in direct proportion to the extent of heterologous cell coupling [7]. Tumor promoters—agents that accelerate the process of carcinogenesis—were found to inhibit GJIC, while cancer preventive agents such as retinoids and carotenoids—agents that delay carcinogenesis—had the opposite effect. Finally, transfection of molecular expression constructs into human and animal neoplastic cells, utilizing either constitutive or inducible promoters, demonstrated that connexin re-expression in these cells decreased their neoplastic potential in \textit{in vitro} and \textit{in vivo} assays. Collectively, these lines of evidence support the conclusion that connexins can function as tumor-suppressor genes [8].

Studies of human and animal cells have demonstrated that connexin 43 (Cx43), the most widely expressed connexin in human and animal tissues, is upregulated at the message and protein level by chemopreventive retinoids and carotenoids. The ability of carotenoids to regulate Cx43 expression appears to be independent of their conversion to retinoids. This is exemplified by compounds such as lycopene, a straight-chain hydrocarbon that is not cleaved to vitamin A in mammals, yet possesses this regulatory ability. It is unclear how much the parent compound contributes to the observed increase in Cx43 expression; for example two oxidation products of lycopene are active in this respect [9; 10]. Moreover, the potential conversion of carotenoids to retinoids—chemopreventive agents which are highly potent inducers of Cx43 and GJIC [11; 12]—cannot be ignored. Indeed, there is evidence that conversion of canthaxanthin
to 4-oxo-retinoic acid, an active retinoid, may be in part responsible for increased Cx43 expression in 10T1/2 cells [13], although we found no evidence of this conversion in the same system [14].

In the 10T1/2 system, upregulated GJIC as a consequence of increased Cx43 expression is highly correlated with the ability of carotenoids to prevent neoplastic transformation of these cells [15;16]. Due to these multiple lines of evidence linking GJIC with inhibition of carcinogenesis and decreased neoplastic potential of established transformed cells, Bertram and others have proposed that GJIC allows for the transmission of growth controlling signals between normal and transformed cells [17]. In this model, the ability of carotenoids and retinoids to inhibit tumor progression is a result of enhanced GJIC between carcinogen-initiated cells and surrounding growth-controlled normal cells. By extension, the ability to upregulate GJIC may be an important indicator of chemopreventative potential.

Astaxanthin, found predominantly as a dietary source in shrimp, lobster and salmon, has been associated with reduced risk of diseases such as age-related macular degeneration and ischemic diseases, effects attributed to its potent antioxidant activity [18]. In addition, the antioxidant activity of astaxanthin has been reported to be 10 times stronger than that of other carotenoids, namely, zeaxanthin, lutein, canthaxanthin, and β-carotene [19;20]. In experimental animal studies astaxanthin has been shown to be capable of inhibiting chemically induced oral and bladder carcinogenesis [21;22]; its usefulness as an immunomodulating agent in experimental cancer studies is also well documented (reviewed [23;24]). Based on these findings, astaxanthin has significant cancer chemopreventative potential.
One problem confronting researchers investigating the effects of carotenoids such as astaxanthin in cell culture and/or whole animals has been the delivery of these highly lipophilic molecules to target cells. For cell culture studies, the Bertram lab developed the use of tetrahydrofuran (THF) as a delivery solvent, the use of which creates a highly bioavailable and non-toxic pseudo-solution of carotenoids in cell culture medium [25]. However, caution must be taken to protect THF from oxidation to toxic species, and this method is unsuitable for use in whole animal studies. Delivery of carotenoids to experimental animals or humans is usually achieved by delivery of carotenoids as a suspension in oil, frequently as a micro-dispersed emulsion. Unfortunately, bioavailability is usually low in animals (e.g. rodents) and can be variable in humans.

To circumvent these problems of drug delivery Hawaii Biotech, Inc. has synthesized a set of novel carotenoid derivatives, disodium salt disuccinate (dAST) and disodium salt diphosphate (pAST) derivatives of synthetic astaxanthin (3,3'-dihydroxy-β, β-carotene-4,4'-dione), in all-trans (all-E) form. Synthetic astaxanthin can be commercially obtained most economically as the statistical mixture of stereoisomers [optically active (3S,3’S) and (3R,3’R) and optically inactive (3R,3’S; meso) in a 1:1:2 ratio]; the statistical mixture of stereoisomers is known as (3RS,3’RS)-astaxanthin or “racemic”astaxanthin (Buckton Scott, India). The individual stereoisomers are also available commercially from Hoffman-LaRoche (Switzerland) and BASF (Germany). The racemic mixture of the novel derivative, as well as purified stereoisomeric forms of the derivative, were successfully synthesized and tested in the current study. The derivatives exhibit several unique characteristics that increase their utility for use in the cancer chemoprevention setting. They are water soluble (critical micelle concentration =
0.3 mg/mL) and water dispersible, with the maximum aqueous dispersibility greater than 8 mg/mL (approximately 10 mM), allowing them to be introduced into cell culture without a co-solvent and delivered parenterally to experimental animals [27;28]; (Lockwood, unpublished results). Additionally, the intact synthetic derivatives retain antioxidant activity prior to enzymatic cleavage to mono-succinates and non-esterified, free astaxanthin [29]. Derivatives that enhance astaxanthin’s water solubility and bioavailability should prove invaluable in assessing the carotenoids’ abilities in vitro and in vivo.

