Elsevier Editorial System(tm) for
Biochemical and Biophysical Research Communications
Manuscript Draft

Manuscript Number:

Title: Inhibition of collagen production delays malignant mesothelioma tumor growth in a murine model

Article Type: Short communication/Full length article

Keywords: malignant mesothelioma; cell proliferation; anti-neoplastic agents; protein synthesis inhibitors; animal models

Corresponding Author: Professor Steven E Mutsaers, PhD

Corresponding Author's Institution: Institute for Respiratory Health

First Author: Steven E Mutsaers, PhD

Order of Authors: Steven E Mutsaers, PhD; Keith S Abayasiriwardana, PhD; Michael K Wood, MBBS PhD; Cecilia M Prele, PhD; Kimberly A Birnie, PhD; Bruce W Robinson, MBBS MD; Geoffrey J Laurent, PhD; Robin J McAnulty, PhD

HIGHLIGHTS

Human and mouse malignant mesothelioma cells synthesize collagen.

Thiaproline inhibits basal and TGF-β-induced mesothelioma cell collagen production.

Thiaproline inhibits mesothelioma tumour growth in a mouse model.

Thiaproline induces apoptosis but not tumour vasculature or inflammatory cell influx.

Collagen inhibitors are a potential therapy for mesothelioma.

INHIBITION OF COLLAGEN PRODUCTION DELAYS MALIGNANT MESOTHELIOMA TUMOR GROWTH IN A MURINE MODEL

Keith S. Abayasiriwardana[1], Michael K. Wood[1], Cecilia M. Prêle[2,3], Kimberly A. Birnie[2], Bruce W. Robinson[4], Geoffrey J. Laurent[2,3], Robin J. McAnulty[1]§, Steven E. Mutsaers[1,2,3]§

[1]Centre for Inflammation and Tissue Repair, Rayne Institute, Department of Medicine, University College London, London, UK;

[2]Institute for Respiratory Health, Centre for Respiratory Health, University of Western Australia, Nedlands, WA, Australia;

[3]Centre for Cell Therapy and Regenerative Medicine, School of Biomedical Sciences, University of Western Australia;

[4]National Centre for Asbestos Related Diseases, Department of Medicine, University of Western Australia, Nedlands, WA, Australia

§ These authors contributed equally to this work

CORRESPONDING AUTHOR

Associate Professor Steven Mutsaers

Institute for Respiratory Health, Harry Perkins Institute of Medical Research, QEII Medical Centre, 6 Verdun Street, Nedlands WA 6009 Australia

TEL: +61 (0)8 6151 0891 FAX: +61 (0)8 6151 1027,

Email: steven.mutsaers@uwa.edu.au

ABSTRACT

Malignant mesothelioma is an aggressive fibrous tumour, predominantly of the pleura, with a very poor prognosis. Cell-matrix interactions are recognized important determinants of tumour growth and invasiveness but the role of the extracellular matrix in mesothelioma is unknown. Mesothelioma cells synthesize collagen as well as transforming growth factor-beta (TGF-β), a key regulator of collagen production. This study examined the effect of inhibiting collagen production on mesothelioma cell proliferation *in vitro* and tumor growth *in vivo*. Collagen production by mesothelioma cells was inhibited by incubating cells *in vitro* with the proline analogue thiaproline (thiazolidine-4-carboxylic acid) or by oral administration of thiaproline in a murine tumor model. Cell cytotoxicity was measured using neutral red uptake and lactate dehydrogenase assays. Proliferation was measured by tritiated thymidine incorporation, and inflammatory cell influx, proliferation, apoptosis and angiogenesis in tumors examined by immunohistochemical labelling. Tumor size was determined by tumor weight and collagen production was measured by HPLC. Thiaproline at non-toxic doses significantly reduced basal and TGF-β-induced collagen production by over 50% and cell proliferation by over 65%. *In vivo* thiaproline administration inhibited tumor growth at 10 days, decreasing the median tumor weight by 80%. The mean concentration of collagen was 50% lower in the thiaproline-treated tumors compared with the controls. There were no significant differences in vasculature or inflammatory cell infiltration but apoptosis was increased in thiaproline treated tumors at day 10. In conclusion, these observations strongly support a role for collagen in mesothelioma growth and establish the potential for inhibitors of collagen synthesis in mesothelioma treatment.

Key words: malignant mesothelioma, cell proliferation, anti-neoplastic agents, protein synthesis inhibitors, animal models

INTRODUCTION

Malignant mesothelioma (MM) is an aggressive tumor occurring primarily in the pleura and usually associated with previous exposure to asbestos [1]. It is projected that in the next 15-20 years MM will kill over a quarter of a million people in Western Europe alone [1, 2] with median survival from diagnosis ranging from 8-14 months. Furthermore, the continued use of asbestos in some geographical regions such as Asia, may lead to a significant increase in MM over the next several decades. MM can be extremely fibrous, particularly in the desmoplastic form of sarcomatoid MM. However, most MM contain fibrous fractions, with an extensive extracellular matrix (ECM), a characteristic which has previously been shown to promote malignant growth and impart resistance to chemotherapy [3-5].

The ECM provides a scaffold for the attachment, migration, growth and differentiation of cells and a reservoir for many growth factors and cytokines [3, 6]. Interactions between cells and ECM are crucial to tumorigenesis and are required for tumor growth beyond 1 to 2 mm in diameter [6]. Enhanced deposition of collagen, the most abundant ECM component, is associated with malignant growth [7-9], and inhibition of collagen production reduces mammary tumor growth [10] and inhibits metastatic spread [11, 12]. In small cell lung cancer (SCLC), SCLC cell adhesion to ECM proteins collagen, fibronectin and laminin increased proliferation and prevented chemotherapy-induced apoptosis promoting tumor spread [5]. In addition, SCLC patients with a disseminated tumor matrix had a significantly shorter survival time than those with localised or no matrix. High levels of collagen and hyaluronan in tumors including breast, lung, ovarian, colorectal and pancreatic also correlate with poor prognosis [4, 13].

