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A novel fused 1,2,4-triazine aryl derivative as antioxidant and nonselective antagonist of adenosine A_{2A} receptors in ethanol-activated liver stellate cells

Agnieszka Szuster-Ciesielska [a,*], Krzysztof Sztanke [b], Martyna Kandefer-Szerszeń [a]

[a] Department of Virology and Immunology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[b] Chair and Department of Medical Chemistry, Medical University, Chodźki 4A, 20-093 Lublin, Poland

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ABSTRACT

It has been detected that hepatic adenosine A_{2A} receptors play an active role in the pathogenesis of hepatic fibrosis and suggest a novel therapeutic target in the treatment and prevention of hepatic cirrhosis. In this paper we examined if our new triazine derivative (IMT) can inhibit ethanol-induced activation of HSCs measured as increased α-SMA, collagen synthesis and enhanced oxidative stress in rat liver stellate cells. We also investigated its influence on cytokines (TGF-β, TNF-α) synthesis, MMP-2 and TIMP-1 production and ethanol-induced intracellular signal transduction. Moreover, with using of known adenosine A_{2A} receptor agonist (CGS 21680), and antagonist (SCH 58261) we examined if this triazine derivative acts on adenosine receptors.

We detected a strong antagonistic action of new triazine derivative (IMT) on ethanol-induced rat liver stellate cells activation, observed as a significant decrease in α-SMA, collagen synthesis, reactive oxygen species production, TGF-β, TNF-α, MMP-2 and TIMP-1 production as well as JNK, p38MAPK, NFκB, IκB, Smad3 phosphorylation. Moreover, IMT strongly inhibited activation of stellate cells by known selective agonist of adenosine A_{2A} receptor (CGS 21680). When known A_{2A} receptor antagonist (SCH 58261) was used together with IMT this effect was not spectacular. Additionally, only slight enhancement of inhibition was observed when cells were pretreated both IMT with SCH 58261, hence we suppose that IMT acts as nonselective antagonist of A_{2A} receptors, and, besides its antioxidant activity, also by this way inhibited ethanol-induced stellate cell activation.

1. Introduction

Hepatic stellate cells (HSCs) play important functions in normal liver, such a retinoid storage, remodeling of extracellular matrix (ECM) and production of growth factors and cytokines. However, in response to liver damage, HSCs undergo a process of activation, developing a myofibroblast-like phenotype associated with increased proliferation and excessive production and deposition of ECM components which is the major pathological feature of hepatic cirrhosis. The main compounds of ECM are smooth muscle α-actin (α-SMA) and collagen type I. The activated HSCs secrete also high levels of metalloproteinase-2 (MMP-2) as well as tissue inhibitor of metalloproteinase-1 (TIMP-1) [1,2]. Because activation of hepatic stellate cells is an early step in hepatic fibrogenesis, therefore, suppression of HSCs activation has been proposed as a therapeutic target against this liver disease and further complications.

Recently the role of adenosine and its receptors in the regulation of hepatic fibrosis [3,4], and glycogen metabolism [5,6] were demonstrated. Moreover, adenosine signaling is contributing to ethanol induced fatty liver in mice, especially role of A_1 and A_{2B} receptors are emphasized. The targeting of those adenosine receptors may be effective not only in the prevention of alcohol-induced fatty liver [7]. It has also been detected that hepatic adenosine A_{2A} receptors play an active role in the pathogenesis of hepatic fibrosis and suggest a novel therapeutic target in the treatment and prevention of hepatic cirrhosis [3]. Mice treated in vivo by antagonist of adenosine A_{2A} receptors (ZM241385) had diminished hepatic fibrosis induced by CCl_4 or thioacetamide. Moreover, it has also been observed that A_{2A} receptor occupancy is involved in activation of liver stellate cells, measured as enhanced collagen expression [8].