This thesis evaluates the ability of the novel derivatives delivered in several aqueous formulations to upregulate Cx43 protein expression, induce functional GJIC, and inhibit carcinogen-induced neoplastic transformation in the 10T1/2 cell culture system described above. The compounds were found to induce expression of functional Cx43 protein, significantly increase GJIC and significantly inhibit MCA-induced neoplastic transformation with enhanced ability over the parent carotenoid astaxanthin. These results indicate that the major hurdle of delivering hydrophobic carotenoids to biological tissues in model systems has been overcome, and that evaluation of these highly bioavailable astaxanthin derivatives in in vivo models of cancer chemoprevention should be pursued.
CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1. Compounds

The racemic disodium salt diphosphate derivative of astaxanthin (racemic pAST; > 90% purity by HPLC) was synthesized from commercial astaxanthin and its structure verified. It was prepared in a formulation of 20% EtOH and sterile water (0.2% final EtOH conc.) to minimize supramolecular aggregation. The racemic disodium salt disuccinate derivative of astaxanthin (racemic dAST; > 90% purity by HPLC) was synthesized from commercial astaxanthin and its structure verified. It was prepared in a formulation of 33% EtOH and sterile water (0.33% final EtOH conc.). Non-esterified, all-E synthetic astaxanthin (AST) (> 96% purity by HPLC; Sigma, St. Louis, MO) was added to tetrahydrofuran (THF). Synthetic canthaxanthin (CTX) (CaroteNatur, Switzerland) was added to THF. The synthetic retinoid TTNPB [p-<(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-napthyl) propenyl>benzoic acid] (Hoffman-LaRoche, Nutley, NJ) and retinol acetate (Sigma, St. Louis, MO) were prepared in acetone (Sigma, St. Louis, MO). TTNPB, canthaxanthin and astaxanthin concentrations were confirmed by comparing their UV absorption and their published extinction coefficients. The chemical structures of the three stereoisomers are shown in Fig. 1A. These are: (3R,3'R)-, (3S,3'S)-, and (3R,3'S; meso)-astaxanthin disuccinate, disodium salt. The structure of the all-E-astaxanthin diphosphate, disodium salt is shown in Figure. 1B. Due to the sensitivity of carotenoids to light, heat and oxygen, special precautions were taken
throughout the study. All compounds were stored under nitrogen at -70° C and care was
taken to ensure minimal exposure to direct sunlight or UV light.

A

3R,3'R disodium salt disuccinate
astaxanthin derivative

3S,3'S disodium salt disuccinate
astaxanthin derivative

3R,3'S disodium salt disuccinate
astaxanthin derivative

B

Racemic disodium diphosphate
astaxanthin derivative

Fig. 1. (A) Structures of the three stereoisomers of the disodium salt disuccinate astaxanthin derivatives
d(AST) evaluated in the current study (shown as the all-\(E\) geometric isomers). The racemic mixture of
stereoisomers, referred to as "racemic dAST" in the text, contains (3S,3'\(S\))-astaxanthin disuccinate,
disodium salt; (3R,3'\(R\))-astaxanthin disuccinate, disodium salt; and (3R,3'\(S\); meso)-astaxanthin disuccinate,
disodium salt in a 1:1:2 ratio. (B) Structure of the racemic disodium salt diphosphate astaxanthin derivative
(pAST), containing (3S,3'S)-astaxanthin diphosphate, disodium salt; (3R,3'R)-astaxanthin diphosphate, disodium salt; and (3R,3'S; meso)-astaxanthin diphosphate, disodium salt in a 1:1:2 ratio. Racemic pAST, racemic dAST and all individual dAST stereo isomers (3S,3'S dAST, meso dAST, and 3R,3'R dAST) were separated to > 90% purity by HPLC.

2.1.2. Cell lines and culture conditions

Mouse embryonic fibroblast 10T1/2 cells were cultured in Eagle's basal medium with Earle's salts supplemented with 4% fetal calf serum (Atlanta Biologicals, Norcross, GA), 25 μg/mL gentamicin sulfate (Sigma, St. Louis, MO), and passaged by trypsin/EDTA. Cells were incubated at 37° C in 5% CO₂ as described previously [1;2], and confluent cells were treated on the 7th day after seeding, unless otherwise indicated in methods.