Malignant mesothelioma (MM), Extracellular matrix (ECM), Small cell lung cancer (SCLC), Transforming growth factor-beta (TGF-β)

The role of ECM proteins in MM has not been fully elucidated, although MM cells migrate on fibronectin, laminin and collagen via β_1 integrin-mediated mechanisms [14]. In addition, MM cells produce higher levels of hyaluronan contributing to invasion [15, 16], and a greater capacity to produce collagen compared with normal mesothelial cells [17].

Several growth factors and cytokines are associated with MM, with levels in tumors higher than in normal mesothelium. Transforming growth factor-beta (TGF-β), a key regulator of ECM production, is increased in malignant pleural effusions caused by MM [18], and may contribute directly to tumor cell growth and the fibrous nature of this tumor. It has been proposed that inhibiting tumor stroma production or collagen crosslinking, or removing ECM with enzymes may be useful in cancer treatment, depriving tumors of support, survival and growth factors [19-21].

In this study the role of collagen in MM proliferation and tumor growth was examined by inhibiting collagen production using the proline analogue thiaproline. Once incorporated into a growing procollagen polypeptide chain, thiaproline prevents further chain elongation leading to truncated peptides which are degraded [22]. Thiaproline has previously been shown to inhibit collagen accumulation in both the glomerular basement membrane and heart ventricles of diabetic db/db mice with no toxicity at the doses used [23, 24].

METHODS

Cell lines: Characterized human (JU77, LO68, NO36 and ONE58) [25], and murine (AB1, AB22 and AC29) [26] MM cell lines and normal human mesothelial cells (NM20) [27] were maintained in standard culture medium containing Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, Scotland) supplemented with 10% fetal bovine serum (FBS;

Imperial Laboratories, Andover, UK), 4mM L-glutamine (Gibco) and antibiotics (penicillin, 100,000 units/l and gentamycin, 50mg/l; Gibco) and grown in a humidified atmosphere of 10% CO_2 in air at 37^{o}C.

Cytotoxicity assay: Cells were seeded in 96 well microtitre plates (4000 cells/well) in DMEM containing 1% FBS, and incubated for 24 hr (subconfluent assays) or until confluent (3 days). The medium was replaced with DMEM containing 1% FBS and thiaproline (0–50mM) (Sigma, Poole, UK) for 24 hr, the medium removed and the wells washed with PBS. The effect of thiaproline on cell viability was assessed by the uptake of neutral red dye (Sigma) [28] and a lactate dehydrogenase release assay: CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, USA).

Assessment of cell proliferation: Cells were seeded in 96 well collagen coated or uncoated microtitre plates (4000 cells/well) in DMEM containing 1% FBS for 24 hr then replaced with 100 μl DMEM containing 1% FBS, 74 KBq/ml [methyl-^{3}H]-thymidine (^{3}H-TdR, Amersham, Buckinghamshire, UK) and 0-50 mM thiaproline. After 24 hr the cells were lysed and incorporation of ^{3}H-TdR into DNA was measured as an index of cell proliferation [29].

Determination of collagen production and non-collagen protein synthesis: Cells were grown to confluence in 2.4 cm diameter wells in standard culture medium then pre-incubated for 24 h in DMEM containing 1% FBS, ascorbic acid (50 μg/ml) and proline (0.2 mM), followed by a further 24 hr in fresh pre-incubation medium containing 74 KBq L-[4-^{3}H]phenylalanine (Amersham), TGF-β_1 (0–10 ng/ml) and/or thiaproline (0–10 mM). Identical cultures lacking the isotope were treated in parallel to determine cell number. Following incubation the cell layer was scraped into the medium and hydroxyproline (hyp) was

measured by HPLC as an index of collagen synthesis [30], and incorporation of ^{3}H-phenylalanine measured on a liquid scintillation counter as an index of non-collagen protein synthesis [31]. To assess the collagen content of tumor samples, frozen powdered tumors were hydrolysed and hydroxyproline measured by HPLC [32].

Animal model of MM: All animal studies received Home Office, UK, ethics approval in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. Tumor growth studies were performed in female 8 week-old CBA mice (Harlan, Oxon, UK) using an established murine model of MM [26]. Briefly, mice were anaesthetised and injected subcutaneously above the thigh in both sides with 10^{6} syngeneic AC29 cells in 100 μl of serum free DMEM. From day 1, one group of injected animals received thiaproline in their drinking water at an approximate dose of 100 mg/kg/day. The control injected group received normal drinking water. At 10 and 18 days after injection of tumor cells, animals were euthanized and the tumors removed, weighed and snap frozen for subsequent collagen analysis.

Assessment of tumor cellularity, vascularity, proliferation and apoptosis: Paraffin sections (5μm) of mouse tumor were rehydrated and immunolabelled by standard biotin-streptavidin immunohistochemistry with diaminobenzidine as a substrate [33] using the following antibodies: 1/200 dilution B220 (B cells; BD Pharminigen, San Diego, USA), 1/200 CD3 (T cells, Serotec, Biorad, Raleigh, USA), 1/200 F/480 (macrophages, Abcam, Cambridge, UK), 1/200 podocalyxin (endothelial cells, R&D Systems, Minneapolis, USA), 1/200 Ki67 (proliferation), 1/200 caspase 3 (apoptosis, Abcam). For controls, sections were incubated either with normal goat serum or species and isotype specific immunoglobulin instead of primary antisera.

Statistical analysis: All cell data were expressed as mean ± standard error of the mean (SEM) for 6 replicates and each experiment repeated at least three times. For single group comparisons statistical analysis was performed using an unpaired Student's t-test. Multiple group comparisons were performed using ANOVA followed by an ad hoc unpaired Student's t-test. *In vivo* tumor data were expressed as medians and range. Statistical analysis comparing the control group to the thiaproline treated group was performed using a Mann-Whitney U test. A P value less than 0.05 was considered statistically significant.

