Ursolic acid, an antagonist for transforming growth factor (TGF)-β1

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Abstract Transforming growth factor- (TGF-), a multifunctional cytokine which is involved in extracellular matrix modulation, has a major role in the pathogenesis and progression of fibrotic diseases. We now report the effects of ursolic acid on TGF-1 receptor binding and TGF-1-induced cellular functions in vitro. Ursolic acid inhibited [^{125}I]-TGF-1 receptor binding to Balb/c 3T3 mouse fibroblasts with an IC_{50} value of 6.9 ± 0.8 μM. Ursolic acid dose-dependently recovered reduced proliferation of Minc Mv1Lu cells in the presence of 5 nM of TGF-1 and attenuated TGF-1-induced collagen synthesis and production in human fibroblasts. Molecular dynamics simulations suggest that ursolic acid may interact with the hydrophobic region of the dimeric interface and thereby inhibit the binding of TGF-1 to its receptor. All these findings taken together show that ursolic acid functions as an antagonist for TGF-1. This is the first report to show that a small molecule can inhibit TGF-1 receptor binding and influence functions of TGF-1.

Keywords: Ursolic acid; Oleanolic acid; Transforming growth factor-β1; Mv1Lu cell; Triterpene; Collagen

1. Introduction

Ursolic acid is a triterpenoid compound widely present in food, medicinal herbs and other plants, and its biological effects include antitumor, hepatoprotectory, and anti-inflammatory activities [1]. In our search for active components from the medicinal plant, *Clerodendranthus spicatus* (Thunb.) C.Y. Wu, which is used as a folk medicine to treat patients with nephritis in China, we identified ursolic acid and oleanolic acid as compounds active against transforming growth factor-β1 (TGF-β1) receptor binding [2].

TGF-β, a key regulatory molecule in controlling the activity of fibroblasts, has been implicated in several diseases states characterized by excess fibrosis [3]. In the kidney, TGF-β promotes tubuloepithelial cell hypertrophy and regulates glomerular production of the extracellular matrix, including collagens, fibronectin, and proteoglycans. TGF-β also blocks destruction of the extracellular matrix by up-regulating the synthesis of protease inhibitors and down-regulates the synthesis of matrix-degrading proteases. Thus, TGF-β plays a pivotal role in the pathogenesis and progression of nephritis [4–7], as well as in fibrotic changes of other tissues [8–10]. The therapeutic benefits of inhibiting TGF-β function by an antibody against TGF-β have been demonstrated in cases of experimental nephropathy [11,12]. Injection of decorin, a small proteoglycan, that binds and neutralizes TGF-β1 function [13], results in suppression of TGF-β1-induced extracellular matrix accumulation in nephritic glomeruli [14]. Similar therapeutic effects on nephritis were noted in rats transfected with the decorin gene [15]. While several successful approaches to inhibiting protein–protein binding by a small molecule have been reported [16,17], we found no documentation concerning the interaction of TGF-β and its receptor. We now report the functional antagonistic effects of ursolic acid in TGF-β-induced inhibition of mink lung epithelial (Mv1Lu) cell proliferation and TGF-β-induced production of collagen in vitro.

2. Materials and methods

2.1. Materials

Recombinant human TGF-β1 was purchased from Austral Biologicals (San Ramon, CA) and Genzyme Techne (Cambridge, MA). [^{125}I]-TGF-β1 and [^{3}H]-thymidine were from Dupont-NEN (Boston, MA). [2.3-^{3}H]-proline was from Amersham (Arlington Heights, IL). EIA Kits for procollagen type I C-peptide (PIP) were from Takara (Kyoto, Japan). Ursolic acid, α,α′-dipyridyl and type IV collagenase were from Sigma Chemical Co. (St. Louis, MO). Minimal essential medium (MEM), fetal bovine serum (FBS), penicillin and streptomycin were from Life Technologies (Rockville, MD). Other chemicals were of analytical grade.