2.2. Methods

2.2.1. Analysis of Cx43 expression

Expression of Cx43 protein in murine fibroblasts was assessed by immuno-(Western) blotting essentially as described [1;2]. Briefly, 10T1/2 cells were treated with the novel astaxanthin derivatives and/or retinoids at confluence on the 7th day after seeding in 100 mm dishes (Fisher Scientific, Pittsburgh, PA). Cells were harvested after 4 days and total protein content was measured using the Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Cell lysates containing 50 μg of protein were analyzed by Western blotting using the NuPage Western blotting kit (Invitrogen, Carlsbad, CA). Cx43 was detected using a rabbit polyclonal antibody (Zymed, San Francisco, CA) raised against a synthetic polypeptide
corresponding to the C-terminal domain common to mouse, human and rat Cx43. Cx43 immunoreactive bands were visualized by chemiluminescence using an anti-rabbit HRP-conjugated secondary antibody (Pierce Chemical Co., Rockford, IL). Images were obtained by exposure to X-ray film as previously described [1;2] or obtained with a cooled CCD camera, and quantitative densitometry was performed (Bio-Rad, Richmond, CA). Equal protein loading of the lanes was confirmed by staining with Coomassie blue protein (Sigma, St. Louis, MO) and digital image analysis.

2.2.2. Immunofluorescence

Expression and assembly of Cx43 into plaques was assessed by immunofluorescence staining essentially as described [3]. Briefly, confluent cultures of 10T1/2 cells were grown on Permanox plastic 4-chamber slides (Nalge Nunc International, Naperville, IL) and treated for 4 days with (1) racemic dAST dissolved in 33% EtOH; (2) TTNPB at 1 x 10^-8 M in acetone (0.1% final acetone concentration in assay) as a positive control; or (3) 33% EtOH (0.33% EtOH final concentration) as a solvent-only control. Cells were fixed with ~20 °C methanol overnight, washed in buffer, blocked in 1% bovine serum albumin (Sigma, St. Louis, MO) in PBS, incubated with the rabbit anti-Cx43 antibody, and finally detected with Alexa568 conjugated anti-rabbit secondary antibody (Molecular Probes, Eugene, OR). Slides were illuminated with 568 nm light and images were acquired at a wavelength of 600 nm using a Zeiss Axioplan microscope and a Roper Scientific cooled CCD camera.
2.2.3. *Gap junctional communication assay*

Junctional permeability was assayed by microinjection of the fluorescent dye Lucifer Yellow CH (10% in 0.1 M LiCl) (Sigma, St. Louis, MO) into confluent cells as described previously [4]. Confluent cultures of 10T1/2 cells were treated for 4 days with: (1) racemic dAST at 10^{-5} M in 33% EtOH; (2) TTNPB in acetone at 10^{-8} M as positive control; or (3) 33% EtOH as solvent-only control as described above. Single cells were injected with the fluorescent dye and digital images were taken under UV illumination after 2 minutes. The number of fluorescent cells adjacent to the injected cell was later determined by digital image analysis, and this number was used as an index of junctional communication, as described previously [4].

2.2.4. *MCA-induced neoplastic transformation assay*

In this experiment, the novel derivatives were assessed for their ability to prevent carcinogenic neoplastic transformation in the C3H/10T1/2 cell assay developed in the Bertram lab [4;5]. Using the protocols previously described, cells were initiated with the potent carcinogen methylcholanthrene (MCA, Sigma, St. Louis, MO) in acetone at a final concentration of 5 µg/mL, and media was replaced 24 hours later. Cells were treated with either the novel carotenoid derivative (pAST) in 20% EtOH (0.2% final EtOH concentration) or the parent carotenoid astaxanthin (AST) in THF (0.1% final THF concentration) beginning 7 days after removal of carcinogen and continuing to the end of the four-week culture period [6]. The ability to inhibit neoplastic transformation was assessed at concentrations of 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M. Negative controls include MCA-treated cells followed by media only treatments, Acetone-treated cells followed by media
only treatments, and MCA-treated cells followed by treatment with THF as a solvent control. A total of 24 dishes/treatment has previously been shown to yield an expected 50 transformed foci in MCA-only controls and 10 foci in canthaxanthin $10^{-5}$ M treated cells, and allows statistical significance to be met over most of the dose range [7].

