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Inhibition of pro-inflammatory cytokines in tumour associated macrophages is a potential anti-cancer mechanism of carboxyamidotriazole

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ABSTRACT

Carboxyamidotriazole (CAI) has not only direct anti-cancer activities, but also anti-inflammation effects in a variety of inflammatory animal models. In the present study, we investigated whether macrophages, which are important both in cancer and inflammation, could be regulated by CAI. The results showed that CAI could inhibit tumour necrosis factor-α (TNF-α) production in macrophages in various environments, including those isolated from peritoneal cavity of adjuvant-induced arthritis (AA) rats, from Lewis lung carcinoma (LLC) transplanted tumours and those induced by LLC cells *in vitro*. Dexamethasone (DEX), one of the pro-inflammatory cytokines inhibitors, could enhance CAI's inhibition of LLC cells proliferation and invasion in macrophages and LLC cells co-culture systems, as well as the tumour growth *in vivo*. However, DEX failed to enhance CAI's inhibition of LLC cells proliferation when LLC cells were cultured alone, suggesting that the combination of CAI and DEX exerted great anti-tumour effects probably by acting on macrophages in the tumour environment. Over all, we found CAI could act on macrophages and regulate the production of TNF-α not only in inflammatory diseases but also in tumour microenvironment, which might be another anti-tumour mechanism of CAI.

1. Introduction

Carboxyamidotriazole (CAI) is a non-cytotoxic anticancer drug in development. It inhibits proliferation and induces apoptosis of some cancer cells.[1–6] A series of clinical trails have shown that the adverse effects of the drug are slight comparing with other anticancer drugs, although its effect is still in investigation.[7–10] It has been suggested that CAI is a voltage-independent calcium channel inhibitor of cancer cells.[11–13] But the exact mechanism remains unclear.

In our previous study, we found that CAI also possessed great anti-inflammation action in a variety of animal models of acute and chronic inflammation. In the adjuvant-induced arthritis (AA) model, the decrease of the pro-inflammatory cytokines at the site of inflammation and in serums by CAI was observed.[14] Macrophages are thought to be responsible for pro-inflammatory cytokines production after activated by the microbial products and inflammatory mediators in the inflammation responses. Thus, in the present study, we investigated the effect of CAI on tumour necrosis factor-α

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(TNF-α) production in peritoneal macrophages isolated from AA rats, and the inhibition was observed.

As CAI is also an anti-cancer drug, we hypothesised that it may also have effects on the pro-inflammatory cytokines production in tumour associated macrophages (TAMs), and this effect may be one of the mechanisms for the anti-cancer actions of CAI. TAM is an important component in tumour microenvironment and usually related to the poor prognosis of many types of cancer.[15,16] Although TAMs are not activated as strongly as macrophages in the inflammatory models, when stimulated with the substances produced by tumour cells and so on, low chronic levels of pro-inflammatory cytokines can also be released from TAMs. Then the pro-inflammatory cytokines can promote tumour growth and form a positive feedback. For example, TNF-α and its downstream pathways in tumour cells, such as NF-κB, can promote tumour proliferation, migration and invasion.[17]

Based on the hypothesis above, we analysed the effect of CAI on TNF-α production in macrophages isolated from Lewis lung carcinoma (LLC) transplanted tumours. Besides, we investigated the effect of CAI on TNF-α production in macrophages co-cultured with LLC cells or LLC cells conditioned medium and observed the effects of macrophages (treated or untreated with CAI) on the proliferation and invasion of LLC cells.

Dexamethasone (DEX) can regulate the expression of inflammatory cytokines effectively and has been widely used in cancer therapies.[18–20] In the previous studies, we found DEX could greatly enhance the anti-inflammation actions of CAI. Besides, it has been reported that glucocorticoids can suppress angiogenesis via inhibiting angiogenic factors, which include many pro-inflammatory cytokines.[21–23] Thus, we investigated whether the combination of DEX and CAI could inhibit TNF-α production and tumour growth more effectively *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

CAI was synthesised by the Institute of Materia Medica, Chinese Academy of Medical Sciences. Polyethylene glycol (PEG) 400 was provided by Beijing Chemical Reagents Company (Beijing, China). DEX sodium phosphate injection was from Tianjin Jinyao Amino Acid Co., Ltd. (Tianjin, China).

2.2. Animals and cells

Male Wistar rats (180–200 g) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Male C57BL/6 mice (18–22 g) were obtained from the Institute of Laboratory Animal sciences, CAMS&PUMC. They were housed in an air-conditioned room (22 ± 2 °C and 40–70% humidity), with a controlled 12-h light/dark cycle (lights on 8:00 AM). Animals had free access to standard chow and water. All animal studies and procedures were approved by the Institutional Animal Care and Use Committee of Peking Union Medical College.

Lewis Lung Carcinoma (LLC) cells were obtained from Shanghai Institute for Biological Sciences, CAS. RAW264.7 was a gift from Professor Chengyu Jiang (Institute of basic medical sciences, CAMS&PUMC). The peritoneal macrophages were isolated from the peritoneal cavity of male Wistar rats in AA model or normal male C57BL/6 mice (18–22 g). All of the cells above grew in DMEM supplemented with 10% FBS, 50 mg/mL penicillin, 100 mg/mL streptomycin, and L-glutamine at 37 °C in a humidified 95% O_2–5% CO_2 atmosphere.

2.3. Adjuvant-induced arthritis model

Male Wistar rats were randomized into five groups, 15 animals per group. On day 0, animals were induced adjuvant arthritis by a single intradermal injection of Freund's complete adjuvant (100 μL) into the plantar region of the right hind paw, except group 5. For group 1, 2, 3 and 4, N.S. (i.p.), Dex (0.2 mg/kg, i.p.), PEG400 (i.g.) and CAI (20 mg/kg, i.g.) were administered daily from days 0 to 21, respectively.