RESULTS

MM collagen production: The murine MM cell line AC29 produced high basal levels of collagen which was further stimulated by TGF-β_1, plateauing at 1 ng/ml TGF-β_1 with values approximately 2.5-fold above medium control (Table 1). Similar results were obtained with all MM cell lines tested (Table 1). The murine cell lines produced significantly higher basal levels of collagen than the human cell lines, the lowest murine cell line (AB1) producing 2.5-fold more collagen than the highest human cell line (JU77). TGF-β_1 treatment increased collagen production in all MM cell lines, the largest increase observed in JU77 at 10 ng/ml TGF-β_1 (approximate 540% increase compared with control). The human normal mesothelial cell line, NM20, exhibited a lower basal collagen production (0.10 ± 0.01nmol hyp/10^6 cells/24 hr) which increased approximately 2-fold following TGF-β_1 (1 ng/ml) treatment (0.28 ± 0.01 nmol hyp/10^6 cells/24 hr). Due to consistent growth characteristics *in vivo*, the cell line AC29 was chosen for subsequent experiments.

Analysis of thiaproline cytotoxicity *in vitro*: The cytotoxic effect of thiaproline on subconfluent and confluent cells was determined by measuring neutral red uptake to identify the range of non-toxic doses for *in vitro* cell proliferation and collagen production assays. Concentrations of thiaproline greater than 10 mM induced cell death in subconfluent cells, whereas confluent cells (as used in collagen production assays) were more resistant to thiaproline, with cell death at concentrations of thiaproline 40 mM and above (Fig. 1).

Effect of thiaproline on collagen and non-collagen protein production: The effect of non-toxic doses of thiaproline on basal and TGF-β_1-induced collagen production was measured in AC29 cells (Fig. 2a). TGF-β_1 increased collagen production by approximately 150% compared with control. Increasing concentrations of thiaproline decreased both basal and TGF-β_1-induced collagen production in a dose dependent manner. At 10 mM, thiaproline reduced basal and TGF-β_1 stimulated collagen production by approximately 50% and 65% respectively.

Collagen and non-collagen protein production were assessed in AC29 cells to evaluate the specificity of inhibiting collagen production with thiaproline (Fig. 2b). At 1 mM, thiaproline inhibited collagen and non-collagen protein production equally by approximately 20% compared with medium control. At 10 mM, thiaproline inhibited collagen production by approximately a further 30% with no additional inhibition of non-collagen protein.

Effect of thiaproline on *in vitro* cell proliferation and tumor growth *in vivo*:

Increasing concentrations of thiaproline caused a dose dependent reduction in ^{3}H-TdR labelling in AC29 cells (Fig. 3a). Concentrations of 1, 5 and 10 mM thiaproline resulted in

decreases of approximately 20, 35 and 75% respectively. There was a clear linear correlation between thiaproline concentration and cell proliferation (Fig. 3b).

The effect of thiaproline on the development of MM in mice is shown in Fig. 4. The tumor growth pattern was characterized by a lag phase of 5–7 days, followed by rapid growth to macroscopic tumors. At 10 days the thiaproline treated group had a median tumor weight of 10.5 mg (range 5–12 mg) compared with 58 mg for controls (range 30–105 mg) (Fig. 4a), which represented a reduction in median tumor weight of over 80% with thiaproline treatment. However, by 18 days the tumor weight in control and thiaproline treated animals were similar (control; 106 mg, range 27–326 mg; thiaproline; 106 mg, range 46-187 mg) (Fig. 4b).

Thiaproline treated tumors contained a lower concentration of collagen per mg tumor weight than the control tumors at 10 days (Fig. 4c) but there was no difference compared with control at 18 days (Fig. 4d). A highly significant correlation (r^2=0.97, P<0.01) was observed between collagen content and tumor weight (Fig. 4e).

Effect of thiaproline on tumor cellularity, vascularity, cell proliferation and apoptosis:

Thiaproline reduced tumor cellularity at 10 days after treatment as evidenced by reduced tumor size. However there was no obvious differences in vascularity, inflammatory cell infiltrate or tumor cell proliferation (data not shown) but there was more apoptosis in thiaproline treated tumors at day 10 (Fig. 4F).

DISCUSSION

The ECM promotes tumorigenesis, both directly and through the sequestration and release of tumor enhancing growth factors and cytokines. Although host stromal cells produce tumor associated ECM, tumor cells can secrete their own ECM. MM cell lines produce ECM components such as proteoglycans, versican and biglycan [34], hyaluronan [15], laminin, fibronectin and type IV collagen [35].

In this study, we show that murine and human MM cells produce significant amounts of collagen basally and can be further stimulated with TGF-β_1, a potent inducer of collagen production [36]. The rates of collagen synthesis varied between cell lines and between species. In addition, human MM cells showed higher rates of collagen synthesis than normal human mesothelial cells and were equally or more responsive to TGF-β_1. Heterogeneity in MM cell lines has been reported previously in terms of their morphology, growth rates [25] and production of various cytokines such as TGF-β [37, 38] and these may contribute to the differences observed in collagen production.

Incubation of AC29 cells with non-toxic concentrations of thiaproline decreased basal and TGF-β_1-induced collagen production. Measurement of TGF-β-induced non-collagen production demonstrated a selective inhibition of collagen production at concentrations of thiaproline above 1 mM. In addition, thiaproline inhibited MM cell proliferation to a similar extent to that of collagen production implying the importance of cell-matrix interactions in MM cell growth.

Ten day thiaproline treatment at a non-toxic dose [24, 39], significantly reduced median MM tumor weight which correlated with a lower collagen concentration. These data clearly

demonstrate that inhibition of collagen production by thiaproline has a significant effect on tumor growth rate up to 10 days post transplantation.

At 18 days there was no significant difference in tumor weight between the treated and untreated groups. As collagen concentrations were lower in the 10 day thiaproline treated tumours, it is possible that there was insufficient collagen producing cells to sustain a rapid rate of growth and that a critical amount of collagen was required to be produced before the rate of growth increased. This is supported by the observation that a large number of cells in the 10 day treated tumors were undergoing apoptosis compared with control. Administration of higher doses of thiaproline may have had a greater effect in inhibiting tumor growth, however higher doses have been associated with *in vivo* toxicity [40, 41].