### 2.2.5 Statistical Analysis

Statistical analyses were conducted with the NCSS 2001 and PASS 2002 statistical software (J. Hintze, Number Cruncher Statistical Systems, Kaysville, UT). All tests were conducted at the $\alpha = 0.05$ level.
CHAPTER 3. RESULTS

3.1. Formulation and Solubility Studies

In pure aqueous formulation, racemic dAST and the purified stereoisomeric dAST derivatives demonstrate supramolecular assembly, a form of 3-dimensional aggregation that can limit solubility and bioavailability [27;31]. The aggregation is of the so called H- or “card-pack” type, and may be disrupted into a completely monomeric solution with the addition of a less polar solvent (such as EtOH) [32]. We observed precipitation of red solid onto the cellular monolayer several days after introduction of dAST from purely aqueous stock solutions, without effect on the cellular viability (data not shown). This phenomenon has also been observed after introduction of non-esterified, free astaxanthin in EtOH to cell culture systems [Lockwood, unpublished results]. To enhance both solubility and bioavailability, several EtOH/water formulations were tested for aggregation with UV/vis spectroscopy. The “solubility” of the derivatives was significantly enhanced by the use of 1:1 (50% EtOH) and 1:2 (33% EtOH) EtOH/water formulations. These formulations have been previously demonstrated to maintain the carotenoid derivatives in monomeric form [26]. The addition of EtOH at 50% final concentration caused the expected red-shifted, hyperchromic changes in the UV/vis absorption spectrum of the racemic dAST derivative, from 423 nm (card-pack aggregate) to 484 nm ($\lambda_{\text{max}}$ of the non-esterified, free astaxanthin chromophore measured in acetone), due to the disaggregation of the compound to molecular monomers (Figs. 2A and 2B) [33]. Therefore, ethanolic formulations were utilized in subsequent analyses in the current study. Non-esterified, synthetic all-E astaxanthin exhibited a lack of
solubility in both the water and EtOH/water formulations as assessed spectrophotometrically (Figs. 2C and 2D), demonstrating the significant enhancement of water solubility achieved by the derivatives over the parent carotenoid.

**Fig. 2.** UV/vis absorption spectra of racemic dAST dissolved in water, 2A, compared to a solution in 50% EtOH, 2B (all solutions at 10^-5 M racemic dAST). The $\lambda_{max}$ in 50% EtOH (484 nm) is red-shifted and hyperchromic relative to the aggregated state in water (423 nm), expected spectral changes for carotenoids which demonstrate supramolecular assembly in aqueous solution. Panel 2C: lack of UV/vis absorption of non-esterified, synthetic all-$E$ astaxanthin in water, or as shown in 2D, in 50% EtOH, illustrating the dramatic increase in aqueous solubility achieved with the disodium disuccinate derivatization of non-esterified, free astaxanthin.

Studies conducted using the disodium diphosphate derivative (pAST) yielded similar disaggregation effects with the use of 20% EtOH. This allowed the compound to be directly dissolved in media without precipitation for up to 24 hours, upon which
phosphatases present in the media and serum presumably resulted in cleavage of the
diphosphates to the parent moiety and subsequent precipitation in solution.

3.2. Molecular Analysis of Cx43

Initial treatments of the 10T1/2 cells were performed with the goal of comparing the
relative biological efficacy of the racemic dAST in both water and in 50% EtOH to
enhance connexin 43 expression. Cells were treated with racemic dAST in water at $10^{-5}$,
$10^{-6}$, and $10^{-7}$ M, as well as in a 50% EtOH formulation at $10^{-5}$ M (0.5% final EtOH
concentration in assay). 50% EtOH was used as a solvent-only control. Racemic dAST
caued induction of Cx43 in comparison with solvent-only treated controls in a dose-
dependent manner when dissolved in sterile water (Fig. 3). Additionally, induction levels
were higher with the EtOH formulation at $10^{-5}$ M than for the formulations in sterile
water alone, demonstrating enhanced biological efficacy using EtOH as a co-solvent, as
suggested by the physico-chemical studies described in Section 3.1 above. Racemic
dAST in pure aqueous formulation was able to upregulate Cx43 expression with
equivalent or greater potency than that previously observed for other carotenoids in
organic vehicle [15;16]. Importantly, this activity was achieved via direct delivery in
sterile water alone, eliminating the absolute need for introduction of a co-solvent into cell
culture.
Fig. 3. Western blot demonstrating Cx43 protein expression in 10T1/2 cells treated for four days with two different formulations of racemic dAST. (A) Lanes 1-3: racemic dAST in water at $10^{-5}$, $10^{-6}$, and $10^{-7}$ M, respectively; Lane 4: racemic dAST at $10^{-5}$ M in 50% EtOH; Lane 5: 50% EtOH solvent-only control. (B) Relative Cx43 induction levels by racemic dAST obtained by digital analysis of data shown in Panel A; Cx43 protein levels in 50% EtOH solvent control set to 1.0. The upper immunoreactive bands in A are believed to represent phosphorylated forms of the protein assembled into gap junctions; lower bands unphosphorylated proteins [34]. Figure is representative of three separate replicates. Immunoblot stained with Coomassie blue and confirmed by densitometry for equal protein loading and transfer.