2.4. Peritoneal macrophages isolation and induction

For the preparation of macrophage monolayer, Wistar rats were sacrificed by cervical dislocation, after which each peritoneal cavity was washed twice with 5 mL PBS perfusion liquid. Peritoneal exudates was harvested and centrifuged at 1000 rpm for 5 min. The pellet of peritoneal cells was re-suspended in complete DMEM and the cells were plated in a 24-well plate and allowed to attach for 2 h, 1×10^6 cells/well. By 2 h, the non-adherent cells were removed and the medium was replaced by the fresh DMED containing 1 μg/mL LPS (Sigma). After 18 h of stimulation, the supernatant was collected for ELISA test described below.

Six percent starch broth was injected into the peritoneal cavity of normal C57BL/6 mice for three days, 1 mL/day/animal. The mice were sacrificed by cervical dislocation on the fourth day. The 2–3 mL PBS perfusion liquid was aspirated out of the cavity. The cells were centrifuged at 1000 rpm for 5 min. The pellet of peritoneal cells was re-suspended in complete DMEM and the cells were plated in a 24-well plate and allowed to attach for 2 h, 1×10^6 cells/well. By 2 h, the non-adherent cells were removed and the medium was replaced by the fresh DMEM containing 50% LLC cells conditioned medium (the LLC cells were plated on a middle-size dish, 1×10^5/mL, 6 mL/dish. By 48 h, the cell-free supernatant was collected as conditioned medium) for 24 h.

2.5. LLC tumour model

LLC cells collected in log phase of growth (1×10^7/mL, re-suspended with PBS) was injected into subcutaneous tissue of the right axillary fossa, 0.1 mL/animal (day 0). The animals were randomly divided into four groups, with 25 animals in each group. Vehicle (PEG400), CAI (20 mg/kg), DEX (1 mg/kg), or combination of CAI and DEX (COM) were administered (i.g. daily for PEG400 and CAI; s.c. twice a week for DEX). The body weight was recorded every 3 days. The length and width were measured with a sliding caliper every three days from days 7 to 13. Tumour volumes (in mm^3) were calculated as length × width^2 × 0.5. Fifteen animals in each group were sacrificed on day 14. The tumour tissues

were isolated and each weight was recorded. The tumour tissues in each group were then immediately dissociated for TAM isolation or stored at −80 °C for preparing tumour homogenates. Continue administering the drugs to the rest animals (10 in each group) until death for survival time recording.

2.6. Tumour tissue macrophages isolation and identification

The tumour tissue macrophages were isolated according to the previous method.[24] For identification, the cells were plated on a cover slip placed in a dish after being re-suspended in complete Hank's balanced salt solution (HBSS). The rest adherent cells were incubated with anti-mouse F4/80 (AbD Serotec, CI: A3-1, 1:50) overnight at 4 °C and stained with the second antibody (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) at room temperature for 2 h. The sections were then covered with DAPI mounting agent and observed with the confocal (TCM-SP2, Leica Instruments, Ltd.) away from the light. Pictures were captured with Volocity software version 5.3.0.

2.7. Tumour tissue homogenates preparation

The tumour tissues, free of blood, fat and connective tissues were put into a homogenizer with PBS (1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) and homogenated every 5 min on ice. By 30 min, the tissue homogenates were centrifuged at 10,000*g* for 10 min. The supernatants were centrifuged at 150,000*g* for 45 min, followed by 40,000*g*, 50 min. All operations were carried out at 4 °C. The supernatants were stored at −80 °C until further analysis. The total protein was measured using Bradford method.

2.8. LLC proliferation test in vitro

The LLC cells collected in log phase of growth were plated in a 96-well plate and allowed to attach overnight, 5×10^3 cells/well. In a half of the wells, the medium was replaced with fresh complete medium containing desired concentration of CAI (5, 10, 20, 40 μM), DEX (0.01, 0.1, 1, 10 μM), COM1 (CAI10 μM + DEX10 μM), or COM2 (CAI20 μM + DEX10 μM). The control group was added with DMSO, three wells per group. The medium in other wells was replaced according to the same protocol on the second day. The plate was incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. On the third day, the LLC cells proliferation was determined with CCK-8 agent (Dojindo Molecular Technologies, Inc.). The absorbance in each well was read at 490 nm.

In the co-culture test, the transwell inserts (0.4 μm) (Millipore Corporation) attached with LLC cells overnight (5×10^4, 1×10^5 and 2×10^5/well, four wells per group) were placed into the 24-well plate with 1300 μL fresh DMEM in each well containing LLC cells conditioned peritoneal macrophages. Another 200 μL fresh DMEM was then added into each insert. By 48 h, the LLC cells proliferation was determined with CCK-8 agent (Dojindo Molecular Technologies, Inc.). The absorbance in each well was read at 490 nm.

In another experiment, CAI (30 μM), DEX (10 μM) or the combination was added in the co-culture system (5×10^4 LLC cells/well). Groups of LLC cells cultured alone were set as controls.

2.9. LLC invasion test in vitro

The RAW264.7 cells collected in log phase of growth were plated in a middle-size dish, 2.5×10^5 cells/dish. Fresh DMEM containing 50% LLC cells conditioned medium and CAI (30 μM), DEX (10 μM) or the combination was added to each dish. By 24 h, the RAW264.7 cells were harvested and re-suspended with fresh DMEM. The RAW264.7 cells of each group were plated in a 24-well plate with 6×10^5 and 1300 μL medium per well. The transwell inserts (8 μm) (Millipore Corporation, the membranes were pre-treated with 0.1% gelatin) were placed into the 24-well plate for several minutes. Two hundred microlitres of LLC cells (4×10^4) suspension was then added into each insert. By 24 h, the inner surface of the insert's membrane was wiped with a cotton bud in order to remove the LLC cells that had not invaded, and then put into a new 24-well plate with 450 μL crystal violet each at 37 °C. By 30 min, the excess crystal violet was removed by washing with PBS. The stained LLC cells were then observed with the microscope (DMI4000B, Leica Instruments, Ltd.) after the membrane was air dried. Pictures were captured with the software of Leica Qwin V3. The inserts were put into a new 24-well plate with 160 μL destaining solution per well. By shaking 7 min, the solution was aspirated into a 96-well plate, and the absorbance was read at 570 nm.