We have already described the synthesis, structure and antitumor properties of several novel fused 1,2,4-triazine aryl derivatives [9]. Some of those derivatives exhibited antiproliferative activity against Jurkat cells but some of them were non-toxic not only for normal skin fibroblasts but also for tumor cells. It has been indicated in the literature that 1,2,4-triazole derivatives inhibited ethanol-induced oxidative stress in mouse brain and liver [10]. Moreover, some new 1,2,4-triazolo[1,5c] pirimidine derivatives were identified as selective A_{2A} receptor antagonist [11]. A_{2A} adenosine receptors expression and occupancy on rat and human liver stellate cells promotes collagen production in these cells [8].

* Corresponding author. Tel.: +48 81 537 59 43; fax: +48 81 537 59 59.
E-mail addresses: szustera@hektor.umcs.lublin.pl (A. Szuster-Ciesielska), krzysztof.sztanke@am.lublin.p (K. Sztanke), kandem@poczta.umcs.lublin.pl (M. Kandefer-Szerszeń).

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Hence, the goal of our study was to examine if our new triazine derivative, described as No13 in our previous paper [9] can inhibit activation of HSCs measured as increased α-SMA, collagen synthesis and enhanced oxidative stress in rat liver stellate cells. We also investigated its influence on cytokines (TGF-β, TNF-α), MMP-2 and TIMP-1 production. Studies performed with a well-characterized HSC clone (CFSC-2G cell line) as a model to investigate liver stellate cell activation, that is comparable to the data obtained from in vivo animal models, as well as human samples [12]. To test hypothesis, that because this derivative possess structure complementary to adenosine so it may act as agonist or antagonist of adenosine A_{2A} receptors, we examined its ability to inhibit α-SMA and collagen synthesis in ethanol-activated liver stellate cells using standardized, commercially available, antagonist and agonist of adenosine A_{2A} receptors.

2. Materials and methods

2.1. Synthesis of triazine derivative (IMT)

Biologically active 8-(4-methoxyphenyl)-4-oxo-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]-triazine-3-carbohydrazide (IMT) has been obtained in one step synthetic approach by treating of the ethyl 1-[4-oxo-8-(4-methoxyphenyl)-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]-triazin-3-yl]formate with hydrazine hydrate. In an attempt to prepare the final compound in the highest yield, three different synthetic methods were applied. Thus the condensation of above mentioned substrates was carried out: (a) in *n*-butanol in the temperature of boiling for 5 h (method i), (b) in ethanol under reflux for 6 h (method ii), and (c) without any solvent by heating in the alloy bath in the temperature of ca. 90 °C for 7 h (method iii). Substrates were mixed together in molar ratio 1:1 (method i), 1:3 (method ii) and 1:10 (method iii). The highest yield (70%) for the desired hydrazide was achieved when the condensation reaction is performed in ethanolic medium in excess of hydrazine hydrate (method ii). Chemical structure of 8-(4-methoxyphenyl)-4-oxo-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]-triazine-3-carbohydrazide was confirmed by elemental analysis, thin layer chromatography (TLC) and spectral data (IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry (EI-MS) [9]. IMT was maintained as 50 mM stock solution in DMSO (final DMSO concentration in experimental cell cultures was less than 0.2%). A comparison of the structure of known A_{2A} receptor agonist and antagonist, and IMT was shown in Fig. 1.