Racemic dAST and the purified stereoisomers 3S,3'S dAST, 3R,3'S (meso) dAST, and 3R,3'R dAST were also added to cell cultures in a formulation of 33% EtOH at 1 x $10^{-5}$ M. The potent chemopreventive retinoids TTNPB at $10^{-8}$ M in acetone (0.1% final acetone concentration in assay) and retinol acetate ("RetAC") at $10^{-5}$ M in acetone (0.1% final acetone concentration in assay) were included as positive controls. All compounds induced increased expression of Cx43 in comparison to cell cultures treated with 33%
EtOH as a solvent-only control (Fig. 4). Treatment with racemic dAST induced the highest level of Cx43 of all novel derivatives tested (~3-fold). As previously observed with unmodified carotenoids, connexin 43 induction by the astaxanthin derivatives (~3-fold or less) was several-fold lower than induction levels observed with the retinoids (up to 9-fold induction). Interestingly, in cells treated with the novel astaxanthin derivatives, the majority of the Cx43 protein was located in the higher molecular weight regions of the gel, whereas the majority of connexin 43 induced by the retinoids remained in the lower regions (Fig. 4A). These higher molecular weight isoforms are believed to represent phosphorylated forms of the protein assembled into gap junctions, whereas the lower bands are comprised of non-phosphorylated, unassembled proteins [39].

A

B

Fold Induction

<table>
<thead>
<tr>
<th></th>
<th>Media</th>
<th>RetAC</th>
<th>TTNPB</th>
<th>Rac</th>
<th>R,R</th>
<th>S,S</th>
<th>Meso</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold</td>
<td>1.00</td>
<td>2.00</td>
<td>3.00</td>
<td>7.00</td>
<td>2.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Fig. 4. Western blot demonstrating induction of Cx43 protein expression in 10T1/2 cells treated for four days by novel astaxanthin derivatives delivered in 33% EtOH, versus positive and negative controls. (A) **Lane 1**: 33% EtOH (final EtOH concentration in assay 0.3%) as solvent-only control. **Lane 2**: TTNPB at 10^{-8} M in acetone (final acetone concentration in assay 0.1%) as positive control. **Lane 3**: Retinol acetate ("RetAC") at 10^{-5} M in acetone (final acetone concentration in assay 0.1%) as positive control. **Lane 4**: racemic dAST at 10^{-5} M; **Lane 5**: 3R,3'R dAST at 10^{-5} M; **Lane 6**: 3S,3'S dAST at 10^{-5} M; **Lane 7**: meso dAST at 10^{-5} M. (B) Relative induction levels of Cx43 expression versus solvent-only control, as in Fig. 3B. Figure is representative of three separate replicates. Racemic dAST at 10^{-5} M in 33% EtOH demonstrates greatest induction of Cx43 protein levels (> 3-fold), similar to that obtained with racemic dAST at 10^{-5} M in 50% EtOH vehicle (Fig. 3B). Figure is representative of three separate replicates. Immunoblot stained with Coomassie blue and confirmed by densitometry for equal protein loading and transfer.

Separate treatments of the 10T1/2 cells were also performed with the goal of comparing the relative biological efficacy of the racemic pAST in a 20% EtOH formulation to enhance connexin 43 expression as compared to the parent carotenoid astaxanthin, the related xanthophyll canthaxanthin and the retinoid control. Cells were treated with racemic pAST in 20% EtOH (0.2% final EtOH conc.), AST in THF (0.1% final THF conc.), and CTX in THF (0.1% final THF conc.) at 10^{-5}, 10^{-6}, and 10^{-7} M. TTNPB in acetone at 10^{-8} M was used as a positive control and media without compound was used as a negative control. Racemic pAST caused induction of Cx43 in comparison with non-treated controls in a dose-dependent manner at concentrations of 10^{-6} and 10^{-7} M, although Cx43 induction was not observed at 10^{-5} M (Fig. 5). Cx43 induction by the astaxanthin derivatives (≈3.5-fold or less) was several-fold lower than induction levels observed with the retinoid (up to 12-fold induction). CTX exhibited potent induction of Cx43 in comparison with non-treated controls in a dose-dependent manner, with strong
induction observed at $10^{-5} \text{ M}$ (≈7-fold). Neither pAST nor AST at $10^{-5} \text{ M}$ demonstrated significant Cx43 induction. This finding was corroborated by Lucifer dye transfer studies, where treatment of cells with pAST and AST at $10^{-5} \text{ M}$ demonstrated a lack of induced gap junctional communication (data not shown). CTX induced greater expression of Cx43 at $10^{-6} \text{ M}$ (≈4.5-fold) as compared to pAST at $10^{-6} \text{ M}$ (≈3.5-fold), and comparable induction at $10^{-7} \text{ M}$ (≈2-fold). AST exhibited very weak induction at $10^{-5}$ and $10^{-6} \text{ M}$ (≈2-fold or less), and no induction at $10^{-7} \text{ M}$.