In another experiment, 0.1 or 0.5 μg/mL TNF-α neutralising antibody (R&D, AF-410-NA) was added into the LLC cells (4×10^3/well) and RAW264.7 cells (pretreated with LLC cells conditioned medium, 6×10^4/well) co-culture system. By 24 h, the invasion of LLC cells was observed as described above and the stained LLC cells of each group were counted in 10 random microscope fields.

2.10. Measurement of TNF-α level

2.10.1. ELISA

Peritoneal macrophages from AA rats were stimulated as described above. Peritoneal macrophages (normal C57BL/6 mice) in co-culture system containing 30 μM CAI or 10 μM DEX or the combination were separated from LLC cells and stimulated with 1 μg/mL LPS for 18 h. RAW264.7 macrophages pretreated with 50% LLC cells conditioned medium (containing 30 μM CAI or 10 μM DEX or the combination) were plated in a 24-well plate with 6×10^5 and stimulated with 1 μg/mL LPS (Sigma) for 18 h. The tumour tissue homogenates, the supernatants of peritoneal macrophages and RAW264.7 macrophages were tested for TNF-α concentration with specific rat and mouse ELISA kits (R&D) according to the manufacturer's instructions. The absorbance was read at 450 nm (Synogen 4, Gene Company, Ltd.).

2.10.2. Realtime RT-PCR

The RAW264.7 cells collected in log phase of growth were plated in a middle-size dish, 2.5×10^5 cells/dish. The medium was replaced by fresh DMEM containing 50% LLC cells

conditioned medium and CAI (30 μM), DEX (10 μM) or the combination on the next day. By 24 h, the RAW264.7 cells were harvested and the total mRNA was extracted with a kit (Bioteke Corporation). The total mRNA of the tumour tissue macrophages mentioned above was also extracted.

Reverse transcription was performed from 2 μg of total RNA using oligo-dT primers and M-MLV reverse transcriptase (Promega Corporation) according to the manufacturer's instructions. Primers for TNF-α and beta-actin were designed as followed: TNF-Forward primer:5-GTCTACTGAACTTCGG GGTGAT-3; TNF-Reverse Primer:5-CACTTGGTGGTTTGCTAC GAC-3. Beta-actin-Forward primer:5-CCTGAGGCTCTTTTCCA GCC-3; Beta-actin-Reverse Primer:5-TAGAGGTCTTTACGGAT GTCAACGT-3. They were synthesised by Shanghai GeneCore BioTechnologies Co., Ltd. Quantitative PCRs were performed on IQ5 (BIO-RAD). The amount of generated DNA was measured by fluorescence detection of the ds-specific DNA-binding dye SYBR Green I (Bioeasy SYBR Green I Realtime PCR Kit). Experiments were performed at least in triplicate.

2.11. Statistical analysis

All data were reported as mean ± SD except where indicated. Comparisons among multiple groups were subjected to a one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference post hoc test. The survival analyses were performed using the Kaplan–Meier survival curve and the log-rank test. The 95% confidence limits ($P < 0.05$) were considered significant.

3. Results

3.1. CAI suppressed TNF-α production in peritoneal macrophages isolated from rats of adjuvant-induced arthritis

In AA model, macrophages are important components and TNF-α is one of the crucial mediators. The adjuvant could induce inflammation systematically and peritoneal macrophages could be affected.[25] In the light of previous discovery of evident repression of TNF-α in paw homogenate following CAI treatment,[14] we checked TNF-α in AA rat peritoneal macrophage culture supernatant using ELISA. CAI and Dex significantly inhibited the TNF-α production in those macrophages (Fig. 1). This result inspired us to investigate the action of CAI on macrophages, especially the TAMs.

3.2. CAI decreased TNF-α in tumour tissues and suppressed the expression of mRNA for TNF-α in tumour tissue macrophages

LLC cells injected into C57BL/6 mice formed tumour tissues in 3–5 days. Two weeks after the inoculation, we prepared the tumour tissue homogenates and found combination treatment of CAI and DEX could significantly decrease the TNF-α concentration (Fig. 2A). As TNF-α could be expressed by both tumour cells and stroma cells like macrophages in tumour microenvironment,[26] we isolated and identified LLC tumour tissue macrophages (the purity is approximately 80% by staining F4/80, Fig. 2B) to study whether CAI or DEX could down-regulate the TNF-α expression in these cells. The result of realtime RT-PCR showed that, compared with PEG group, mRNA for TNF-α in CAI, DEX and combination group was decreased by 75%, 88% and 91%, respectively (Fig. 2C).

3.3. CAI inhibited TNF-α production in macrophages induced by LLC cells conditioned medium

In vitro, the macrophages can be induced to different phenotype by various inducers. The tumour cells may switch macrophages to a phenotype similar to those found in tumour tissues.[27] Peritoneal macrophages and RAW264.7 cells are often used for TAM study.[28–30] In our study, the result of realtime RT-PCR showed that TNF-α mRNA level in RAW264.7 cells induced by LLC cells conditioned medium and treated with CAI, DEX or the combination were much lower compared with the vehicle (Fig. 3A). These results indicated that CAI or DEX could inhibit the TNF-α expression in macrophages induced by tumour environment *in vitro*. The combination of CAI and DEX could significantly decrease the TNF-α level compared with CAI alone.

