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Research Paper

Enhanced anticancer activity of glutamate prodrugs of all-trans retinoic acid

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Abstract

Objectives All-trans retinoic acid (ATRA), an active metabolite of vitamin A, is widely used in the treatment of acute promyelocytic leukaemia and myelodysplastic syndrome. However, its high lipophilicity is thought to be responsible for the slow dissolution and low bioavailability following oral administration. In order to obtain compounds with better solubility characteristics to improve the transportation and bioavailability of ATRA, derivatives of ATRA containing glutamic acid or its sodium salt were synthesised.

Methods The ATRA derivatives synthesised – all-trans retinoyl glutamate (RAE) and all-trans retinoyl sodium glutamate ($RAENa_2$) – were characterised in terms of melting point, optical rotation, mass spectrometry, NMR and partition coefficient. A liposomal preparation formed from RAE was characterised by particle size and zeta potential. The anti-tumour activity of RAE and $RAENa_2$ was compared with that of ATRA in mice bearing S_{180} tumours and their effects on the cell cycle were determined in human pro-myelocytic leukaemia HL-60 cells.

Key findings RAE and $RAENa_2$ were more active than ATRA against tumour growth. Flow cytometry indicated that RAE and $RAENa_2$ induced HL-60 cell cycle arrest, similar to ATRA. DNA fragmentation studies suggested that apoptosis may be one of the mechanisms responsible for the anti-tumour activities.

Conclusions The two derivatives of ATRA, RAE and $RAENa_2$, exhibited improved aqueous solubility and were more effective in mice bearing S_{180} tumours.

Keywords all-trans retinoic acid; anti-tumour; cell cycle; glutamic acid; pro-drug

Introduction

Retinoic acid (RA) is an oxidative metabolite of vitamin A and plays important roles in embryonic development. It acts through Hox genes and ultimately controls anterior/posterior patterning in early development.[1] RA also regulates cell proliferation and differentiation by binding to heterodimers of the RA receptor (RAR) and the retinoid X receptor (RXR), which then bind to RA response elements in the regulatory regions of direct targets (including Hox genes), thereby activating gene transcription.[2] RAR mediates transcription of different sets of genes involved in cell differentiation.[3]

All-trans retinoic acid (ATRA) is widely used in the treatment of various skin conditions including keratoderma and photo-aged skin. It is also used in the prevention and treatment of different types of cancer such as breast cancer, skin cancer, cervical cancer and leukaemia. More recently, ATRA has been used in the treatment of acute pro-myelocytic leukaemia, resulting in better rates of complete remission and cure – the most significant step forwards in the treatment of this disease in the last 25 years. From a drug delivery point of view, however, ATRA has relatively low water solubility (< 0.2 mg/l), which is probably responsible for the low dissolution and poor bioavailability following oral administration.

Various strategies have been tried in attempts to improve the anti-tumour activities of ATRA, including preparation of nanoparticles[4,5] and chemical modifications.[6,7] In the present study, derivatives of ATRA containing glutamic acid, a water-soluble amino acid, or its sodium salt (Figure 1) have been synthesised in an effort to improve solubility in water and ultimately bioavailability. In addition to improved water solubility, which may

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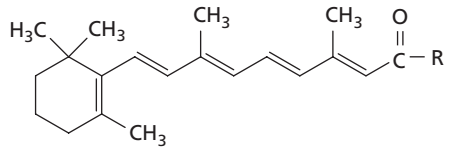

ATRA, R = OH; RAE, R = Glu; $RAENa_2$, R = $GluNa_2$ (ENa_2)

Figure 1 Structures of all-trans retinoic acid (ATRA), all-trans retinoyl glutamate (RAE) and all-trans retinoyl sodium glutamate ($RAENa_2$)

provide flexibility in terms of formulation, incorporation of an amino acid moiety may lead to increased cellular uptake, since many tumour cells express high levels of amino acid transporters.[8–13]

Anticancer activities of the ATRA derivatives synthesised – all-trans retinoyl glutamate (RAE) and all-trans retinoyl sodium glutamate ($RAENa_2$) – were evaluated *in vivo*. To understand the mechanisms involved, the effects of the derivatives on the cell cycle in HL-60 cells were studied. A DNA fragmentation assay of HL-60 cells was carried out following exposure to RAE and $RAENa_2$. The results obtained were compared with those of ATRA.

