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Original article

Development of a novel furocoumarin derivative inhibiting NF-κB dependent biological functions: Design, synthesis and biological effects

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ABSTRACT

Nuclear Factor kappaB (NF-κB) plays a very important role in the control of gene expression and is deeply involved in several human pathologies. Accordingly, molecules targeting NF-κB dependent biological functions are considered of great interest. Virtual screening of furocoumarin libraries against NF-κB p50 allowed to rank compounds in respect to their expected ability to bind NF-κB and the identified compound might be considered for the development of analogs to be tested for biological activity on inhibition of NF-κB/DNA complex formation. The data reported in the present paper suggest that, following this approach, the best ranked compounds identified by virtual screening (a) strongly bind *in silico* to NF-κB and (b) efficiently inhibit the molecular interactions between ^{32}P-labeled NF-κB double stranded DNA and p50 or p50/p65 complex. These data allowed to develop a novel lead of great interest for inhibiting NF-κB dependent biological functions. This novel molecule (compound **2**), bearing a methyl group in the 9 position of the psoralen nucleus, exhibits high efficiency in inhibiting NF-κB/DNA interactions. In addition, we found that compound **2** is a potent inhibitor of IL-8 gene expression in TNF-α treated IB3-1 cystic fibrosis cells. Taken together, our data indicate that compound **2** might find an important place in the set of molecules of interest for the development of pharmaceutical strategies against the inflammatory phenotype of cystic fibrosis.

1. Introduction

Nuclear factor kappaB (NF-κB) plays a critical role in several biological processes, including cell cycle regulation [1–3], expression of specific genes [4,5], regulation of cell differentiation [6,7] and apoptosis [8–11]. On the other hand, alteration of NF-κB activity is associated with several human pathologies, including osteoporosis [12], rheumatoid arthritis [13] and cancer [14,15]. In addition, NF-κB is one among the master transcription factors responsible for inflammation in cystic fibrosis (CF) cells infected with *Pseudomonas aeruginosa* [16–20]. Accordingly, targeting NF-κB appears to be a relevant therapeutic strategy, as recently reviewed [21–23]. Unfortunately, targeting NF-κB is not a simple task. First of all, several proteins belong to the NF-κB family, including RelA (also known as p65), RelB, cRel/Rel, p50 and p52, originating homo- and hetero-dimers, the most common of them being p50/p65 and p52/RelB. Moreover, the metabolic regulation of NF-kB biological functions involves several control levels, one of the most important being the interaction with inhibitory proteins belonging to the IκB (inhibitor of NF-κB) family [24,25]. Among these, IκBα plays a major role as recently reviewed by Ferreiro and Komives [24], generating a complex with the NF-κB homo- or hetero-dimers; this molecular interaction prevents NF-κB to translocate to the nucleus and exert its regulatory functions on transcription of target genes [24–26]. Activation of NF-κB is operated by the IκB kinase (IKK) complex, which is composed by the two catalytic subunits IKKα and IKKβ and a regulatory subunit, the NF-kappaB essential modulator (NEMO, also known as IKKγ) [27]. Upon different stimuli, IKK phosphorilates the N-terminal signal response domain of NF-κB-bound IκBα, causing subsequent

Abbreviations: VS, virtual screening; PSO, psoralen; ANG, angelicin; TMP, 4,5',8-trimethylpsoralen; TMA, trimethylangelicin; IL-8, interleukin-8; EMSA, electrophoretic mobility shift assay; CF, cystic fibrosis; TNF-α, tumor necrosis factor α; NF-κB, nuclear factor kappaB; IκBα, inhibitory protein α of nuclear factor kappaB; PCR, polymerase–chain reaction; RT-qPCR, reverse transcription quantitative PCR; FBS, fetal bovine serum.

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polyubiquitinylation and proteasome-directed degradation, thus leading NF-κB to be translocated to the nucleus. At the nuclear level, NF-κB binds to the DNA target elements present in NF-κB regulated genes, as well as to co-activators of gene transcription [28]. In any case, the availability of NF-κB inhibitors is considered a major objective of therapeutic intervention.

In this respect, the concerted actions of researchers involved in bioinformatics, medicinal chemistry, cellular and molecular biology appear to be crucial for the development of novel drug candidates for important human pathologies [29–31]. In this context we have recently reported the possible use of a structured based virtual screening (VS) procedure to identify possible NF-κB binders [32–34], demonstrating that this *in silico* screening approach is suitable for the identification of low-molecular-weight compounds that inhibit NF-κB/DNA interactions and NF-κB dependent functions [32–34]. VS against NF-κB p50 using docking simulations was applied starting from a three-dimensional (3D) database containing more than 4.6 million commercially available structures. Docking simulations to p50 NF-κB were performed with a test set of six known inhibitors of NF-κB-DNA interactions [32,33]. In agreement with docking results, the highest-scored compound displayed a high level of inhibitory activity in EMSA experiments (inhibition of NF-κB/DNA interactions) and on biological functions dependent on NF-κB activity (inhibition of IL-8 gene expression in IB3-1 CF cells). In a more recent study, we constructed a focus library of differently substituted furocoumarins and analogs for an *in silico* screening against NF-κB with the aim of finding more potent NF-κB inhibitors belonging to the furocoumarin family [34]. We identified several furocoumarin derivatives expected to be active on NF-κB dependent functions: four of the five best ranked compounds (the commercially available ones) displayed interesting activities (inhibition of NF-κB/DNA interactions and IL-8 gene expression [34]), demonstrating the success of our VS approach.