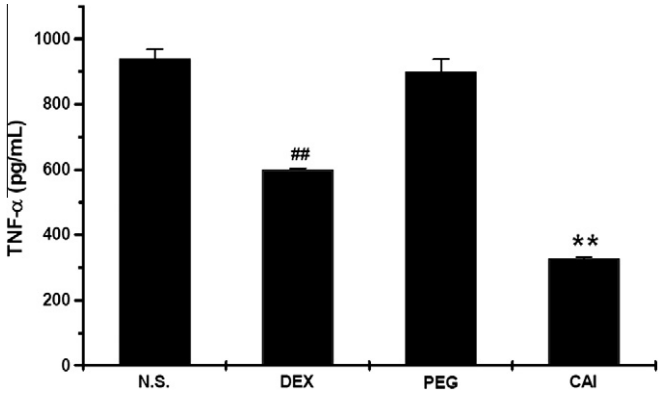

Fig. 1 – CAI significantly decreased TNF-α level in AA rat peritoneal macrophage culture supernatant. Peritoneal macrophages were collected, incubated for adherence, and activated by addition of LPS-containing DMEM media (1 μg/mL). The culture supernatant was analysed for TNF-α. The values represent mean ± SD ($n = 3$). $^{**}p < 0.001$, significantly different from PEG400 group. $^{\#\#}p < 0.01$, significantly different from saline group.

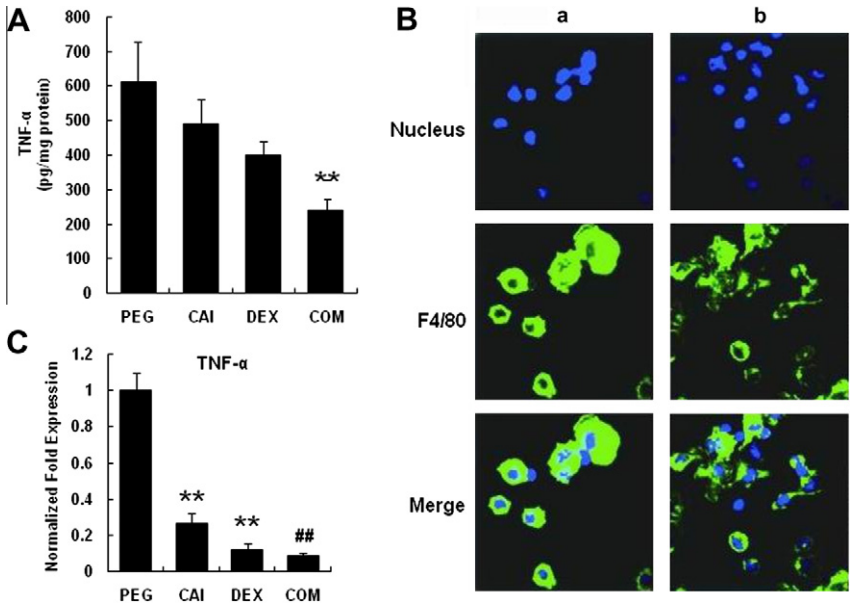

Fig. 2 – CAI significantly decreased mRNA for TNF-α in tumour tissue macrophages and TNF-α concentration in tumour tissue. (A) TNF-α concentration in tumour tissue homogenates. The values represent mean ± SD (n = 3). (B) Two fields (a and b) of F4/80 and DAPI staining of tumour tissue macrophages (60×). (C) TNF-α mRNA in tumour tissue macrophages. The values represent the normalised fold expression, mean ± SD (n = 3). PEG, vehicle control group; COM, combination treatment of CAI and DEX. $^{**}p < 0.01$, significantly different from PEG. $^{\#\#}p < 0.01$, significantly different from CAI.

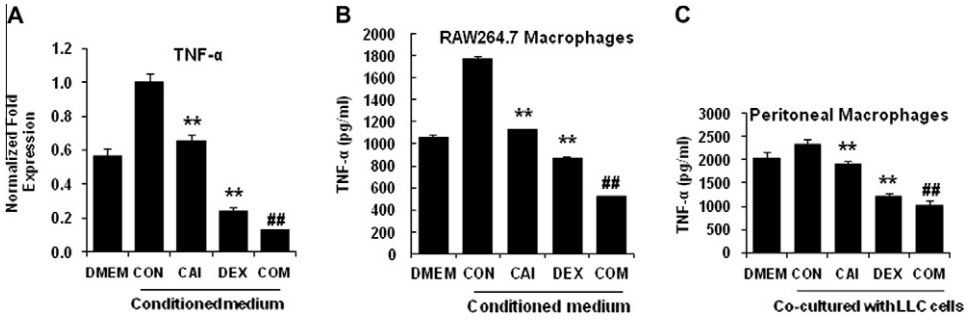

Fig. 3 – CAI significantly inhibited TNF-α production in macrophages induced by LLC or LLC conditioned medium. (A) The mRNA of TNF-α in RAW264.7 macrophages induced by LLC conditioned medium was inhibited by CAI, DEX and the combination. The values represent the normalised fold expression, mean ± SD (n = 3). (B and C) The concentrations of TNF-α in RAW264.7 macrophages (B) and peritoneal macrophages (C) induced by LLC or LLC cells conditioned medium were down-regulated by CAI, DEX and the combination. The values represent TNF-α concentration, mean ± SD (n = 3). DMEM, with fresh DMEM medium; CON, vehicle control (with LLC conditioned medium); COM, combination of CAI and DEX. $^{**}p < 0.01$, significantly different from CON; $^{\#\#}p < 0.01$, significantly different from CAI.

Both CAI and DEX could decrease the level of TNF-α secreted from macrophages (RAW264.7 macrophages induced by LLC cells conditioned medium or peritoneal macrophages co-cultured with LLC cells, stimulated by LPS for 18 h). Combination treatment could decrease TNF-α production to a lower level (Fig. 3B and C).