Materials and Methods

Reagants

ATRA was obtained from Beijing Brilliance Biochemical Ltd (Beijing, China). L-Glutamic acid was from Sichuan Sangao Biochemical Ltd (Sichuan, China). Dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide were from GL Biochem Ltd (Shanghai, China). Egg lecithin (molecular weight 750), sodium chloride for injection and sodium carboxymethyl cellulose (CMC-Na) were purchased from Shandong Qilu Pharmaceutical Co. Ltd (Zibo, Shandong, China). All chemicals were of chemical grade unless otherwise specified.

RPMI 1640 cell culture medium was from Gibco (Grand Island, NY, USA). Fetal calf serum (FCS) and HyQ trypsin 0.25% were purchased from HyClone (Logan, UT, USA). Penicillin and streptomycin were from Sigma (St Louis, MO, USA). DMSO was purchased from Acros Organics (Geel, Belgium).

Cell culture

Human promyelocytic leukaemia HL-60 cells were from the Peking University Medical Center. Cells were maintained in RPMI 1640 supplemented with 10% FCS, 100 units/ml penicillin and 100 units/ml streptomycin.

Animals

Male Kunming mice (18–22 g) were purchased from the Animal Services, Peking University Medical Center and were supplied with food and water *ad libitum*. Animal experiments were carried out according to a protocol approved by the Experimental Animal Care Committee of Capital Medical University.

Synthesis of RAE and $RAENa_2$

Conjugation of ATRA with glutamate was carried out according to the general method reported previously.[14] As shown in Figure 2, the carboxylic group of RA was first activated by forming a succinimide ester, which was allowed to react with the amino group of glutamate to form $RAENa_2$. RAE was obtained from $RAENa_2$ in acidic conditions.

Characterisation

Melting points were determined using an XT5 melting point analyser (Beijing Scientific Instrument Ltd, Beijing, China). Optical rotation was determined using a P-1020 Polarimeter (Jasco, Tokyo, Japan). NMR spectra were recorded on an Avance II 500 (Bruker, Fällanden, Switzerland). Mass spectra were recorded on a Quattro Micro 2000 mass spectrophotometer (Waters, Milford, MA, USA).

Determination of partition coefficient

Partition coefficient was determined using a reported method.[15] Briefly, about 0.5 mg ATRA, RAE or $RAENa_2$ was dissolved in 5 ml water-saturated 1-octanol. An equal volume of 1-octanol-saturated water was added. The mixture was shaken for 2 h at 25 ± 5ºC and then centrifuged at 3500 rpm for 20 min to separate the two phases. For $RAENa_2$, the water phase was collected and diluted with water before measuring UV absorbance at 350 nm. For RAE and ATRA, the 1-octanol phase was collected and diluted with chloroform before measuring UV absorbance at 350 nm. The UV absorbance determined at 350 nm was defined as A_x. The 1-octanol/water partition coefficient (P) for $RAENa_2$ was calculated from $P = ([n_o A_o V_o] - [n_x A_x V_x])/(n_x A_x V_x)$ and for RAE and ATRA from $p = (n_x A_x V_x)/([n_o A_o V_o] - [n_x A_x V_x])$ where n_x was the dilution factor of the sample after partition, A_x was the absorbance of the sample and V_x was the volume of the sample used for the measurement of absorbance after partition, n_o was the dilution factor of the initial sample, A_o was the absorbance of the initial sample before partition and V_o was the volume of the initial sample. Log P values were calculated as the average of three measurements.

Preparation of RAE liposomes

RAE liposomes were obtained according to a reported method.[5,16] Briefly, RAE and egg lecithin (at equal molar ratio) were dissolved in chloroform in a flask. The mixture was subject to evaporation using a rotary evaporator at 35°C under nitrogen until a thin film was formed in the flask. Phosphate-buffered saline (PBS) was then added and the hydrated mixture sonicated at 0°C under argon protection for 20 min to obtain RAE liposomes.