2.2. TGF-β1 binding assay

Binding experiments were done as described [18,19]. Briefly, Balb/c 3T3 cells (Dainippon, Osaka, Japan) were seeded in a 24 well-plate at a density of 5×10^4 cells per well and cultured for 3 days in MEM supplemented with 10% FBS and antibiotics (100 IU/ml penicillin and 50 μg/ml streptomycin). When cells were at a near-confluent stage, the medium was replaced with binding buffer containing 50 mM Hepes, 128 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, and 1.2 mM $CaCl_2$. The assay was initiated by adding 50 pM [^{125}I]-TGF-β1 and increasing the concentrations of ursolic acid. After 4 h at 4 °C, the medium was removed and cells were washed five times with ice-cold binding buffer. The cells were then solubilized using buffer containing Triton X-100 and the radioactivity was measured. Ursolic acid was dissolved in

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Abbreviations: TGF-β, transforming growth factor-β; MEM, minimal essential medium; FBS, fetal bovine serum; Mv1Lu, mink lung epithelial; TCA, trichloroacetic acid; DMEM, Dulbecco's modified essential medium

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dimethylsulfoxide (DMSO). The final concentration of DMSO was under 0.6%. Non-specific binding was determined in the presence of an excess (10 nM) of unlabeled TGF-β1. The IC_{50} value of [^{125}I]-TGF-β1 binding inhibition by ursolic acid was determined in five separate experiments.

2.3. Cell growth assay

Mv1Lu cells (Dainippon, Osaka, Japan) were grown and maintained in MEM supplemented with 10% FBS, antibiotics (100 IU/ml penicillin and 50 μg/ml streptomycin) and non-essential amino acids. For growth inhibition assays, Mv1Lu cells were seeded at 3×10^4 cells/well in 24-well plates in 500 μl/well [20]. After incubation for 1 day, medium was replaced with fresh MEM supplemented with 0.2% FBS, maintained for 24 h, then replaced with the same fresh medium containing the indicated concentration of ursolic acid and 10 pM TGF-β1. Cultures were incubated for an additional 24 h, the last 2 h being in the presence of 0.5 μCi/ml of [^{3}H]-thymidine. Ursolic acid was dissolved in DMSO at a final concentration of 1%. Cells were washed twice with phosphate-buffered saline and solubilized with 0.5 M NaOH. Incorporation of [^{3}H]-thymidine into DNA was measured using liquid scintillation counting.

2.4. Measurement of collagen synthesis

The synthetic activity of collagen was assessed by incorporation of [^{3}H]-proline into collagenase degradable protein, as described [21,22]. Human skin fibroblasts (Dainippon, Osaka, Japan) were seeded into 24-well plates at a density of 4×10^4 cells per well in CS-C Medium [Dulbecco's modified essential medium (DMEM)-HamF12 1:1] containing 10% FBS, 15 mM Hepes, acidic-FGF, and heparin (Dainippon, Osaka, Japan). After 24 h of serum deprivation, quiescent medium was then replaced by 500 μl of DMEM containing 50 μg/ml β-aminopropionitril, 50 μg/ml ascorbic acid, 10 μg/ml [^{3}H]-proline, and 5 ng/ml TGF-β1, in the presence or absence of compounds, then the cells were incubated for 24 h and cell layers and medium were analyzed separately. Trichloroacetic acid (TCA)-precipitated material of incubation medium was solubilized in 0.2 ml of 0.2 N NaOH. For cell collagen assay, cells were removed and the protein-containing pellet was solubilized in 0.2 ml of 0.2 N NaOH. After neutralization with 0.15 N HCl, 40 μl of purified collagenase solution (type IV from *Clostridium histolycum*, Sigma, 1250 U/ml in 50 mM Tris–HCl, pH 7.4, and 10 mM $CaCl_2$) was added to each 0.46 ml sample from the medium and cells and the samples were digested at 37 °C for 2 h. The samples were then reprecipitated with 10% TCA containing 0.5% tannic acid and radioactivity released into the supernatant was determined using liquid scintillation counting. Collagenase-resistant radioactivity in the pellet was similarly determined after solubilization in 0.2 ml of 0.2 N NaOH.

2.5. Measurement of collagen production

Production of type I collagen was estimated based on the accumulation of PIP in the medium, using an enzyme immunoassay. Human skin fibroblasts (Dainippon, Osaka, Japan) were seeded into 24-well plates at a density of 1.5×10^4 cells per well in CS-C Complete Medium (DMEM-HamF12 1:1, Gibco) containing 10% FBS, 15 mM Hepes, acidic-FGF, and heparin, and then grown for 48 h to reach confluency. After 24 h of serum deprivation in DMEM/F-12 medium, quiescent medium was then replaced with fresh DMEM/F-12 containing 50 μg/ml ascorbic acid and 5 ng/ml TGF-β1, in the presence or absence of drugs. After 48 h of incubation, medium was collected by centrifugation and PIP was determined using an enzyme immunoassay and Takara PIP EIA Kits. Cell viability was assessed microscopically after staining cells with Alamar Blue.