3.4. *Proliferation of LLC cells were promoted by peritoneal macrophages; DEX enhanced the inhibition of CAI on the proliferation of LLC cells co-cultured with peritoneal macrophages instead of LLC cells cultured alone*

It is believed that TAMs and TNF-α can promote tumour cell proliferation and invasion.[15,31] As CAI could inhibit TNF-α production in TAMs and the activity could be enhanced by DEX, we next investigated whether CAI could influence tumour cell proliferation and invasion by targeting TAMs and whether the activity could also be enhanced by DEX.

The results showed that the proliferation of LLC cells could be significantly enhanced by peritoneal macrophages. As shown in Fig. 4A, the action of macrophages in the left group (LLC cells 5×10^4/well, peritoneal macrophages 1×10^6/well, L:P = 1:20) was the most significant, indicating that peritoneal macrophages could enhance LLC cells proliferation and the action was positively related with the proportion of macrophages. In this co-culture system, DEX gained the ability to inhibit LLC cells proliferation. The LLC cells proliferation

was pulled to the baseline (cultured alone) while treating macrophages with CAI and DEX (Fig. 4B).

3.5. RAW264.7 cells promoted LLC cells invasion and the activity was inhibited if RAW264.7 cells were pre-treated with CAI

In the invasion test, we observed the stained LLC cells with a microscope after the membrane was air dried. The invasion of LLC cells was significantly enhanced by RAW264.7 cells induced by LLC cells conditioned medium for 24 h. RAW264.7 cells induced by LLC cells conditioned medium added with CAI and/or DEX promoted less LLC cells to invade (Fig. 5A). The results were quantified by dissolving the crystal violet and measuring the absorbance at 570 nm. RAW264.7 macrophages pretreated with the combination of CAI and DEX could hardly promote LLC cells to invade (Fig. 5B).

We also tested the proliferation of LLC cells co-cultured with RAW264.7 macrophages for 24 h without wiping the inner surface of the trans-well inserts. The LLC proliferation was not affected by RAW264.7 macrophages (treated or not treated with CAI).

3.6. TNF-α was crucial in the invasion of LLC cells

We co-cultured LLC cells and RAW264.7 cells (induced by LLC cells conditioned medium for 24 h), with 0.1 or 0.5 μg/mL TNF-α neutralising antibody in the co-culture system. By 24 h, the migrated LLC cells were stained with crystal violet and observed after air dried. The LLC cells invasion promoted by RAW264.7 cells was inhibited by TNF-α neutralising antibody (Fig. 6A). The LLC cell numbers per field were recorded, 10 fields/group. The LLC cell numbers of those cultured alone, co-cultured with RAW264.7 cells, added with 0.1 μg/mL antibody and 0.5 μg/mL antibody groups were 33 ± 8, 105 ± 15, 49 ± 10 and 38 ± 10, respectively (Fig. 6B). This result showed that TNF-α played an important role in the invasion process of LLC cells.

3.7. DEX did not affect the direct inhibition of CAI on the proliferation of LLC cells cultured alone

The results above showed that CAI could act on TAMs and thus inhibit tumour indirectly. The activity could be augmented by DEX. Then we tested the direct inhibition on LLC cells of CAI. The LLC cells were plated in a 96-well plate and allowed to attach overnight were treated with various concentrations of CAI, DEX or both for 24 and 48 h. CCK-8 tests showed that CAI inhibited LLC cells proliferation in a dose and time dependent manner, consistent with the known anti-cancer activity of CAI. Interestingly, in this culturing system in which LLC cells were cultured alone, DEX could hardly affect the proliferation of LLC cells, and the addition of DEX could not enhance the inhibition of CAI (Fig. 7), suggesting that the anti-cancer enhancement by DEX observed above was through regulating the TAMs.

3.8. Combination treatment of CAI and DEX significantly inhibited the LLC tumour growth in mice

As we found DEX could enhance the activity of CAI in cell co-culture systems *in vitro*, we then treated the LLC bearing C57BL/6 mice with the combination of CAI and DEX. By calculating the tumour volume, we found that CAI or DEX could inhibit the tumour growth and the tumour in COM group grew slowest. The tumour volumes in COM group calculated on days 7, 9, 11 and 13 were all significantly different from the PEG group by statistic analysis ($p < 0.01$) (Fig. 8A). Part of the mice was sacrificed on day 14, and the tumour weight was recorded. The tumour weight in the COM group recorded on day 14 was significantly different from the PEG group by statistic analysis ($p < 0.01$) (Fig. 8B). Besides, combination treatment significantly prolonged the survival time by Kaplan–Meier analysis ($p < 0.05$) (Fig. 8C). We recorded the body weights once every 3 days from days 0 to 27. The data became inaccurate after 30 days because death occurred and the tumour weight's proportion