Characterisation of the RAE liposomal preparation

Transmission electron microscopy studies

RAE liposomal preparation (5 μl) was placed on a copper grid laid on filter paper and the sample allowed to dry at room temperature before transmission electron microscopy (TEM). Images were taken using a JEM-1230, JEOL transmission electron microscope operating at 80 kV.

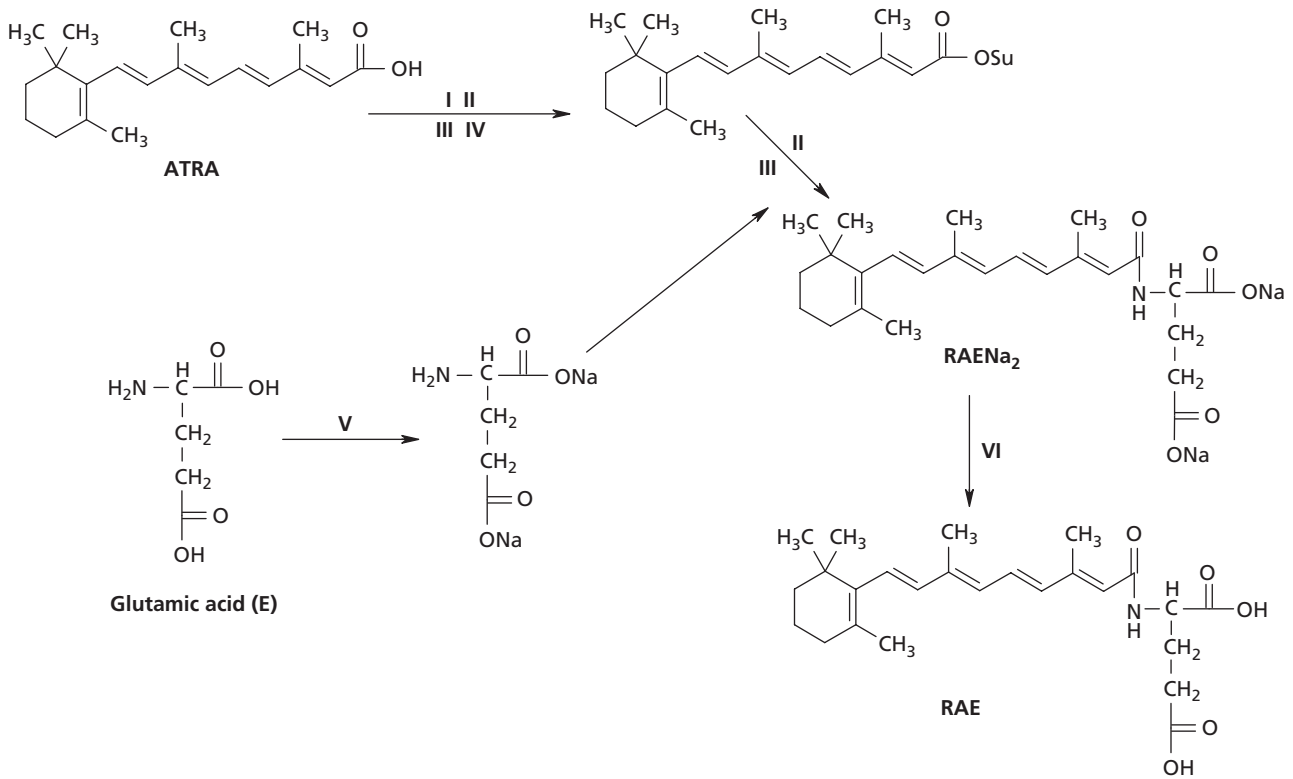

Figure 2 Synthesis of all-trans retinoyl glutamate (RAE) and all-trans retinoyl sodium glutamate ($RAENa_2$) from all-trans retinoic acid (ATRA). I = dicyclohexylcarbodiimide, ice bath; II = protection from light; III = protection under argon; IV = *N*-hydroxysuccinimide, V = Na_2CO_3; VI = 2 M HCl.

Determination of particle size and zeta potential

The RAE liposomal preparation was diluted with PBS. The particle size (determined using a Nano ZS-90 Zetasizer; Malvern Inc. Malvern, UK) and zeta potential of the preparation were then determined at 25ºC every 24 h for 8 days. Averages from three determinations were calculated.