A

B

Fold Induction

<table>
<thead>
<tr>
<th></th>
<th>43 kD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 5. Western blot demonstrating induction of Cx43 protein expression in 10T1/2 cells treated for four days by novel astaxanthin derivatives, versus the related carotenoids astaxanthin and canthaxanthin and positive and negative controls. (A) Lane 1: TTNPB at $10^{-8}$ M in acetone (final acetone concentration in assay 0.1%) as positive control. Lane 2: CTX at $10^{-5}$ M in THF (final THF concentration in assay 0.1%). Lane 3: CTX at $10^{-6}$ M in THF (final THF concentration in assay 0.1%). Lane 4: CTX at $10^{-7}$ M in THF (final THF concentration in assay 0.1%). Lane 5: AST at $10^{-5}$ M in THF (final THF concentration in assay 0.1%). Lane 6: AST at $10^{-6}$ M in THF (final THF concentration in assay 0.1%). Lane 7: AST at $10^{-7}$ M in THF (final THF concentration in assay 0.1%). Lane 8: Racemic pAST at $10^{-5}$ M in 20% EtOH (final EtOH concentration in assay 0.2%). Lane 9: Racemic pAST at $10^{-6}$ M in 20% EtOH (final EtOH concentration in assay 0.2%). Lane 10: Racemic pAST at $10^{-7}$ M in 20% EtOH (final EtOH concentration in assay 0.2%). Lane 11: Non-treated sample as negative control. (B) Relative induction levels of Cx43 expression versus non-treated control. Figure is representative of two separate replicates. Racemic pAST at $10^{-6}$ M in 20% EtOH demonstrates greater induction of Cx43 protein levels ($\approx$$3.5$-fold) over the parent AST carotenoid at $10^{-6}$ M ($\approx$2-fold), although less than the related carotenoid CTX at $10^{-6}$ M ($\approx$4.5-fold). Figure is representative of two separate replicates. Immunoblot stained with Coomassie blue and confirmed by densitometry for equal protein loading and transfer.

3.3. Cellular Analysis of Cx43

Racemic dAST increased assembly of immunoreactive Cx43 into plaques in regions of the cell membrane in direct contact with adjacent cells. Such localization is consistent with formation of functional gap junctions. Cells treated with TTNPB at $10^{-8}$ M in acetone (0.1% final acetone concentration in assay) also exhibited more extensive immunoreactive plaques than untreated cells. In solvent-only treated cultures, such immunoreactive plaques were infrequent, and were smaller than those detected in cells treated with racemic dAST or with TTNPB as a positive control. The frequency of these plaques and their size is consistent with the functional differences in gap junctional
permeability as detected by the Lucifer Yellow dye transfer experiments (described below), and with the degree of induction of Cx43 as detected in the immunoblot experiments described in Section 3.2 above. Representative photomicrographs are shown in Fig. 6.

**Fig. 6.** Racemic dAST increases assembly of Cx43 immunoreactive junctional plaques. Confluent cultures of 10T1/2 cells were treated for 4 days as in Figs. 3-5, and immunostained with a Cx43 antibody. **Panels:** (A) racemic dAST at $10^{-5}$ M in 33% EtOH; (B) 33% EtOH as solvent-only control; (C) TTNPB at $10^{-8}$ M in acetone (final acetone concentration in assay 0.1%). **Panels D, E, F:** digital analysis of micrographs in Panels A, B, and C, respectively, demonstrating pixels above a fixed set threshold positive for fluorescent intensity. Yellow arrows: immunoreactive junctional plaques; red arrows: position of cell nuclei. Note the greater number and intensity of junctional immunoreactive plaques in the cultures treated with racemic dAST in comparison with solvent-only treated controls. The junctional plaques shown in
Panels B and E represent infrequent plaques seen in controls; most cells in these cultures were negative for Cx43 staining, in contrast to the extensive immunoreactivity in racemic dAST- and TTNPB-treated cells.

3.4. Induction of Gap Junctional Communication

To assess the functional capacity of these junctional plaques for direct intercellular communication, Lucifer Yellow dye microinjection studies were performed to assess the ability of racemic dAST to enhance dye transfer among 10T1/2 cells. As discussed above, this ability has been previously highly correlated with the ability of carotenoids to inhibit carcinogen-induced neoplastic transformation [15;16]. Racemic dAST in 33% EtOH formulation at a concentration of $1 \times 10^{-5}$ M effectively increased the extent of junctional communication over that seen in solvent-only treated controls. Of 22 microinjected treated cells, 15 (56%) were functionally coupled by gap junctions in contrast to only 3 out of 11 (27%) solvent-only treated control cells. These differences were statistically significant ($P < 0.03$; Fisher’s Exact test). Representative photomicrographs are shown in Fig. 7.
Fig. 7. Racemic dAST enhances functional GJIC in 10T1/2 cells as shown by transfer of microinjection dye. Confluent cultures were treated for 4 days as described in Fig. 6, then assayed for the ability to transfer the fluorescent dye Lucifer Yellow. Arrows indicate the cell injected with Lucifer Yellow. Panels: (A) racemic dAST at 10^{-5} M in 33% EtOH; (B) 33% EtOH solvent-only control; (C) TTNPB at 10^{-8} M in acetone (final acetone concentration in assay 0.1%). Panels D, E, F: digital analysis of micrographs in Panels A, B, and C, respectively, demonstrating pixels above a set threshold positive for Lucifer Yellow fluorescence. Because cell nuclei have the most volume, they accumulate the most Lucifer Yellow and exhibit the greatest fluorescence. Racemic dAST-treated cells demonstrate significantly increased functional coupling (56%) over solvent-only treated controls (27%); P < 0.03, Fisher's Exact test.