We demonstrated biochemically that thiaproline predominantly inhibits collagen rather than non-collagen proteins. However, inhibition of angiogenesis has been reported following thiaproline treatment in the chick chorioallantoic membrane [42], although these could be indirect effects due to inhibition of collagen production. Furthermore, the lymphoproliferative response, lymphocyte motility and the natural killer activity of leukocytes in mice are stimulated by ingested thiaproline [43] and thiaproline may enhance murine macrophage function [39]. Thus effects of thiaproline unrelated to collagen may have contributed to the lower tumor mass seen in the thiaproline treated group. However, histological analysis did not reveal any obvious differences in vasculature or inflammatory cell infiltration (data not shown). Additionally, collagen analysis revealed a lower concentration of collagen per mg of tumor in the thiaproline treated group compared with the control group. This observation suggests that despite other possible effects of thiaproline, decreased collagen production is likely to be a major cause of delayed tumor growth.

This study shows that thiaproline significantly reduces MM cell proliferation *in vitro* and tumor growth in an animal model of MM. Collectively, these data indicate the importance of collagen in MM growth, and suggest that by inhibiting collagen production, cell proliferation and tumor growth can be delayed. Agents capable of inhibiting collagen production may provide an additional approach in the treatment of MM. This data, together with evidence from other studies showing that blocking integrin-mediated cell-matrix interactions increases susceptibility to chemotherapy-induced apoptosis [5], suggests that further research should be carried out to assess multimodal approaches to treatment that may involve tumor debulking surgery, collagen inhibitors and chemotherapy/immunotherapy.

FUNDING

This work was supported by The British Lung Foundation, Cancer Research U.K., The Medical Research Council U.K., The Raine Medical Research Foundation, Western Australia and the Cancer Council Western Australia.

REFERENCES

[1] B.W. Robinson, R.A. Lake, Advances in malignant mesothelioma, N Engl J Med, 353 (2005) 1591-1603.

[2] J. Peto, A. Decarli, C. La Vecchia, F. et al, The European mesothelioma epidemic, Br J Cancer, 79 (1999) 666-672.

[3] N. Boudreau, M.J. Bissell, Extracellular matrix signaling: integration of form and function in normal and malignant cells, Curr Opin Cell Biol, 10 (1998) 640-646.

[4] M. Fang, J. Yuan, C. Peng, et al, Collagen as a double-edged sword in tumor progression, Tumour Biol, 35 (2014) 2871-2882.

[5] T. Sethi, R.C. Rintoul, S.M. Moore, et al, Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo, Nat Med, 5 (1999) 662-668.

[6] S. Crotti, M. Piccoli, F. Rizzolio, et al, Extracellular Matrix and Colorectal Cancer: How Surrounding Microenvironment Affects Cancer Cell Behavior?, J Cell Physiol, (2016).

[7] H.F. Dvorak, Tumors: wounds that do not heal-redux, Cancer Immunol Res, 3 (2015) 1-11.

[8] K. Esbona, D. Inman, S. Saha, et al, COX-2 modulates mammary tumor progression in response to collagen density, Breast Cancer Res, 18 (2016) 35.

[9] S. Kauppila, F. Stenback, J. Risteli, et al, Aberrant type I and type III collagen gene expression in human breast cancer in vivo, J Pathol, 186 (1998) 262-268.

[10] W.M. Lewko, L.A. Liotta, M.S. Wicha, et al, Sensitivity of N-nitrosomethylurea-induced rat mammary tumors to cis-hydroxyproline, an inhibitor of collagen production, Cancer Res, 41 (1981) 2855-2862.

[11] D.M. Gilkes, P. Chaturvedi, S. Bajpai, et al, Collagen prolyl hydroxylases are essential for breast cancer metastasis, Cancer Res, 73 (2013) 3285-3296.

[12] L. Zhang, Y. Wang, T. Xia, et al, Suppression for lung metastasis by depletion of collagen I and lysyl oxidase via losartan assisted with paclitaxel-loaded pH-sensitive liposomes in breast cancer, Drug Deliv, 23 (2016) 2970-2979.

[13] E. Karousou, M.L. D'Angelo, K. Kouvidi, et al, Collagen VI and hyaluronan: the common role in breast cancer, Biomed Res Int, 2014 (2014) 606458.

[14] J. Klominek, S. Sumitran Karuppan, D. Hauzenberger, Differential motile response of human malignant mesothelioma cells to fibronectin, laminin and collagen type IV: the role of beta1 integrins, Int J Cancer, 72 (1997) 1034-1044.

[15] Y. Li, P. Heldin, Hyaluronan production increases the malignant properties of mesothelioma cells, Br J Cancer, 85 (2001) 600-607.

[16] W. Shigeeda, M. Shibazaki, S. Yasuhira, et al, Hyaluronic acid enhances cell migration and invasion via the YAP1/TAZ-RHAMM axis in malignant pleural mesothelioma, Oncotarget, 8 (2017) 93729-93740.

[17] C.W. Castor, B. Naylor, Characteristics of normal and malignant human mesothelial cells studied in vitro, Lab Invest, 20 (1969) 437-443.

[18] J. Maeda, N. Ueki, T. Ohkawa, et al, Transforming growth factor-beta 1 (TGF-beta 1)- and beta 2-like activities in malignant pleural effusions caused by malignant mesothelioma or primary lung cancer, Clin Exp Immunol, 98 (1994) 319-322.

[19] A. Dolor, F.C. Szoka, Jr., Digesting a Path Forward: The Utility of Collagenase Tumor Treatment for Improved Drug Delivery, Mol Pharm, (2018).

[20] H.F. Dvorak, Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing, N Engl J Med, 315 (1986) 1650-1659.

[21] L. Rossow, S. Veitl, S. Vorlova, et al, LOX-catalyzed collagen stabilization is a proximal cause for intrinsic resistance to chemotherapy, Oncogene, (2018).

[22] J. Uitto, D.J. Prockop, Incorporation of proline analogues into collagen polypeptides. Effects on the production of extracellular procollagen and on the stability of the triple-helical structure of the molecule., Biochim Biophys Acta, 336 (1974) 234-251.

[23] B. Lubec, M. Hjelm, H. Hoeger, et al, 4-Thiaproline reduces heart lipid peroxidation and collagen accumulation in the diabetic db/db mouse. , Amino Acids, 12 (1997) 343-352.

[24] B. Lubec, A. Rokitansky, M. Hayde, et al, Thiaproline reduces glomerular basement membrane thickness and collagen accumulation in the db/db mouse, Nephron, 66 (1994) 333-336.