Anti-tumour activity *in vivo*

The murine sarcoma cell line S_{180} was a gift from Peking University Medical Center. The cell line was cultured in 90% RPMI 1640 medium supplemented with 10% heat-inactivated FCS, pH 7.4, at 37°C in a CO_2 incubator with a humidified atmosphere of 5% CO_2. S_{180} tumour cells passaged in mouse abdomen were harvested on the eighth day and suspended at 2.0×10^7/ml. The tumour cell ascites (0.2 ml) were implanted to each mouse subcutaneously. Mice inoculated with S_{180} tumour cells were randomly divided into four groups of 10.

RAE, $RAENa_2$ and ATRA were suspended in 0.5% CMC-Na at 3.3×10^{-2} mol/l. A solution of 0.5% CMC-Na solution was used as a negative control. Mice were injected (i.p.) with 0.2 ml RAE, $RAENa_2$ or ATRA suspension (3.3×10^{-2} mol/l) or 0.5% CMC-Na solution every day for 8 days. At the end of the 8th day the mice were sacrificed and the tumour dissected from surrounding tissues and weighed. The percentage inhibition of tumour growth was calculated from: [(average tumour weight of the negative control – average tumour weight of the test group)/average tumour weight of the negative control] × 100%. The results are expressed as means ± SD. Statistical analysis was performed using the Student's *t*-test.

Flow cytometry and DNA fragmentation analysis

RAE, $RAENa_2$ and ATRA were first dissolved in ethanol and then diluted in PBS at 1×10^{-5} mol/l (final ethanol concentration 0.1%). PBS containing 0.1% ethanol was used as negative control.

HL-60 cells (2.7 ml, 1×10^5/ml) were seeded into six-well plates and incubated for 4 h. Cells were treated with 300 μl RAE, $RAENa_2$ or ATRA (1×10^{-5} mol/l), or PBS containing 0.1% ethanol and incubated at 37°C. The final drug concentration was 1×10^{-6} mol/l.

For cell cycle analysis, cells (1×10^5) were collected at 24, 48 and 72 h, washed twice with ice-cold PBS and then fixed in ice-cold 70% (v/v) ethanol at 4°C overnight. Cells were washed twice in PBS to remove fixative, and 10 μl RNase A (400 μg/ml) added. Cells were incubated at 37°C for 30 min then stained with 1 ml propidium iodide solution (20 μg/ml). After incubation at 4°C for 30 min, cells were resuspended in PBS to a final volume of 500 μl and analysed by flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA). For each sample 10 000 events were stored. The effects of RAE, $RAENa_2$ and ATRA at 1×10^{-7} mol/l on the cell cycle of HL-60 cells were also examined. The Kruskal–Wallis non-parametric test was used to determine statistical significance.

For the DNA fragmentation assay, cells (2×10^5) were collected at 24, 48, 72, 96 and 120 h, washed twice with ice-cold PBS and then centrifuged at 9000 rpm for 10 min. DNA was extracted using a commercial DNA extraction kit (Sigma-Aldrich, St Louis, MO, USA) and stored at −20°C until use. Lysates were fractionated by DNA ladder electrophoresis using 8% polyacrylamide gels for 45 min at 100 V. The gel was then stained with 0.5 μg/ml ethidium bromide (EB) for 20 min, and photos were taken with a gel-imaging system.

Results

Chemistry

Synthesis of RAENa$_2$

Glutamic acid (0.294 g) and Na_2CO_3 (0.212 g) were dissolved in water to obtain sodium glutamate. ATRA (0.6 g; 2 mmol) was mixed with 0.454 g (2.2 mmol) DCC and 0.230 g *N*-hydroxysuccinimide in anhydrous tetrahydrofuran (THF). The mixture was stirred at 0°C under argon protection for 20 min in the dark, following by stirring at room temperature for 12 h. Dicyclohexylurea formed was removed by filtration. The sodium glutamate prepared was added gradually with stirring, in the dark and protected by argon. The mixture was left stirring for 2 days before the reaction was terminated. THF was removed by evaporation under reduced pressure and the reaction mixture was filtered. The filtrate was stored at −20ºC to dry. The yellow powder formed was then purified using a C_{18} column with 10% methanol in water as eluent to obtain 82 mg of the title compound as a light-yellow powder. Yield: 12.8%; melting point: 289.2–291.7ºC. Log *P*: −0.11.