2.6. Molecular dynamics simulations

The coordinates of TGF-β and ursolic acid were taken from PDB databank (code: 1KLC) and Cambridge Structural Database [23] (code: TARDEV), respectively. Molecular dynamics (MD) calculations were performed under the system in which 10,362 water molecules (correspond to 44 angstrom radii) were spherically attached around the proposed complex model of TGF-β and ursolic acid. The total number of atoms was 34 784. AMBER6 program was used for calculations. The atomic charges of ursolic acid were calculated with HF/6-31G* calculations and RESP fitting. 1ns MD simulation was performed using the time step of 2 fs. Temperature was controlled at 300 K during simulations and no coulomb cut-off was applied. To accelerate calculations, a special-purpose parallel computer for non-bonded force sum, MD-Engine was used [24]. Total computing times were about 11 days.

2.7. Statistical analysis

The results are expressed as mean ± S.E.M. Statistical significance was evaluated using Dunnett's multiple comparison test.

3. Results and discussion

In the present study, we obtained evidence that naturally occurring triterpene, ursolic acid, is an inhibitor of TGF-β1 receptor binding. The fact that ursolic acid blocked TGF-β1-induced antiproliferative activity and suppressed TGF-β1-induced collagen production also supports antagonistic actions of ursolic acid against TGF-β1.

In Balb/c 3T3 fibroblasts, [^{125}I]-TGF-β1 bound to the cells with a high affinity, with a K_d of 99 pM and B_{max} value of 33 fmol/10^6 cells (Fig. 1). Under this assay condition, ursolic acid inhibited the specific binding of [^{125}I]-TGF-β1 to 3T3 fibroblasts, in a dose-responsive manner with an IC_{50} value (mean ± S.E.M.) of 6.9 ± 0.8 μM ($n = 5$) (Fig. 2). The K_i value (mean ± S.E.M.) of ursolic acid was determined to be 3.7 ± 0.4 μM ($n = 5$). In the previous study, to determine the specificity

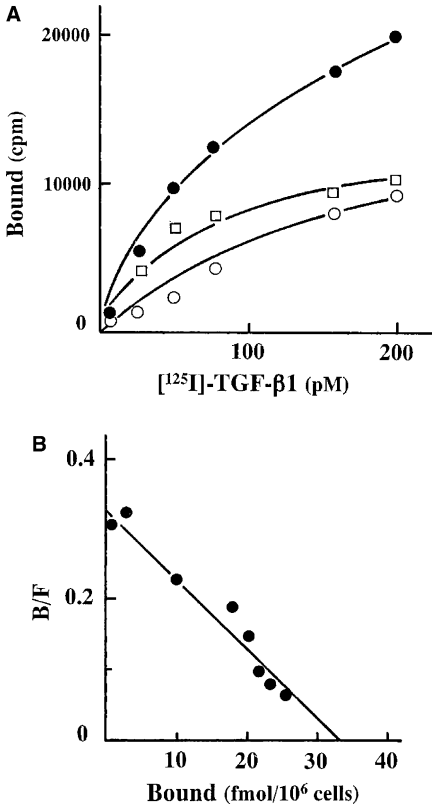

Fig. 1. Characterization of [^{125}I]-TGF-β1 binding to Balb/c 3T3 mouse fibroblasts. (A) Equilibrium binding of [^{125}I]-TGF-β1 to Balb/c 3T3 mouse fibroblasts. The cells were incubated in the presence of various concentrations of [^{125}I]-TGF-β1, as described under Section 2. Specific binding (□) was estimated by subtracting the non-specific binding of [^{125}I]-TGF-β1 to Balb/c 3T3 mouse fibroblasts (○) from the total binding (●). Data represent means obtained from measurement in triplicate wells. (B) Scatchard analysis for the binding of [^{125}I]-TGF-β1 to Balb/c 3T3 mouse fibroblasts.

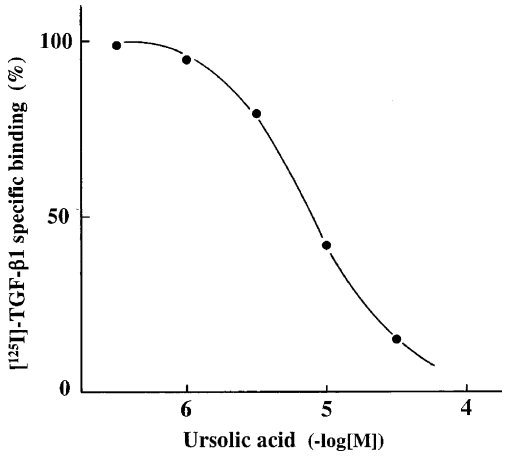

Fig. 2. Effect of ursolic acid on [^{125}I]-TGF-β1 binding to mouse Balb/c 3T3 fibroblasts. Results are expressed as percentage of specific binding in the absence of ursolic acid. Each point represents the mean of duplicate determinations.