[25] L.S. Manning, D. Whitaker, A.R. Murch, et al, Establishment and characterization of five human malignant mesothelioma cell lines derived from pleural effusions, Int J Cancer, 47 (1991) 285-290.

[26] M.R. Davis, L.S. Manning, D. Whitaker, et al, Establishment of a murine model of malignant mesothelioma, Int J Cancer, 52 (1992) 881-886.

[27] A.W. Langerak, P.A. De Laat, C.A. Van Der Linden-Van Beurden, et al, Expression of platelet-derived growth factor (PDGF) and PDGF receptors in human malignant mesothelioma in vitro and in vivo, J Pathol, 178 (1996) 151-160.

[28] E. Borenfreund, J.A. Puerner, Toxicity determined in vitro by morphological alterations and neutral red absorption, Toxicol Lett, 24 (1985) 119-124.

[29] S.E. Mutsaers, R.J. McAnulty, G.J. Laurent, et al, Cytokine regulation of mesothelial cell proliferation in vitro and in vivo, Eur J Cell Biol, 72 (1997) 24-29.

[30] J.S. Campa, R.J. McAnulty, G.J. Laurent, Application of high-pressure liquid chromatography to studies of collagen production by isolated cells in culture, Anal Biochem, 186 (1990) 257-263.

[31] R.C. Chambers, R.J. McAnulty, A. Shock,et al, Cadmium selectively inhibits fibroblast procollagen production and proliferation, Am J Physiol, 267 (1994) L300-308.

[32] W.A. Dik, R.J. McAnulty, M.A. Versnel, et al, Short course dexamethasone treatment following injury inhibits bleomycin induced fibrosis in rats, Thorax, 58 (2003) 765-771.

[33] S.E. Mutsaers, M.L. Foster, R.C. Chambers, et al, Increased endothelin-1 and its localization during the development of bleomycin-induced pulmonary fibrosis in rats, Am J Respir Cell Mol Biol, 18 (1998) 611-619.

[34] M. Gulyas, A. Hjerpe, Proteoglycans and WT1 as markers for distinguishing adenocarcinoma, epithelioid mesothelioma, and benign mesothelium, J Pathol, 199 (2003) 479-487.

[35] S. Scarpa, A. Giuffrida, M. Fazi, et al, Migration of mesothelioma cells correlates with histotype-specific synthesis of extracellular matrix, Int J Mol Med, 4 (1999) 67-71.

[36] E. Tahara, Growth factors and oncogenes in human gastrointestinal carcinomas, J Cancer Res Clin Oncol, 116 (1990) 121-131.

[37] D.R. Fitzpatrick, H. Bielefeldt-Ohmann, R.P. Himbeck,et al, Transforming growth factor-beta: antisense RNA-mediated inhibition affects anchorage-independent growth, tumorigenicity and tumor-infiltrating T-cells in malignant mesothelioma, Growth Factors, 11 (1994) 29-44.

[38] M. Kuwahara, M. Takeda, Y. Takeuchi, et al, Transforming growth factor beta production by spontaneous malignant mesothelioma cell lines derived from Fisher 344 rats, Virchows Arch, 438 (2001) 492-497.

[39] R. Correa, B. Blanco, M. Del Rio, et al, Effect of a diet supplemented with thioproline on murine macrophage function in a model of premature ageing, Biofactors, 10 (1999) 195-200.

[40] M. Gosalvez, Thioproline, Lancet, 1 (1980) 597.

[41] S. Nasca, V. Galichet, J.C. Jardillier,et al, Thioproline toxicity, Lancet, 1 (1981) 778.

[42] D. Ingber, J. Folkman, Inhibition of angiogenesis through modulation of collagen metabolism, Lab Invest, 59 (1988) 44-51.

[43] M. De la Fuente, M.D. Ferrandez, M. Del Rio, et al, Enhancement of leukocyte functions in aged mice supplemented with the antioxidant thioproline, Mech Ageing Dev, 104 (1998) 213-225.

FIGURE LEGENDS

Table 1. Basal and TGF-β_1 induced collagen production in murine and human mesothelioma cell lines. Confluent cultures of cells were incubated with 0–10 ng/ml TGF-β_1 in DMEM supplemented with 1% FCS. Collagen production was assessed 24 hr later. Values were corrected for cell number and collagen production expressed as nmol hyp/10^6 cells/24 hr. The figures in brackets represent the percent change ± SEM compared with medium control. *****P< 0.05, **P< 0.01 and ***P< 0.001. Data are representative of at least two independent experiments.

Figure 1. Assessment of mesothelioma cell viability following thiaproline treatment. Subconfluent (**A**) and confluent (**B**) AC29 cell viability was assessed by neutral red uptake 24 hr after treatment with 0-50 mM thiaproline. Each point represents the mean ± SEM of six replicate cultures. *P<0.05, **P<0.001 compared with medium control. Data are representative of three separate experiments.

Figure 2. Effect of thiaproline on mesothelioma collagen production. (A) Thiaproline decreases basal and TGF-β_1-induced mesothelioma cell collagen production in a dose dependent manner. Confluent cultures of AC29 cells were incubated with 0-10 mM thiaproline with and without 1 ng/ml TGF-β_1 in DMEM supplemented with 1% FCS for 24 hr. Values were corrected for cell number and collagen production expressed as nmol hyp/10^6 cells/24 hr. Each bar represents the mean±SEM for six replicate cultures. *****=P<0.001 compared with the medium control. The data are representative of four independent experiments. **(B)** Effect of increasing concentrations of thiaproline on mesothelioma cell collagen and non-collagen protein production. Confluent AC29 cells were incubated with DMEM containing 1% FCS supplemented with thiaproline at 0, 1, and 10 mM. Collagen and

non-collagen protein production were assessed 24 hr later. The level of protein production was expressed as percentage inhibition compared with the medium control. Each bar represents the mean ± SEM for six replicate cultures. *****=P<0.001. Data are representative of two independent experiments.

Figure 3. Thiaproline inhibits AC29 mesothelioma cell proliferation. (A) Thiaproline decreases mesothelioma cell DNA synthesis in a dose dependent manner. AC29 DNA synthesis was assessed 24 hr after culture with 0-10 mM thiaproline. Results are expressed as disintegrations per minute (dpm). Each bar represents the mean ± SEM for six replicate cultures. *P<0.005, **P<0.001 compared with medium control. Data are representative of two separate experiments. **(B)** Linear regression curve showing correlation between thiaproline concentration and cell proliferation.