Synthesis of RAE

Sixty mg RAENa$_2$ prepared above were dissolved in water and 2 M HCl added to adjust the pH to 2. Chloroform was then used to extract the title compound formed. Anhydrous Na_2SO_4 was added to the chloroform layer collected to remove water. The filtrate was subject to rotary evaporation under reduced pressure to remove chloroform; 41 mg of the title compound was obtained as a yellow powder. Yield: 75.3%; melting point: 156.3–157.8ºC; $[\alpha]_D^{25} = -24.70°$ (c = 0.667, MeOH); Si MS (*m/z*): 428.3 $[M\text{-}H]^+$, 450.3 [M+Na-H], 857.4 [2M-H], 571.3 $[2M+H]^+$, 593.2 $[2M+Na]^+$. ^{1}H-NMR (DMSO-d_6, 500 MHz), δ (ppm): 8.33 (s, 1H, NH), 6.78 (t, H), 6.73 (d, J = 10 Hz, 1H), 6.15 (d, J = 10 Hz, 1H), 6.02 (d, d, J = 15 Hz, J = 15 Hz, 1H), 4.51 (s, H), 2.35 (t, 2H), 2.35 (t, 2H), 2.097 (m, 2H), 1.93 (m, 2H), 1.63 (m, 2H), 1.44 (s, 3H). ^{13}C-NMR (DMSO-d_6, 125 MHz), δ (ppm): 163.01 (CONH), 154.94 (C), 112.25 (CH), 56.50 (CH), 29.37 (CH_2), 25.10 ($3CH_3$), 17.06 ($2CH_3$). Log *P*: 0.48.

Characterisation of RAE liposomal preparation

The morphology of RAE vesicles in its liposomal preparation was revealed by TEM. Individual vesicles assumed a spherical shape (figure not shown). The diameter of the RAE liposomal vesicles formed was about 200 nm and changed little over the 8 days of monitoring (data not shown). Vesicles had a negative charge and the zeta potential was about −60 mV.

RAENa$_2$ failed to form liposome-like vesicles under the same conditions.

Anti-tumour activity *in vivo*

The anti-tumour activity of RAE, RAENa$_2$ and ATRA in mice bearing S_{180} tumours is shown in Table 1. RAE and RAENa$_2$ were more potent inhibitors of tumour growth than ATRA as determined by % inhibition of tumour growth.

Effects on cell cycle

The effects of ATRA, RAE and RAENa$_2$ on HL-60 cells are shown in Figure 3. As the exposure time increased, the percentages of cells in the G_1 and S phases for the negative control changed little. However, ATRA treatment resulted in an increase in the percentage of cells in the G_1 phase and a decrease in the percentage of cells in the S phase, which is consistent with the results of previous studies.[17] RAE and RAENa$_2$ had similar effects on the cell cycle (Figures 3a and 3c). The percentages of cells at G_1 and S phases following 96 h' exposure to ATRA, RAE or RAENa$_2$ were significantly different from that of the negative control with an asymptotic significance value of 0.009 (Kruskal–Wallis non-parametric test).[18] Ten-fold lower concentrations of ATRA, RAE and RAENa$_2$ (1×10^{-7} mol/l) still had significant effects on the cell cycle following 96 h' exposure (Figures 3b and 3d; asymptotic significance value 0.009).

DNA fragmentation assay

DNA fragmentation was seen in cells exposed to ATRA, RAE and RAENa$_2$, indicative of cell apoptosis (data not shown).

Discussion

Although ATRA has been used in the prevention and treatment of different types of cancer, its relatively low solubility in water (< 0.2 mg/l, Log *P* 0.66) limits its clinical success. We aimed to synthesise more water-soluble derivatives to enhance the antitumour activity and broaden the anti-tumour spectrum of ATRA, for example in the management of localised solid tumours. To improve the water solubility, a polar amino acid, glutamic acid, was conjugated to ATRA to form RAE. The di-sodium salt of RAE, RAENa$_2$, was also prepared. Amidon and colleagues have demonstrated that derivatives containing amino acids