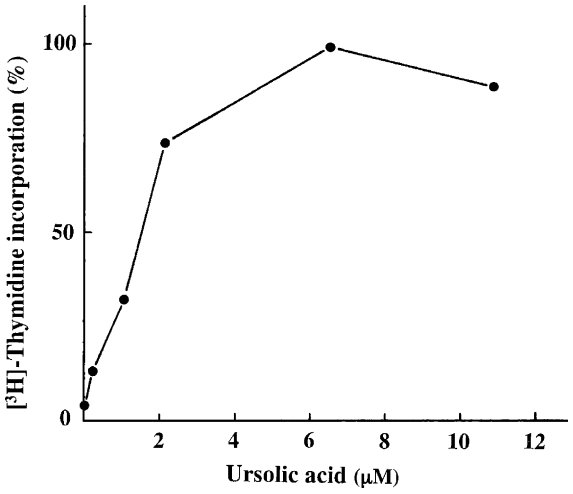

Fig. 3. Effect of ursolic acid on TGF-β1-induced inhibition of Mv1Lu cell proliferation. Mv1Lu cells were incubated with TGF-β1 (10 pM) and various concentrations of ursolic acid for 24 h, the last 2 h being in the presence of [^{3}H]-thymidine. The results are expressed as a percentage of the incorporation of [^{3}H]-thymidine in control cells incubated without TGF-β1 and ursolic acid. Each point represents the mean of duplicate determinations performed in duplicate.

of actions, we examined effects of ursolic acid on receptor binding of cytokines and growth factors, and activities of enzymes that are considered to be involved in the pathogenesis and development of nephritis, including platelet-derived growth factor (PDGF)-BB, tumor necrosis factor (TNF)-α, epidermal growth factor (EGF), interleukin (IL)-6, angiotensin II (AT_1), endothelin (ET_A), leukotriene B_4, thromboxane A_2, platelet activating factor (PAF), and angiotensin converting enzyme. Ursolic acid had no apparent activity to these receptors and enzymes up to 10 μg/ml (6.6 μM) [2]. Therefore, inhibitory actions of ursolic acid are specific to TGF-β at least among these possible factors involved in nephritis. Thus, therapeutic effects of Chinese herbal medicine, *C. spicatus* (Thunb.) C.Y. Wu, on nephritis may be related to antagonistic actions of ursolic acid and oleanolic acid against TGF-β1.

The functional efficacy of ursolic acid was determined in Mv1Lu cells. These cells are highly sensitive to the antiproliferative action of TGF-β and widely used for determination of TGF-β activity [25]. Fig. 3 shows the dose response for neutralization of TGF-β1 activity by ursolic acid. Although cell proliferation was almost completely suppressed by exogenously added TGF-β1, as assessed by incorporation of [^{3}H]-thymidine, this compound overcame TGF-β1-induced reduction in cell proliferation in a dose-dependent manner, with an IC_{50} value of approximately 1.5 μM. Higher concentrations were required to cause a rightward shift in the dose–response curve of TGF-β1-induced inhibition of cell proliferation (Fig. 4).

In addition to proliferation of Mv1Lu cells, we made another functional evaluation against TGF-β using collagen assay systems, since TGF-β is a potent stimulus for collagen formation. Effects of ursolic acid on collagen production were assessed using two different methods, measurement of [^{3}H]-proline incorporation into collagen in both cell layer and medium and measurement of type I collagen content secreted into the medium. TGF-β1 markedly stimulated collagen synthesis from [^{3}H]-proline in human fibroblasts (Fig. 5). [^{3}H]-labeled collagen was increased by TGF-β1 more than five times in both cell and medium. Ursolic acid (0.67–6.6 μM) dose-dependently decreased [^{3}H]-proline incorporation into collagen