Figure 4. Effect of thiaproline on mesothelioma growth and collagen concentration 10 days after treatment. Effect of thiaproline on subcutaneous mesothelioma tumor growth. Tumors were excised at **(A)** 10 and **(B)** 18 days and weighed. Each point represents an individual tumor. The horizontal bar indicates the median tumor weight. *****P<0.001. The results are representative of data obtained in three independent experiments. Thiaproline reduces mesothelioma tumor collagen concentration at **(C)** 10 but not **(D)** 18 days. **(E)** Tumor collagen content at 10 days was plotted against tumor weight and the correlation coefficient ($\mathbf{r^2}$) calculated. Each point represents an individual tumor. *P<0.01 between thiaproline treated and control tumor groups. Data are representative of two separate experiments. **(F)** Apoptosis, as shown by caspase 3 expression, is increased in 10 day thiaproline treated tumors compared with control.

Table

	Cell Line	Hydroxyproline (nmol hyp / 10^6 cells / 24 hr) 0 ng / ml TGF-β_1	0.1 ng / ml TGF-β_1	1 ng / ml TGF-β_1	10 ng / ml TGF-β_1
Murine	AB1	1.37 ± 0.09	1.61 ± 0.12	2.23 ± 0.40	2.79 ± 0.29 *
			(17.79 ± 9.03)	(62.73 ± 28.99)	(103.77 ± 21.67)
	AB22	1.58 ± 0.07	3.88 ± 0.12 ***	6.29 ± 0.10 ***	8.43 ± 0.11 ***
			(146.13 ± 7.75)	(298.84 ± 6.58)	(434.54 ± 6.84)
	AC29	1.55 ± 0.12	2.09 ± 0.06 **	3.83 ± 0.23 ***	4.34 ± 0.33 ***
			(34.72 ± 4.10)	(146.47 ± 15.03)	(179.56 ± 21.44)
Human	NM20	0.10 ± 0.01	-	0.28 ± 0.01 ***	-
				(185.71 ± 5.89)	
	JU77	0.57 ± 0.16	0.84 ± 0.27	1.83 ± 0.94 *	3.66 ± 0.24 ***
			(46.56 ± 47.01)	(220.32 ± 73.19)	(540.48 ± 42.26)
	LO68	0.26 ± 0.08	0.37 ± 0.03	0.55 ± 0.02 **	0.47 ± 0.03 *
			(41.16 ± 11.31)	(106.14 ± 8.09)	(77.94 ± 13.16)
	NO36	0.52 ± 0.28	0.74 ± 0.09	0.87 ± 0.14	0.83 ± 0.11
			(41.28 ±16.42)	(56.51 ± 29.27)	(58.89 ± 21.31)
	ONE58	0.34 ± 0.04	0.81 ± 0.01 ***	1.21 ± 0.09 ***	1.67 ± 0.11 ***
			(134.59 ± 0.82)	(241.71 ± 28.71)	(387.37 ± 31.21)