Table 1 Inhibition of tumour growth by all-trans retinoic acid (ATRA), all-trans retinoyl glutamate (RAE) and all-trans retinoyl sodium glutamate (RAENa$_2$) (3.3×10^{-2} mol/l in suspension) in mice bearing S_{180} sarcoma

Compound	Tumour weight (g)	% inhibition of tumour growth
ATRA	1.23 ± 0.68*	35.6
RAE	0.59 ± 0.36†	69.1
RAENa$_2$	0.83 ± 0.49†	56.5
Vehicle	1.91 ± 1.11	–

Values are means ± SD (n = 10). *$P < 0.05$; $^\dagger P < 0.01$ vs vehicle (0.5% sodium carboxymethyl cellulose).

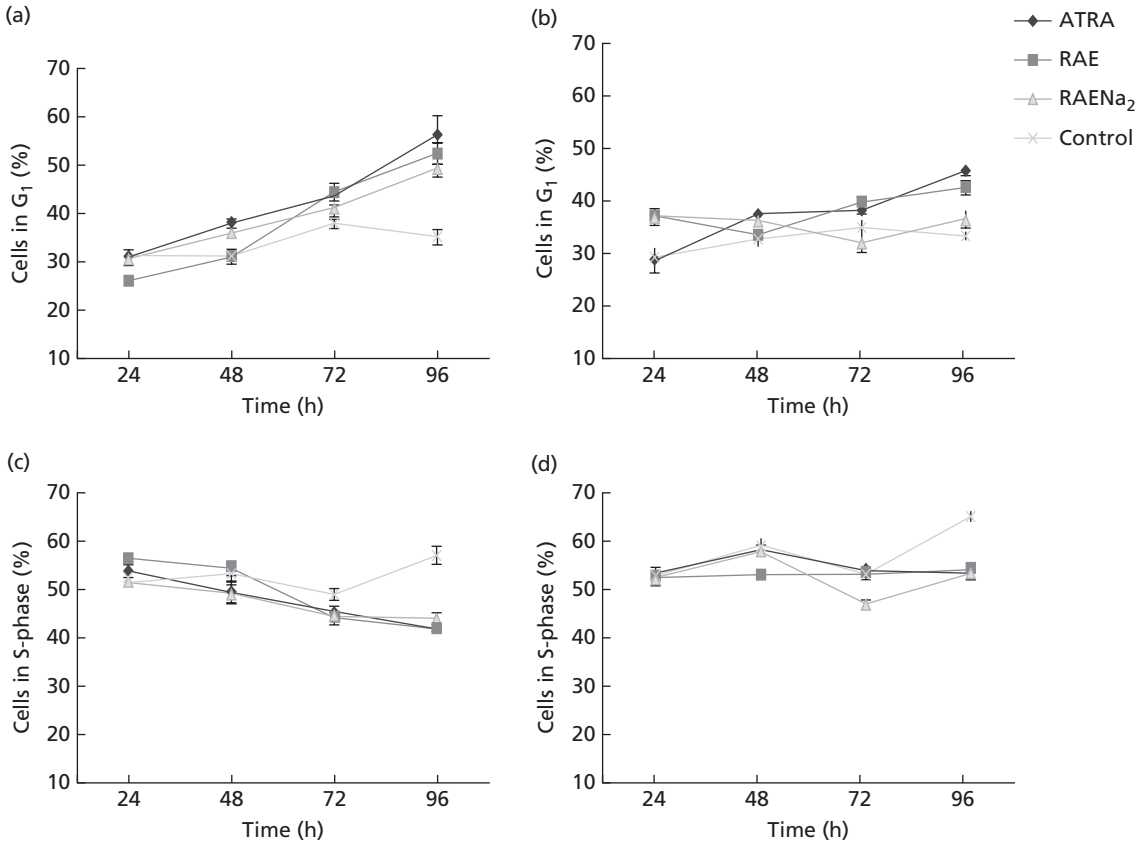

Figure 3 Effects on the cell cycle in human promyelocytic leukaemia HL-60 cells. Effects of all-trans retinoic acid (ATRA), all-trans retinoyl glutamate (RAE) and all-trans retinoyl sodium glutamate ($RAENa_2$) (1×10^{-6} mol/l for a and c; 1×10^{-7} mol/l for b and d) on the percentage of HL-60 cells in G_1 (a and b) and S (c and d) phases.

have improved oral bioavailability, since amino acid transporters are ubiquitous in the gastrointestinal tract.[12,13] The more desirable solubility characteristics may provide flexibility in terms of formulation, such as in preparing liposomes – as demonstrated in this study.