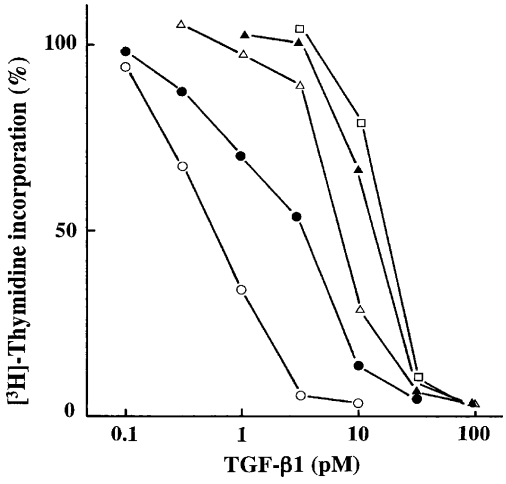

Fig. 4. Effect of TGF-β1 on Mv1Lu cell proliferation in the presence of various concentrations of ursolic acid. Mv1Lu cells were incubated with increasing concentrations of TGF-β1 in the presence of 0 (○), 0.2 (●), 1.1 (△), 2.2 (▲), and 6.6 (□) μM of ursolic acid for 24 h, the last 2 h being in the presence of [^{3}H]-thymidine. The results are expressed as a percentage of the incorporation of [^{3}H]-thymidine in control cells incubated without TGF-β1 and ursolic acid. Each point represents the mean of duplicate determinations performed in duplicate.

in both cell layer and medium. Collagen content secreted into the medium, as measured by EIA assay, was markedly elevated by TGF-β1 (Fig. 6). Type I collagen content in the medium was also dose-dependently decreased by ursolic acid. Ursolic acid up to 6.6 μM had no evident toxic action toward human fibroblasts, as assessed by cell viability assay, thus inhibitory effect of ursolic acid on collagen production was apparently not associated with toxicity of this compound. A well-known inhibitor of prolyl hydroxylase, α,α′-dipyridyl, at 100 μM markedly inhibited collagen synthesis and secretion in both assay systems. Therefore, inhibition of TGF-β1 receptor

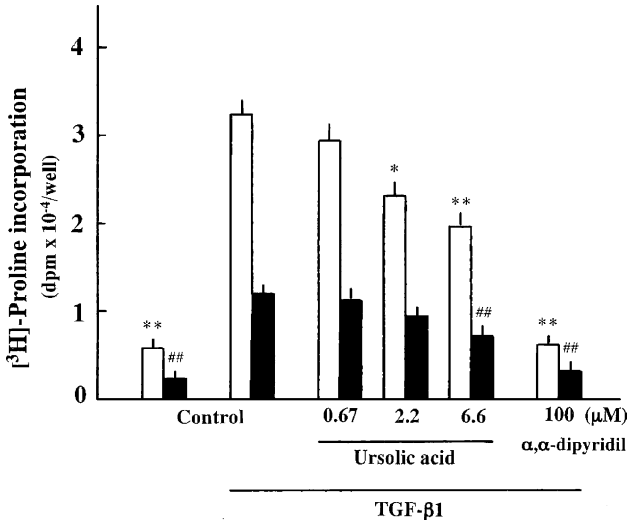

Fig. 5. Effect of ursolic acid on TGF-β1-stimulated collagen synthesis in human fibroblasts. Confluent cells were incubated with TGF-β1 (5 ng/ml) for 24 h in the presence or absence of compounds. Incorporation of [^{3}H]-proline into collagenase degradable protein was assessed in the cell (□) and medium (■), as described under Section 2. Each bar represents the mean ± S.E.M. of three separate determinations. $^*P < 0.05$, $^{**}P < 0.01$ (vs control with TGF-β1 in the cell). $^{\#\#}P < 0.01$ (vs control with TGF-β1 in the medium).

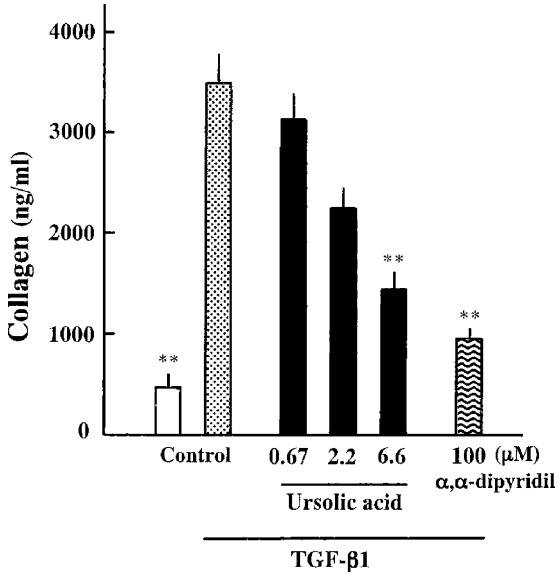

Fig. 6. Effect of ursolic acid on collagen production in human fibroblasts. Confluent cells were incubated with TGF-β1 (5 ng/ml) for 48 h in the presence or absence of compounds and secretion of type I collagen into the incubation medium was determined using PIP EIA kits. Each point represents the mean of four separate determinations done in duplicate. $^{**}P < 0.01$ (vs control with TGF-β1).