Figure

Click here to download high resolution image

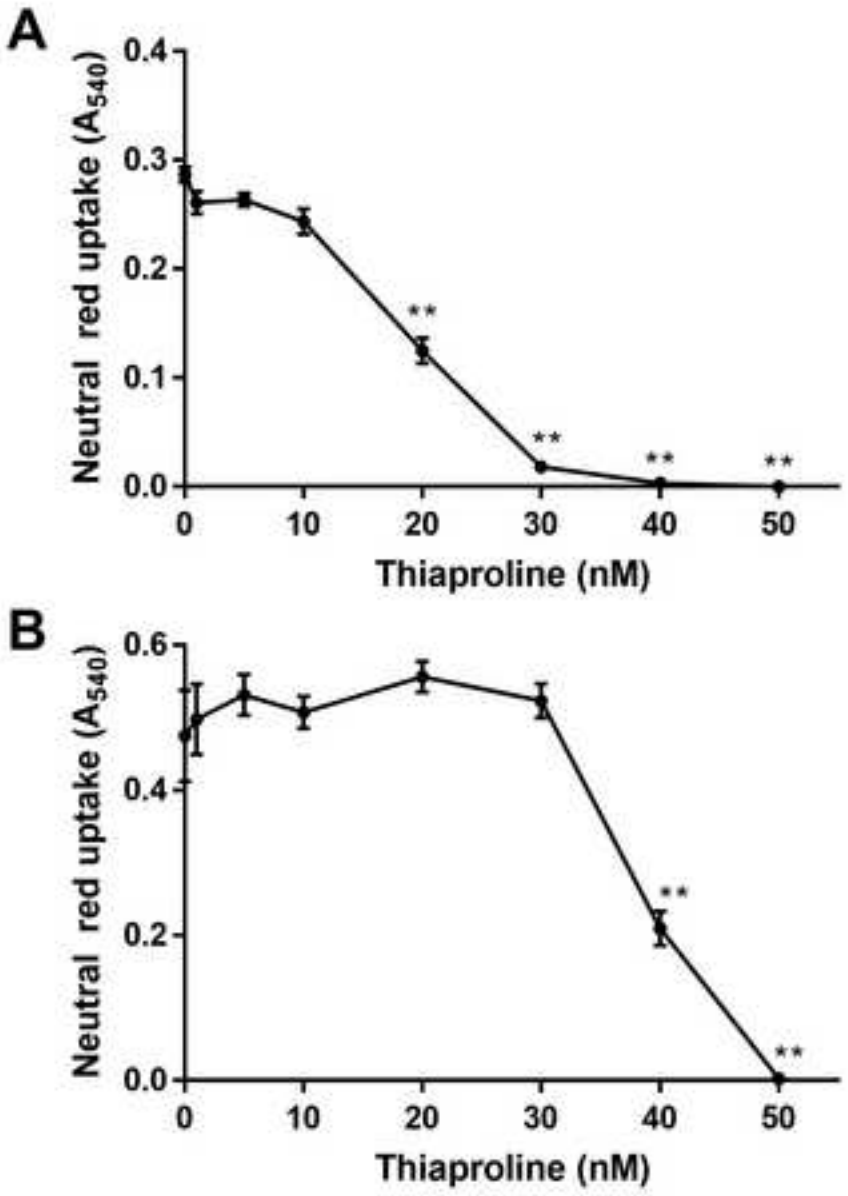


Figure
Click here to download high resolution image

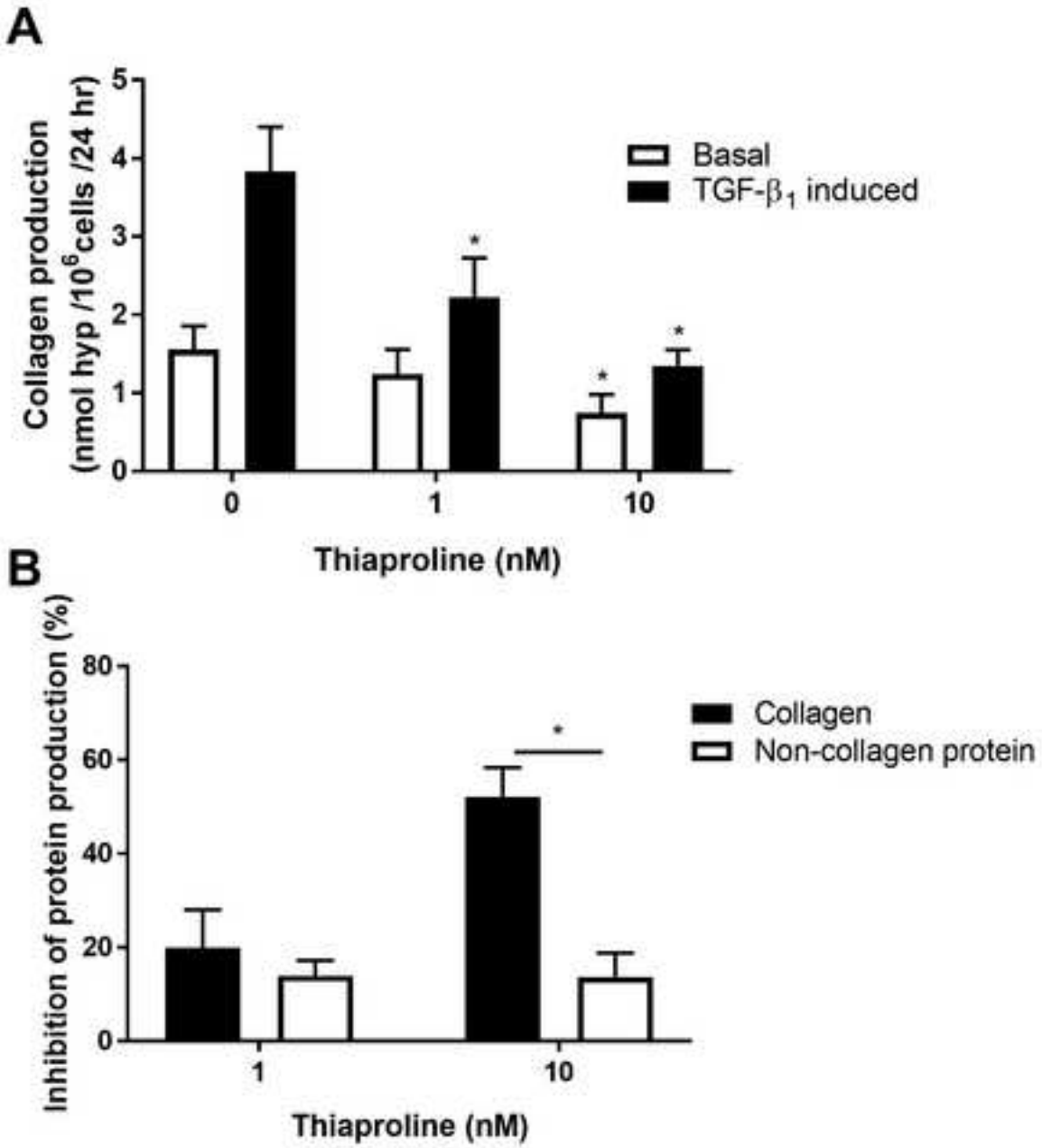
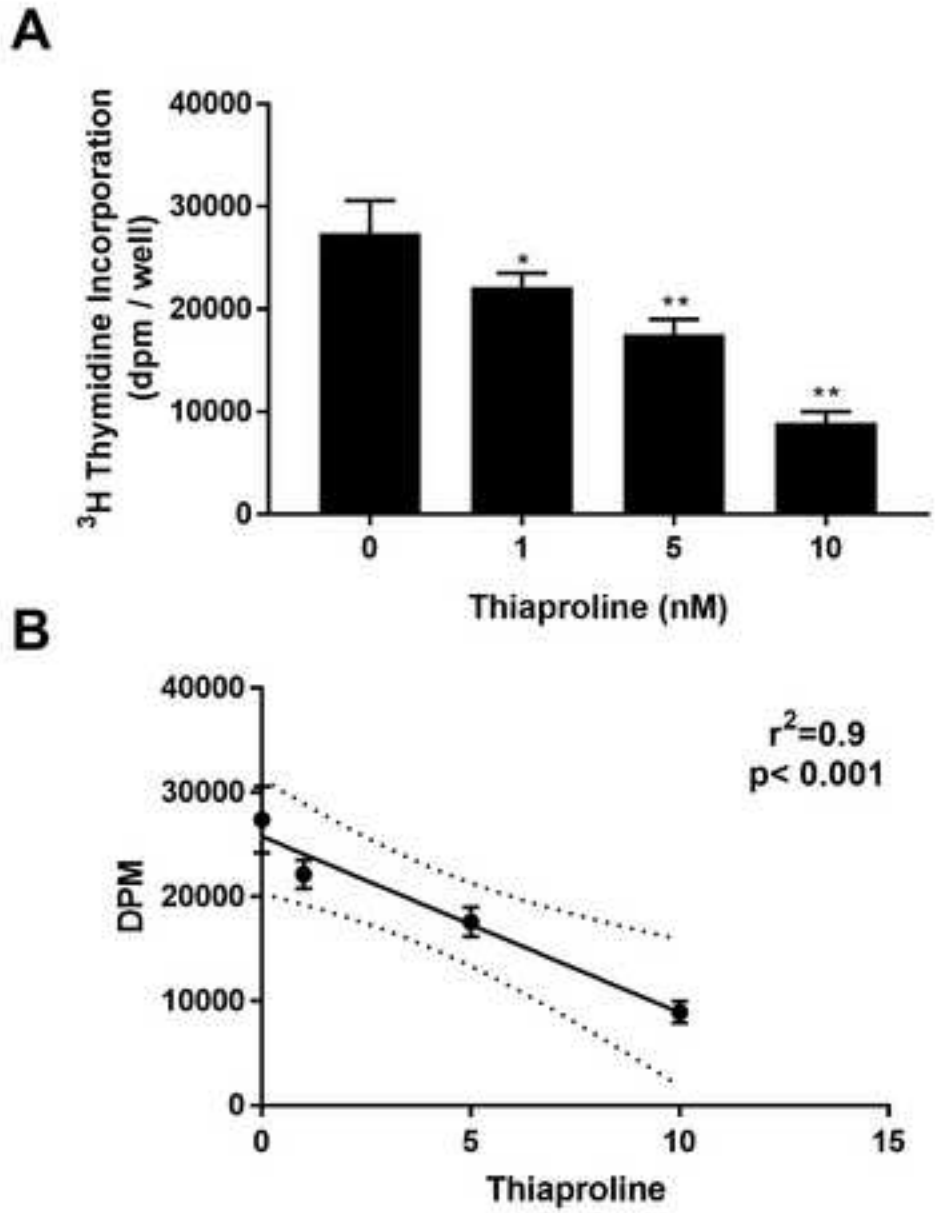
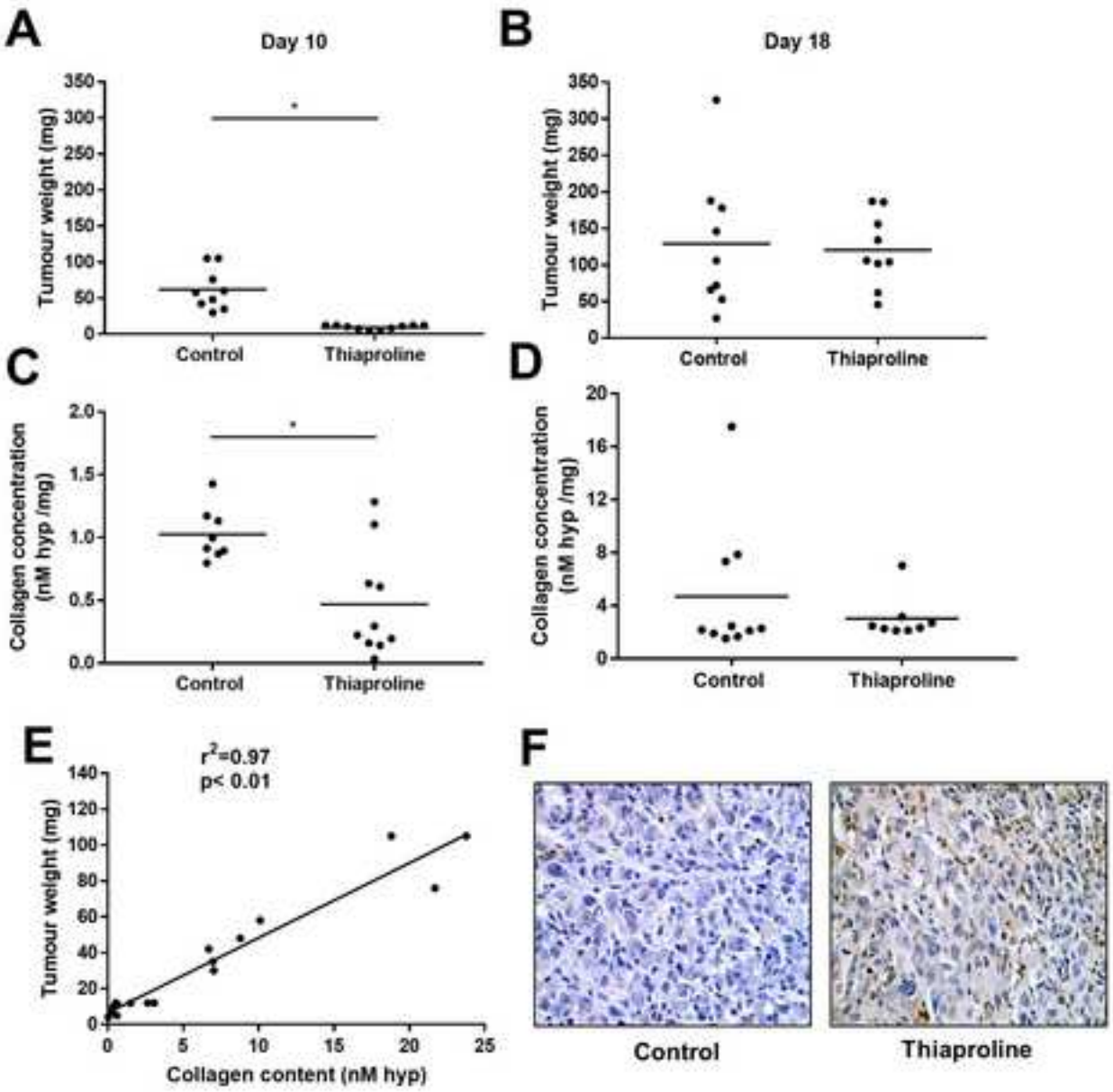


Figure
Click here to download high resolution image



Figure

Click here to download high resolution image



***Conflict of Interest**

Please wait...

If this message is not eventually replaced by the proper contents of the document, your PDF viewer may not be able to display this type of document.

You can upgrade to the latest version of Adobe Reader for Windows®, Mac, or Linux® by visiting http://www.adobe.com/go/reader_download.

For more assistance with Adobe Reader visit http://www.adobe.com/go/acrreader.