3.5. Inhibition of MCA-induced Neoplastic Transformation

The Bertram lab has previously reported that carotenoids such as canthaxanthin and β-carotene act as potent inhibitors of transformation when added in the postinitiation phase of carcinogenesis [36]. Here we examine the ability of the pAST derivative and its parent carotenoid moiety astaxanthin to similarly inhibit carcinogen-induced neoplastic transformation in a dose-dependent manner. As per Bertram lab protocols, compounds were added in all cases 7 days after the removal of the carcinogen so as not to potentially interfere with the production of carcinogen-initiated cells. Treatments were continued at weekly intervals after re-feeding with fresh media 24 hours after MCA treatment. Results are presented as the average number of foci per dish (Fig. 8). Cells treated with pAST and AST at 10^{-5} M demonstrated highly significant inhibition of transformation (P < 0.0001), however visualization of the cell monolayers for both treatments by microscopy revealed regions of incomplete monolayer formation. This is most likely due to cell toxicity at high concentrations, and such toxicity acts to inhibit transformation.
This may also account for their lack of Cx43 protein expression and induced GJIC at these concentrations. This effect was not observed at the lower concentrations. At $10^{-6}$ M pAST demonstrated 100% inhibition of neoplastic transformation, and at $10^{-7}$ M pAST exhibited significant inhibition of transformation ($P > 0.04$). At $10^{-8}$ M pAST did not demonstrate significant inhibition. AST at concentrations of $10^{-6}$ M, $10^{-7}$ M, and $10^{-8}$ M did not significantly demonstrate inhibition of transformation, which corroborates earlier findings of a significant lack of Cx43 expression and induction of GJIC for all doses of AST.

![Graph](image)

**Fig. 8.** Dose-dependent effects of pAST and AST on MCA-induced neoplastic transformation. Results are presented as average number of foci per dish. As a negative control, cells were treated with acetone and followed by media-alone treatments as per protocol. Positive controls were treated with MCA and followed by either media-alone or THF as a solvent control. Statistical significance was calculated by student’s paired t-test.
CHAPTER 4. DISCUSSION

The accumulated body of evidence demonstrates that tumor cells exhibit a lack of communication with surrounding normal cells. This is either a consequence of lack of connexin gene expression, or a loss of functional gap junctional communication due to faulty trafficking or assembly into the plasma membrane [37]. Unlike other tumor suppressor genes, inactivating mutations of connexins are apparently rare and lack of expression in tumors appears a consequence of increased DNA methylation of the connexin promoter [38]. While fully established neoplastic cells can be expected to be non-responsive to carotenoid or retinoid treatments, pre-neoplastic cells may retain some responsiveness. Indeed, in the 10T1/2 cell system, isolated carcinogen-initiated cells were shown to be less junctionally coupled than normal cells, yet able to respond to retinoids with increased GJIC [39]. In contrast, once transformation has occurred, retinoids—and one would expect carotenoids as well—are not able to induce junctional communication between these and surrounding normal cells, nor suppress their ability to proliferate and form transformed foci [11]. A similar situation may exist clinically. The Bertram lab has shown that in cervical dysplasia and leukoplakia of the oral cavity, Cx43 expression is strongly downregulated in these pre-neoplastic lesions induced by HPV infection and tobacco exposure respectively, in contrast to its strong expression in normal epithelium [40;41]. In the cervix, retinoic acid, and in the oral cavity retinoic acid and β-carotene, have separately been shown to have chemopreventative activity. In this context it is of interest that retinoic acid only suppresses oral tumors after a delay of six months to one year. This suggests a lack of effect on initially latent, fully transformed cells and
instead an inhibition of progression of pre-neoplastic cells [42;43]. In the Bertram model of gap junctional communication and proliferation control, the restoration of GJIC by retinoids or carotenoids would lead to suppression of aberrant proliferation and inhibition of the development of cells bearing multiple mutations leading to full carcinogenic potential.