Conjugation of ATRA with glutamate was achieved by reaction of *N*-hydroxysuccinimide-activated ATRA with the α-NH_2 of glutamate (ENa_2) using DCC as a condensation reagent. Because of the chemical instability of ATRA, the overall yield of $RAENa_2$ was low. RAE was obtained from $RAENa_2$ in acidic conditions. RAE and $RAENa_2$ showed lower partition coefficient than ATRA (Log *P* 0.48, −0.11 and 0.66, respectively), indicating better solubility in water.

Since liposomal formulations have certain advantages over conventional formulations and have been used in cancer treatment,[19,20] the propensity of RAE and $RAENa_2$ to form stable liposomal formulations was evaluated. RAE was able to form liposome-like vesicles in the presence of egg lecithin, probably because of its good solubility in both organic solvents and water, as suggested by its partition coefficient, whereas $RAENa_2$ did not form stable liposomes under the same conditions, probably because it was too hydrophilic. Given the structure of RAE, which contains ATRA and a glutamate, it is reasonable to assume that the ATRA portion is inserted in the hydrophobic bilayer of the liposome vesicles and the glutamate end is exposed on the surface of the liposomes. The RAE liposomal preparation was further characterised by a laser scattering particle size analyser. The liposomal vesicles formed had a diameter of about 200 nm. The vesicles had a negative charge, and the zeta potential was about −60 mV. The negative charge on the surface of liposomal particles prevents the aggregation of liposomes as a result of electrical repulsion. The small particle size and surface charge of the particles in the liposomal preparation favour the stability of the formulation. Indeed, the particle size of the RAE liposomal preparation showed little change in the 8 days following preparation. Liposomal formulation may provide a better means to deliver RAE to tumour cells. However, its potential in the delivery of RAE requires further studies.

The ultimate goal of the current study was to enhance the anti-tumour activity of ATRA through the use of prodrugs. Both RAE and $RAENa_2$ were more effective in inhibiting the growth of S_{180} tumours in mice than ATRA, with RAE being more effective than $RAENa_2$. This probably reflects the solubility characteristics of RAE in both lipid and water, which may lead to increased cellular uptake by cancer cells. The improved anti-tumour activity of RAE and $RAENa_2$ over ATRA probably reflects improved water solubility, which may facilitate transportation *in vivo*. The improved anti-tumour activities of RAE and $RAENa_2$ in the solid tumour model may broaden the anticancer spectrum of ATRA.

It is known that ATRA exerts its anticancer activities by inducing cell cycle arrest.[17] ATRA is a potent inhibitor of cell proliferation and induces cell differentiation. As it diffuses into the cell, ATRA binds to RAR/RXR dimers to exhibit its regulatory functions in transcription.[17,21–23] Similar to ATRA, RAE and $RAENa_2$ inhibited the transition from G_1 to S phase in HL-60 cells. In addition, DNA ladder analysis showed that ATRA, RAE and $RAENa_2$ induced DNA fragmentation in HL-60 cells, characteristic of apoptosis.[17,21–23]

Conclusions

The high lipophilicity of ATRA is believed to hinder its transportation, which may lead to accumulation in fatty tissues. To improve aqueous solubility, we linked glutamic acid and its bi-sodium salt to the ATRA molecule to obtain RAE and $RAENa_2$, respectively. These derivatives showed improved aqueous solubility, as evidenced by partition coefficients determined in the octanol/water system. RAE was able to form liposome-like vesicles, probably due to this more desirable solubility feature. RAE and $RAENa_2$ were more potent inhibitors of tumour growth than ATRA. Cell cytometric analysis showed that, in common with ATRA, RAE and $RAENa_2$ induced cell arrest, retarding the transition from G_1 to S in HL-60 cells. In addition, RAE and $RAENa_2$ induced DNA fragmentation in HL-60 cells, suggesting that apoptosis may be involved in their anti-tumour activity.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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