The rationale of this report is based on the evidence that VS, in combination with molecular biology approaches, allows the identification of lead compounds that can be further modified to generate bioactive molecules useful in experimental therapy of human pathologies [29,34]. Following a recently published study [29], in the present work we have synthesized the most active compound identified through VS and a structurally-related derivative. Their activity on NF-κB/DNA interactions was determined by EMSA (Electrophoretic Mobility Shift Assay) and TNF-α induced expression of interleukin-8 (IL-8) in cystic fibrosis (CF) cells was evaluated by quantitative reverse transcription and polymerase–chain reaction (RT-PCR). This cellular system is very attractive, since it is well known that the hallmark in CF airway pathology is a characteristic elevated concentration of pro-inflammatory cytokines and chemokines, the most important of which is IL-8 [35–37]. On the other hand, it is firmly established that downstream activation of nuclear transcription factors, including NF-κB [17–19], is required for a cascade of pro-inflammatory cytokines and chemokines, first of all the NF-κB dependent IL-8, which is well known to play a role of master gene in PMN recruitment in CF lung [20,38,39]. Accordingly, candidate drugs interfering with NF-κB/DNA interactions and inhibiting IL-8 expression in CF cells are of great importance [38,40–45].

2. Results and discussion

2.1. Chemistry

Compound **1**, identified by VS as the best ranked ligand to p50-p50 dimer [34], was synthesized along with its closely related analog **2**, bearing a methyl group in the 9 position of the psoralen nucleus (Fig. 1). The rationale for synthesizing compound **2** was related with the observation that the introduction of a methyl group increases the inhibitory activity of furocoumarins (for instance psoralen and angelicin) on NF-κB/DNA interactions. As reported in Figs. 2 and 4, 5′,8-trimethylpsoralen (TMP) and trimethylangelicin (TMA) inhibit the interactions between p50 NF-κB and target ^{32}P-labeled DNA with higher efficiency in comparison to non-methylated psoralen and angelicin.

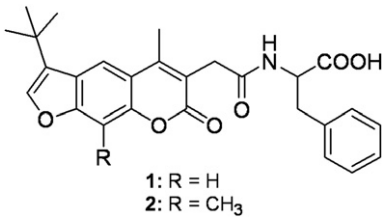

Fig. 1. Structures of synthesized compounds.

Methyl (7-hydroxy-2-oxo-2H-3-benzopyranyl)acetates **3**–**4** [46] were condensed with 1-chloropinacolone using a new MAOS (microwave-assisted organic synthesis) protocol to give the ethers **5**–**6** (Scheme 1). In this way, starting products were irradiated at 130 °C for 20 min in triethylamine and water to give the desired ethers with higher yield and reduced reaction times, if compared to previously reported methods [46]. Subsequent cyclization of the ketoalkyloxy side chain in alkaline medium afforded psoralenacetic acids **7**–**8**, which were finally reacted with DL-phenylalanine through activation of the carboxyl group *via* acyl chloride to give the amides **1a** and **2**. Special attention should be paid to this step because decarboxylation of psoralen acids can easily occur at temperature higher than room temperature. Finally, since a chiral center is present in the amide moiety of compound **1a**, the L-enantiomer **1b** was also synthesized starting from L-phenylalanine, in order to evaluate the influence of the stereochemistry on NF-κB-DNA interactions.

In order to obtain a first indication on biological activity, electrophoretic mobility shift assay (EMSA) was performed. This approach allows to rank even large set of newly synthesized compounds in respect to the effects on molecular interactions between NF-kB and target DNA sequences [32–34].

2.2. Biological activity of the compounds *1a–b* and *2*: EMSA studies

To determine biological activity of compounds **1a, 1b** and **2** in EMSA studies purified p50 NF-κB protein was first employed. The results obtained, shown in Fig. 3A, demonstrate that all the tested compounds are active in inhibiting NF-κB/DNA interactions, being compound **2** the most efficient. Accordingly, further analysis was performed on this compound (Fig. 3B) demonstrating an IC_{50} of about 30 μM and an efficiency in inhibiting the interaction of NF-κB-DNA to a reconstituted p50/p65 heterodimer (Fig. 3C). Interestingly, compound **2** is far more active than psoralen, TMP, angelicin and TMA (Table 1) in inhibiting NF-κB/DNA interactions. This set of experiments suggests that compound **2** might be a good candidate for developing molecules inhibiting the expression of NF-κB regulated genes. Interestingly, compound **2** shows docking activity to NF-κB (Fig. 4). Tyr57, Thr143, Lys144 and Lys145 are the amino acids apparently involved in the interactions between NF-κB and compound **2**.

On the basis of these observations, compound **2** was further characterized for its possible activity on the expression of interleukin-8 (IL-8) gene. It is firmly established that IL-8 gene expression is regulated by NF-κB [20–23]; therefore, since molecules inhibiting NF-κB/DNA interactions might exhibit inhibitory activities on NF-κB regulated genes [35–37], we were interested to determine the activity of compound **2** on IL-8 gene expression.