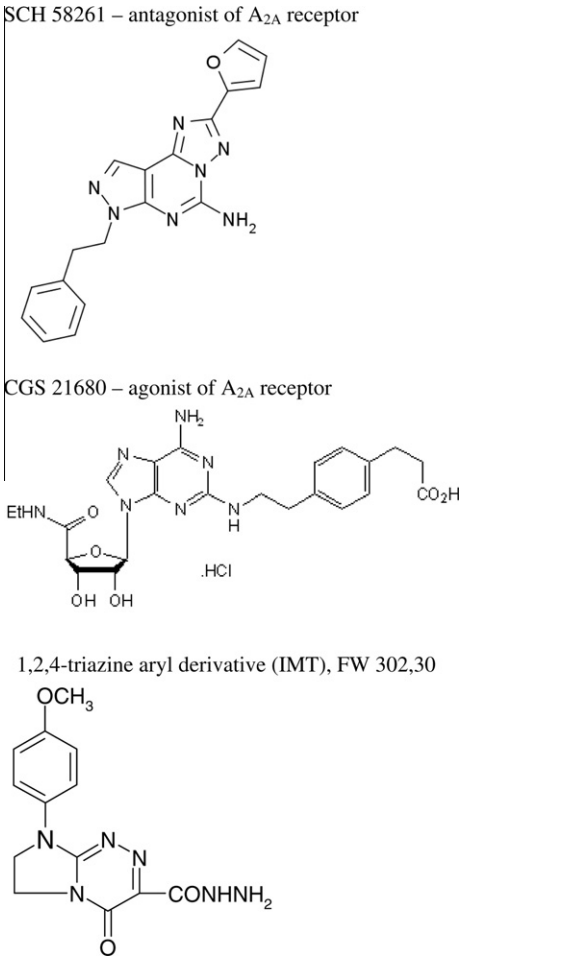

Fig. 1. Chemical structures of SCH 58261 (A_{2A} receptor antagonist), CGS 21680 (A_{2A} receptor agonist) and 1,2,4-triazine aryl derivative (IMT).

2.2. Cell cultures

Rat liver stellate cell line CFSC-2G was kindly provided from Dr. Marcos Rojkind (Department of Clinical Investigation, Walter Reed Army Medical Center, Washington). CFSC-2G cells were cultured in Eaglea's Medium (MEM), supplemented with 5% heat-inactivated fetal calf serum (FCS), 1% nonessential amino acids (NEAA), and 1% Antibiotic–Antimycotic, pH 7.4. Cells were seeded in tissue culture plates (Falcon, Bedford, MA, USA) and incubated at 37 °C in a humidified atmosphere of 5% CO_2. CFSC-2G cells were subcultured twice a week by trypsinization in 0.25% trypsin–EDTA solution after washing with Ca-Mg-free saline. Culture media, antibiotics, 0.25% trypsin–EDTA, FCS and NEAA were obtained from Sigma–Aldrich (Steinheim, Germany). In some experiments Hanks' Balanced Salt Solution (HBSS) (Sigma–Aldrich) was used.

2.3. 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

CFSC-2G cells were grown in 96-well plastic plates (Nunc, Roskilde, Denmark), 2×10^4 cells/well in Eaglea's Medium (MEM), supplemented with 5% FCS for 24 h. Then the medium was replaced by fresh one with addition of 0.1% FCS and 1–100 μM IMT. After 24, 48 and 72 h incubation at 37 °C in a humidified atmosphere of 5% CO_2 the MTT assay was performed in which the yellow tetrazolium salt is metabolized by viable cells to purple formazan crystals. CFSC-2G cells were incubated for 3 h with MTT solution (5 mg/ml). Formazan crystals were solubilized overnight in SDS buffer (10 SDS in 0.01 N HCl) and the product was quantified spectrophotometrically by measuring absorbance at 570 nm wavelength using E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA). Data were presented as % of control cells viability from four independent experiments each with eight separate cell cultures.

2.4. Influence of different ethanol concentration on CFSC-2G cell viability after IMT preincubation

CFSC-2G cells were grown in 96-well plastic plates (Nunc, Roskilde, Denmark), 2×10^4 cells/well in Eaglea's Medium (MEM), supplemented with 5% FCS. After 24 h incubation, the media was replaced by fresh one with addition 0.1% FCS and 1, 5 or 10 μM IMT After next 24 h incubation different ethanol (100–500 mM) concentration was added. Ethanol was purchased from Merck (Darmstad, Germany) and maintained as 1 M stock solution. The cells treated with ethanol were maintained in a humidified CO_2-incubator at 37 °C for 24 h. The toxicity of those chemicals was determined by MTT assay.