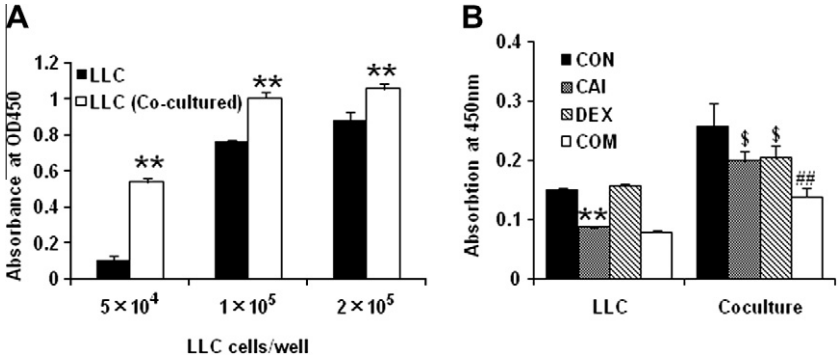

Fig. 4 – DEX enhanced the activity of CAI on the proliferation of LLC cells co-cultured with peritoneal macrophages. (A) LLC cells proliferation in co-culture systems (5×10^4, 1×10^5 and 2×10^5 LLC cells/well and 1×10^6 peritoneal macrophages/well) was promoted by macrophages. The values represent absorbance at 450 nm, mean ± SD ($n = 3$). $^{**}p < 0.01$, significantly different from LLC cells cultured alone. (B) DEX enhanced the activity of CAI on the proliferation of LLC cells only when the peritoneal macrophages were present. The values represent absorbance at 450 nm, mean ± SD ($n = 4$). CON, vehicle control; COM, combination of CAI and DEX. $^{**}p < 0.01$, significantly different from CON (LLC cells cultured alone). $^{\$}p < 0.05$, significantly different from CON (LLC cells co-cultured with macrophages); $^{\#\#}p < 0.01$, significantly different from CAI (LLC cells co-cultured with macrophages).

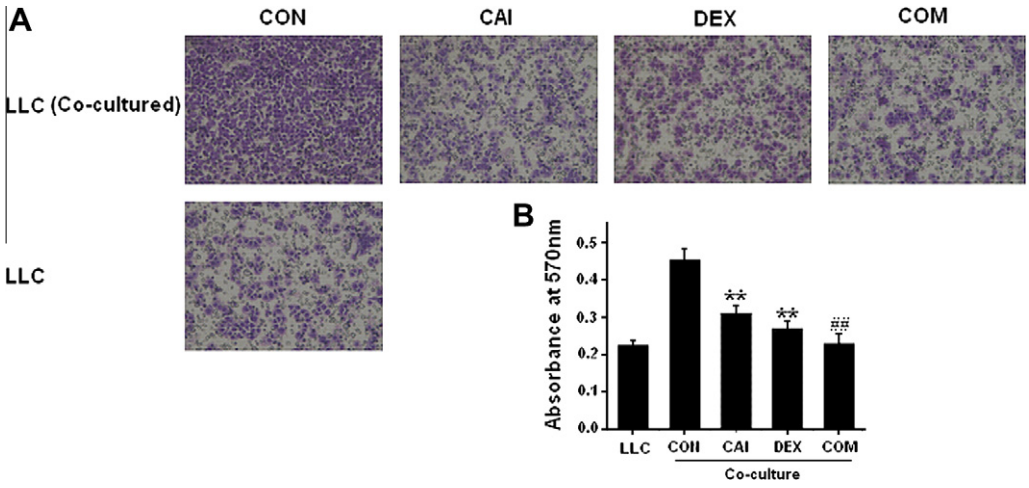

Fig. 5 – CAI significantly inhibited invasion of LLC cells co-cultured with RAW264.7 cells *in vitro*. (A) In the invasion experiment, the stained LLC cells (violet) were observed with a microscope after the membrane was air dried (10×). (B) Invasion of LLC cells co-cultured with RAW264.7 macrophages compared with LLC cultured alone were quantified by dissolving the crystal violet and measuring the absorbance at 570 nm. The values represent absorbance at 570 nm, mean ± SD ($n = 4$). CON, vehicle control; COM, combination of CAI and DEX. $^{**}p < 0.01$, significantly different from CON. $^{\#}p < 0.05$, significantly different from CAI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

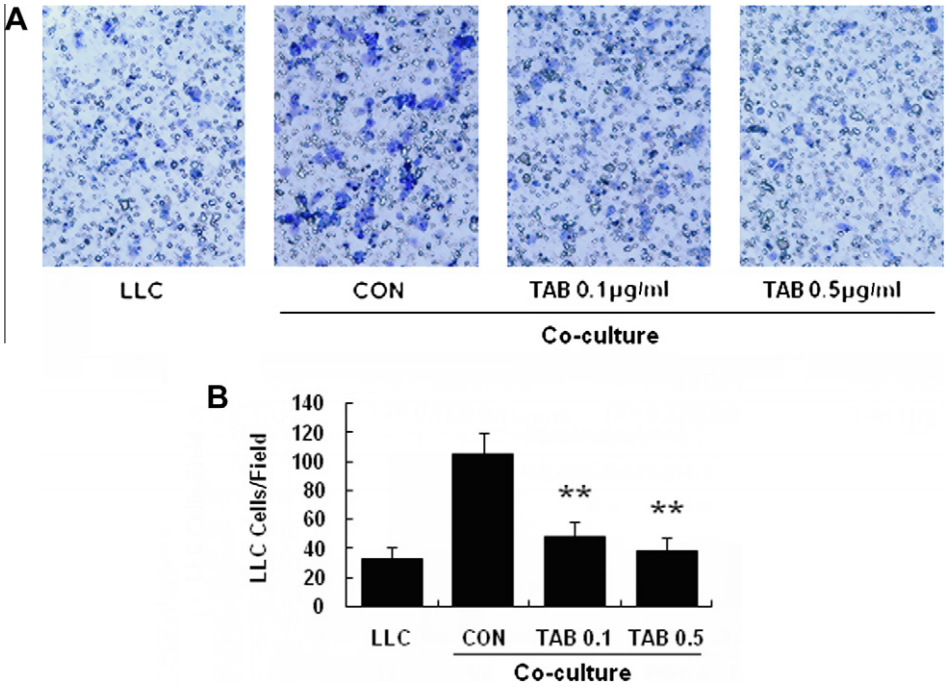

Fig. 6 – TNF-α neutralising antibody inhibited the LLC cells invasion promoted by RAW264.7 cells. (A) In the invasion experiment, the stained LLC cells (violet) were observed with a microscope after the membrane was air dried (10×). (B) Invasion of LLC cells co-cultured with RAW264.7 macrophages compared with LLC cultured alone were quantified by dissolving the crystal violet and measuring the absorbance at 570 nm. The values represent absorbance at 570 nm, mean ± SD ($n = 4$). CON, vehicle control; TAB, TNF-α neutralising antibody. $^{**}p < 0.01$, significantly different from CON. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increased. The results showed that there was no significant difference among body weights in five groups (Fig. 8D). The direct inhibition of tumour cells by CAI and the inhibition of pro-inflammatory cytokines in TAMs by both drugs may explain why the combination could significantly delay the tumour growth *in vivo*.