binding by ursolic acid was demonstrated in cell proliferation and collagen production. Oleanolic acid, a structurally related triterpenoid to ursolic acid, had similar effects on TGF-β1 binding with an IC_{50} value of 21 μM, neutralization of Mv1Lu cell proliferation and collagen production (data not shown).

Some traditional herbal medicines containing ursolic acid and oleanolic acid have been used to treat subjects with hepatitis [1]. Moreover, ursolic and oleanolic acids were reported to be effective in several animal models of liver injury [26,27]. However, the mechanism responsible for hepatoprotective action of these triterpenes remains unclear. Based on our data, it is plausible that preventive effects of ursolic acid and oleanolic acid in cirrhosis and fibrosis of the liver may in part be attributed to antagonism against TGF-β, since many studies demonstrated the important role of TGF-β in fibrotic processes of the liver [7–9], as well as the kidney.

For a detailed examination of mechanisms by which ursolic acid inhibits the binding of TGF-β1 to its receptor, we analyzed the interaction of ursolic acid with TGF-β1 using MD calculations, since the preliminary experiments showed that this compound binds to TGF-β1, not the TGF-β receptor. TGF-β1 is a homologous dimeric protein containing interchain disulfide bonds, and the dimeric interface region consists of the WXXD motif (Trp52–X–X–Asp55) which is important for the growth inhibitory activity of TGF-β1 [28], and several other solvent-exposed hydrophobic residues such as Tyr50, Leu64, Trp28 and Trp32. We first analyzed the binding of ursolic acid to the WXXD motif region. We found that the binding was unstable, because part of the ursolic acid was dissociated with the protein surface and wafted into the water during the simulations (data not shown). The surface of the WXXD region is flat and unsuitable for binding of a small molecule such as ursolic acid. In contrast, another possible binding model in which ursolic acid binds the hydrophobic site close to the WXXD motif was stable during the simulations. As shown in Fig. 7, the shape and hydrophobicity of ursolic acid made for a good fit with TGF-β1. Our findings may account for the inhibition of receptor binding by ursolic acid with the IC_{50} value of micromolar range.

Our results demonstrate that ursolic acid is an antagonist for TGF-β1, suggesting that ursolic acid competes with TGF-β1 binding to its receptor, and thereby neutralizes anti-proliferative effects of TGF-β1 and suppresses TGF-β1-induced collagen production. This is the first report to show that even this small molecule exhibits antagonistic action against TGF-β1,

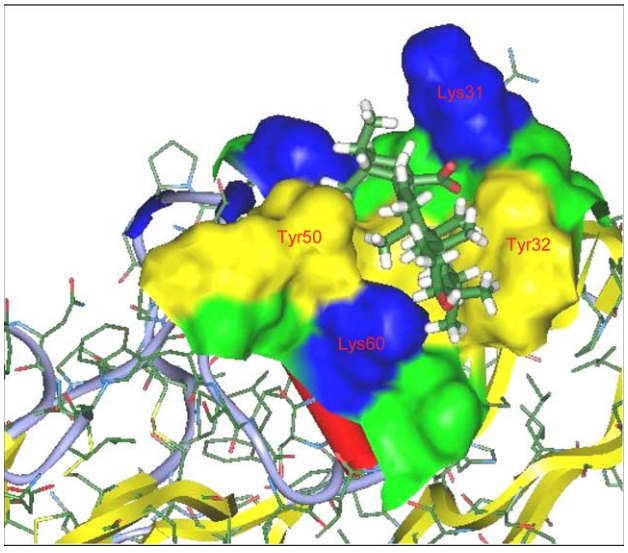

Fig. 7. Modeling of ursolic acid bound to TGF-β1. Molecular surfaces of the binding region are shown. Surface color definitions are as follows; red (acidic), blue (basic), green (hydrophilic), yellow (hydrophobic). Ursolic acid is shown as a bald stick.

although macromolecules such as decorin [8,9] and peptide antagonists [29] have been shown to antagonize TGF-β.

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