Unlike carotenoids, the use of retinoids as chemopreventative agents is limited by toxicity. β-carotene, a pro-vitamin A carotenoid whose consumption in foods and presence in serum strongly correlates in epidemiological studies with decreased lung cancer risk in smokers, has been evaluated most thoroughly. In two interventional studies of individuals at high risk of lung cancer as a consequence of tobacco and/or asbestos exposure, supplemental β-carotene at levels approximately 10-fold higher than found in a "healthy" diet, actually increased lung cancer rates over placebo controls [42;43]. However, in a third study of low-risk physicians, few of whom were smokers, β-carotene did not influence lung cancer rates [44]. In a fourth study, also in current and former smokers, β-carotene tended to decrease cancers of the oral cavity but increase those of the lung [45]. This suggests a direct interaction between tobacco smoke and β-carotene. This was confirmed in animal studies as in which β-carotene or its breakdown products were shown to enhance smoke-induced pathological changes and to interfere with retinoic acid signaling [32]. Such interactions appear unlikely with non-pro-vitamin A carotenoids; a conclusion confirmed in a follow-up study. Here lycopene replaced β-carotene which resulted in protection against smoke-induced lung pathology without associated toxicity [46].

Carotenoids may act as effective antioxidants at low oxygen tension and low
physiologic concentration, in comparison to other chain-breaking antioxidants such as vitamin E. Certain carotenoids may be pro-oxidant under high oxygen tension and high physiologic concentration environments (e.g. β-carotene) [47;48]. Therefore, supplementation of β-carotene at high levels could add an additional oxidative stress insult (particularly in the lung) to these individuals. In contrast, astaxanthin has been described as a “class three” antioxidant; that is an excellent antioxidant that effectively quenches excited molecular states as well as ground state radicals, without evidence of pro-oxidant activity [48]. If pro-vitamin A activity, retinoid toxicity, and pro-oxidant behavior are subsequently definitively implicated in the increased lung cancer rates in supplemented human populations, then the astaxanthin derivatives described here may be particularly well suited for clinical cancer chemoprevention. The influence on GJIC demonstrated in the current study suggests that novel astaxanthin derivatives with increased bioavailability may be especially useful chemopreventative agents.

Lycopene, a non-pro-vitamin A carotenoid found in tomatoes, may also have both therapeutic and preventive properties. Epidemiological evidence suggests that tomato consumption is protective against prostate cancer [49]. When administered as a supplement to men scheduled for radical prostatectomy after diagnosis of prostate cancer, a dose of 30 mg/day for approximately three weeks apparently decreased indices of neoplasia in excised tumors and increased expression of connexin 43 [50]. When lycopene was administered in conjunction with α-tocopherol to patients at high-risk of liver cancer as a consequence of infection with hepatitis C, this combination reduced the incidence of hepatocellular carcinoma after a delay of approximately one year [51].

Carotenoids have been shown to enhance antibody production, increase T-helper
cell numbers, T- and B-cell proliferation and even to increase the cytotoxicity of natural killer cells [52]. Experimental evidence on tumor immunity suggests that β-carotene's suppressive effects on tumor growth may be attributed to enhanced production of tumor specific antigens [53]. Astaxanthin has also demonstrated the ability to inhibit tumor formation in the livers of mice through enhanced T cell-mediated immune response [54;55]. While enhanced immune function may play a role during tumorigenesis in the whole animal, cell culture studies demonstrating inhibition of cancer during the promotion phase of carcinogenesis suggest that it is unlikely to be the central mechanism of action. Rather, the current body of evidence suggests that induction of Cx43 expression and increase in GJIC is the major mechanism by which initiated cells prevent transformation. In the current study, astaxanthin did not significantly induce Cx43 expression, increase GJIC, or prevent carcinogen-induced neoplastic transformation, in contrast to the novel compounds derived from astaxanthin. This apparent discrepancy may be attributed to differences in uptake or cleavage of the derivatives as compared to the parent astaxanthin moiety. Further studies need to be performed to elucidate the mechanism behind this discrepancy. It is noteworthy that the diphosphate derivative was demonstrated to have enhanced biological efficacy over the parent carotenoid despite the precipitation of the compound in solution 24 hours post treatment.

The availability of a water-soluble, highly bioavailable derivative of astaxanthin should facilitate the evaluation of its cancer chemopreventative properties in experimental animals, and if successful, will simplify its evaluation as a chemopreventative agent in the clinic. The demonstration that novel astaxanthin derivatives are able to upregulate the expression of Cx43, increase the size and number of
Cx43 immunoreactive gap junctional plaques, functionally increase GJIC and inhibit carcinogen-induced neoplastic transformation with enhanced activity over the parent carotenoid suggests that these derivatives will have potent chemopreventive properties in vivo. In view of this potential, further cell culture and animal studies utilizing these derivatives are warranted.
APPENDIX

REFERENCES


44. Sakr, W., Tabaska, P., Kucuk, O., and Bertram, J. S. Differential expression of connexin 43 in normal, preneoplastic and neoplastic squamous epithelium of the upper aerodigestive tract. Proc.AACR 37, 269. 1996.