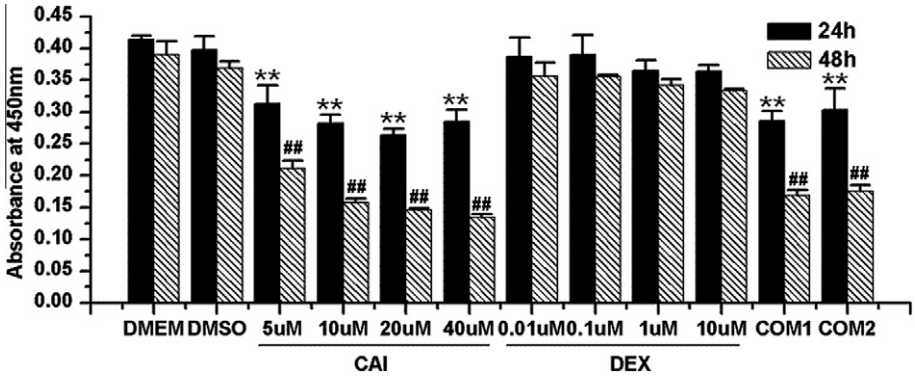

Fig. 7 – CAI directly inhibited LLC cells proliferation, which was not affected by DEX. The values represent absorbance at 450 nm, mean ± SD (n = 3). DMEM, with fresh DMEM medium; DMSO, vehicle control; COM 1, CAI 10 μM + DEX 10 μM; COM 2, CAI 20 μM + DEX 10 μM. $^{**}p < 0.01$, significantly different from DMSO (24 h); $^{\#\#}p < 0.01$, significantly different from DMSO (48 h).

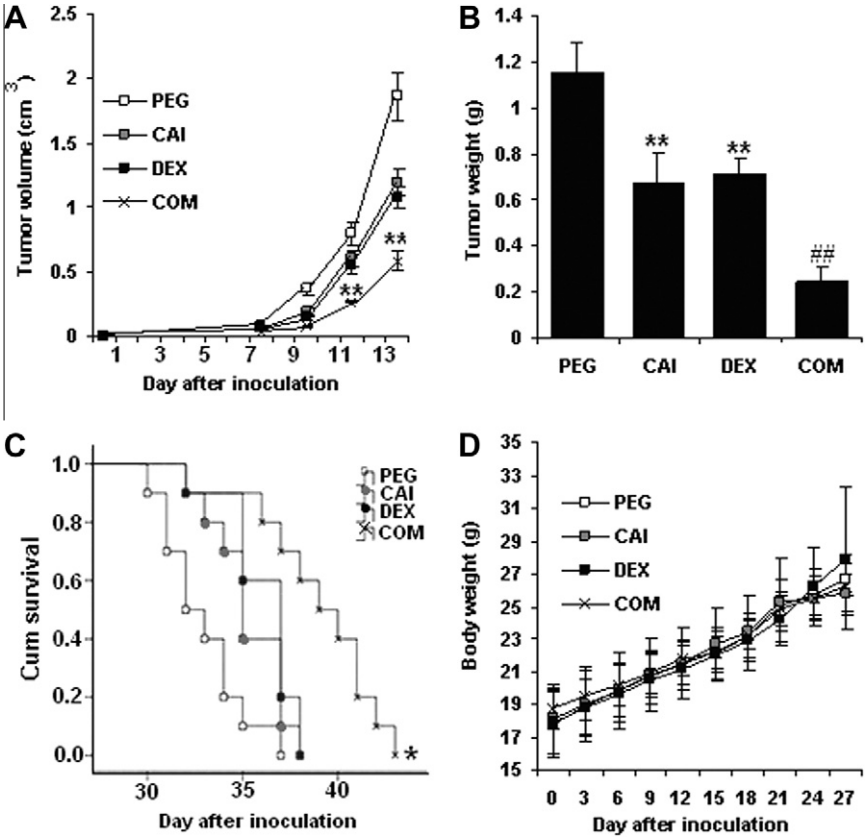

Fig. 8 – CAI significantly inhibited LLC tumour growth. (A) Tumour volumes in LLC model. The values represent mean ± SE (n = 15). (B) Tumour weights in LLC model on day 14. The values represent mean ± SE (n = 15). (C) Kaplan–Meier survival curve in LLC model (n = 10). (D) Body weights in LLC model. The values represent mean ± SD (n = 15). PEG, vehicle control group; COM, combination treatment of CAI and DEX. $^{*}p < 0.05$, significantly different from PEG. $^{**}p < 0.01$, significantly different from PEG. $^{\#\#}p < 0.01$, significantly different from CAI.

4. Discussion

CAI is a potential anti-cancer drug, possessing anti-proliferation, anti-metastasis, anti-angiogenesis and pro-apoptosis activities. It is believed that those activities can be attributed to the inhibition of calcium entry.[11–13] Antoine Enfissi et al. pointed out that capacitative calcium entry was the main pathway involved in regulation of the processes leading to cell proliferation.[32] Mitochondrial inner-membrane potential could be altered by CAI treatment, suggesting that CAI antagonizes mitochondrial calcium import and thus local calcium clearance, which is crucial for the maintenance of capacitative calcium entry.[33]

In our previous studies, we demonstrated that CAI inhibited inflammation processes using a variety of animal models, including the croton oil-induced ear oedema, the

cotton-induced granuloma and the rat adjuvant-induced arthritis. We showed CAI significantly decreased TNF-α level at the site of inflammation and in serums, which might contribute to the anti-inflammatory effect.[14]

Considering CAI has both anti-cancer and anti-inflammation activities, we then focused on its effects on macrophages, which were important both for tumour growth and inflammation processes. Macrophages can be classified as either M1 cells, which are considered to be inflammation promoting and tumoricidal, or M2 cells, which are thought to be anti-inflammatory and tumour promoting.

In the AA model, the adjuvant can induce systematic inflammation and the peritoneal macrophages can be affected.[22] We found that CAI could inhibit TNF-α production in peritoneal macrophages of AA rats. IL-6 production could also be reduced. Thus, M1 macrophage is one of the targets of CAI in this inflammatory disease.

The prevailing cytokine profile in TAMs was found to be similar to that of M2 cells, in which the pro-inflammatory cytokines levels were thought to be decreased. However, a detailed molecular analysis supporting the role of these different macrophage subtypes in tumour promotion and progression is lacking.[34] Although the prevailing cytokine profile is more like M2 phenotype, TAMs can also induce 'smoldering' inflammation in tumours by producing pro-inflammatory cytokines. Manuela Banciu et al. used liposomal clodronate to deplete macrophages in tumour-bearing mice and found i.v. administered liposomal clodronate inhibited tumour growth by approximately 55% compared with control animals.[35] The specific TNF-α or IL-6 antagonists are undergoing clinical trials to improve cancer treatment.[36–38] As thalidomide could inhibit TNF-α expression in several ways, it was also tried to be used in cancer therapy.[39–41] Thus, it is meaningful to inhibit the inflammation by antagonizing or suppressing the pro-inflammatory cytokines production in TAMs.

We isolated macrophages in tumour tissues to analyse the TNF-α mRNA expression. CAI showed greater inhibitory effect on expression of TNF-α mRNA in macrophages than decreasing TNF-α concentration in tissue. It has been reported that TNF-α was expressed and released not only by tumour infiltrated monocytes, but also by tumour cells themselves.[42] In our immunohistochemistry study, we also found that TNF-α could be expressed in tumour cells, which could hardly be affected by carboxyamidotriazole. Thus, we hypothesise that carboxyamidotriazole may inhibit TNF-α expression in macrophages more effectively. *In vitro*, the macrophages can be induced to different phenotype by various inducers. The tumour cells may switch macrophages to a phenotype similar to those found in tumour tissues.[24] In 1986, Plytycz et al. demonstrated that syngeneic as well as semisyngeneic and allogeneic macrophages support the proliferation of the ascite subline of the murine sarcoma MC1M *in vitro*.[25] Consistent with that, we found that the proliferation of LLC cells was promoted by peritoneal macrophages isolated from C57BL/6 mice. Apart from the primary cultured macrophages, the mice macrophage cell line RAW264.7 induced by LLC cells conditioned medium could greatly promote LLC cells invasion. RAW264.7 is a mouse macrophage-like cell line, which is often used for TAM study. Suzuki et al. chose RAW264.7 to study whether dehydroxymethylepoxyquinomicin (DHMEQ) could inhibit tumour growth via inhibition of macrophage activation. Chad E. Green et al. demonstrated that treatment of CT26 cells with RAW 264.7 conditioned medium induces cell migration, invasion and metastasis.[27] TNF-α and IL-6 are considered to be major factors which can promote tumour cells proliferation and invasion.[43–48] The present study shows that CAI can also inhibit TNF-α production in macrophages isolated from LLC transplanted tissues and those co-cultured with LLC cells or LLC cells conditioned medium. We also found that the mRNA expression for IL-6 in tumour induced RAW264.7 macrophages could be inhibited by CAI, indicating the inhibition of CAI on macrophages is not limited to TNF-α. Reviewing the anti-angiogenesis activity of CAI in tumours, although angiogenesis is another downstream effect of calcium influx antagonism,[49] the anti-inflammation activity may provide another explanation, as it is clear that TNF-α and IL-6 are also important pro-angiogenic factors.

In the LLC cells and macrophages co-culture system, DEX could enhance the anti-tumour action of CAI. RAW264.7 macrophages pretreated with CAI plus DEX promoted less LLC cells invasion and expressed less TNF-α than the macrophages pre-treated with CAI alone. Besides, DEX could enhance the anti-tumour action of CAI *in vivo* rather than *in vitro* when LLC cells were cultured alone. These results indicated that DEX enhanced the actions of CAI by regulating TAMs. That the combination of the drugs inhibited the tumour growth most significantly could be attributed to the direct inhibition of tumour cells by CAI and the inhibition of pro-inflammatory cytokines in TAMs by both. Recently, it was found that lenalidomide plus low dose DEX (1.5 mg/kg, 1qw) is highly effective in multiple myeloma xenograft models,[50] and the effect has been tested in an open-label randomized control trial.[51] In several clinical trials, cancers responded poorly to CAI alone or combined with cyto-toxic drugs,[52–54] promoting a need to identify other agents to sensitise tumours to treatment. In this study, we found that low dose DEX could greatly enhance the anti-cancer activity of CAI without steroid related side-effects. This attempt of combination may be developed to a better regimen for CAI.

In summary, we have found that CAI cannot only act on tumour cells directly, but also on macrophages, including those in arthritis model and TAMs. It can suppress TNF-α and IL-6 production, break the 'smoldering' inflammation balance in TAMs, and thus inhibit tumour growth indirectly. The findings suggest that CAI exerts anti-cancer activities may be due to its direct actions on both tumour cells and TAMs. The newly found anti-inflammation activity may make CAI one of effective tools to study the relationship between inflammation and cancer, and the combination with low dose DEX may be of great importance for clinical application of CAI.

Conflict of interest statement

None declared.

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