Cellular Signalling 26 (2014) 2614–2620

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Oxysterols act as promiscuous ligands of class-A GPCRs: *In silico* molecular modeling and *in vitro* validation

Cristina Sensi [a], Simona Daniele [c], Chiara Parravicini [b], Elisa Zappelli [c], Vincenzo Russo [d], Maria L. Trincavelli [c], Claudia Martini [c], Maria P. Abbracchio [b], Ivano Eberini [a,*]

[a] Laboratorio di Biochimica e Biofisica Computazionale, Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, Italy
[b] Laboratorio di Farmacologia Molecolare e Cellulare della Trasmissione Purinergica, Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, Italy
[c] Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Università degli Studi di Pisa, Pisa, Italy
[d] Cancer Gene Therapy Unit, Program of Immunology and Bio Immuno Gene Therapy of Cancer, Division of Molecular Oncology Scientific, Institute San Raffaele, Milan, Italy

ARTICLE INFO

Article history:
Received 15 July 2014
Accepted 15 August 2014
Available online 22 August 2014

Keywords:
Oxysterols
Cell signaling
G proteins
Cholesterol
Receptors/seven transmembrane domain
Molecular recognition

ABSTRACT

According to classical pharmacology, each neurotransmitter/hormonal receptor, including GPCRs, is exclusively activated by highly specific ligands. However, recent evidence challenges this dogma. Oxysterols are produced at inflammatory sites and can surprisingly potently activate the Epstein Barr virus induced gene receptor-2 (EBI2), a GPCR involved in adaptive immune responses. Similarly, oxysterols promiscuously operate CXCR2, a chemokine receptor participating to immune reactions and cancer development. Both EBI2 and CXCR2 are phylogenetically related to GPR17, another GPCR implicated in inflammatory/immune neurodegenerative events. Here, we used an integrated approach combining comparative modeling, molecular docking and *in vitro* experiments to investigate their potential interactions with oxysterols. All three receptors share the binding site to allocate oxysterols with different local arrangements, higher sensitivity to specific oxysterols and different activation thresholds. Such differences may dictate the diverse biological effects induced by oxysterols, depending on production site, concentration, specific spatiotemporal features and receptor expression on targeted cells. Thus, EBI2, CXCR2 and GPR17 are promiscuously operated by oxysterols making this class of ligands a '*fil rouge*' linking oxidative stress, inflammation and neurodegeneration. Such a transversal role may represent a conserved, "unspecific" (but selective) signaling mode, by which emergency molecules activate multiple receptors involved in inflammatory/immune responses.

1. Introduction

G-protein-coupled receptors (GPCRs) are integral cell membrane receptors organized in seven transmembrane α-helices associated to heterotrimeric G-proteins. They are involved in a plethora of physiological processes through a very efficient, specific and selective control of cell functions. In several pathologies, a dysregulation of their expression and/or activity has been described, and their pharmacological targeting is at the basis of the most up-to-date therapeutic strategies for many relevant human diseases. Indeed, most marketed drugs have been developed for their ability to target GPCRs, preferentially the class-A ones [1].

In the last decades, classical *in vitro* and *in vivo* studies suggested that each GPCR has a specific pharmacological profile and is operated by highly specific ligands. All the pharmacological efforts of the last years have been focused on the development of drugs selective for a single class of GPCR, or for a specific receptor, in order to achieve a successful therapeutic strategy without serious and limiting side effects. However, recent evidence challenges the currently accepted dogma that each receptor responds to a single endogenous ligand or a single family of related signaling molecules.

In this respect, oxysterols are well-known as natural and specific ligands of nuclear liver X receptor (LXR) α and β belonging to the cytoplasmic family of steroid receptors, whose activation controls lipid and cholesterol homeostasis inducing several target gene products. Furthermore, activation of LXRs by oxysterols can favor tumor progression by dampening native immune response [2] *via* activation of specific target genes and trans-repression of pro-inflammatory genes.

In 2011, Nature published two letters, in which it was demonstrated that EBI2, an orphan class-A GPCR involved in the immune response, can be operated by oxysterols [3,4]. Very recently, our research group contributed to demonstrating the ability of oxysterols to also operate a completely different class-A GPCR, CXCR2, a chemokine receptor involved in the control of the immune system and of cancer development [5]. All together, these observations suggest that a second level of operability, less specific and more transversal, exists, at least for some class-A GPCRs and for some ligands. The different affinity of

* Corresponding author at: Via Giuseppe Balzaretti, 9, 20133 Milan, Italy. Tel.: +39 02 50318362; fax: +39 02 50318284.

http://dx.doi.org/10.1016/j.cellsig.2014.08.003
0898-6568/

oxysterols with respect to CXCR2 endogenous ligands also suggests that this second activation mode can become of great interest under specific pathological conditions, *e.g.* when oxysterols are produced locally at very high concentrations. These results also raise the hypothesis that activation of some class-A GPCRs by oxysterols represents a new way to modulate some specific immune system function.

At the moment, no molecular data about the activation mechanism of CXCR2 by oxysterols have been reported. In order to carefully analyze at an atomistic level this mechanism, and to evaluate if oxysterols can act as transversal ligands also for other structurally related class-A GPCRs, we modeled, through comparative *in silico* modeling, the 3D structures of human CXCR2 and of two phylogenetically and structurally related receptors, EBI2 and GPR17, a P2Y-like receptor involved in central nervous system function [6,7]. We then investigated the mechanism of molecular recognition between these three receptors and oxysterols through *in silico* molecular docking and well-established *in vitro* experimental approaches. Results show that oxysterols bind to and activate transversally the three receptors, thus extending the number of GPCRs and tissues involved in this new type of signaling, with significant implications for the biology and pharmacology of these systems.

2. Materials and methods

2.1. Comparative modeling

The tridimensional structures of the human forms of G-protein coupled receptor 183 (EBI2), C-X-C chemokine receptor type 2 (CXCR2), and uracil nucleotide/cysteinyl leukotriene receptor (GPR17) were built through a classical homology/comparative modeling, by using the MOE Homology Model program of the MOE Protein module of the Molecular Operating Environment (MOE 2012.10, Chemical Computing Group, Montreal, Canada). We selected, among the human class-A GPCRs, the primary structures which present two conserved pairs of cysteines involved in putative disulfide bridges: one linking the extracellular loop (EL) 2 to transmembrane helix (TM) 3, the other connecting N-terminus with EL3. This subgroup includes members of the P2Y, CysLT, AGT, and chemokine (CCR, CXCR, CCRL, XCR, CX3CR) receptor families together with APJ, EBI2, GP174, GPR4, GP132, SPR1, G109A, G109B, GP171, GPR31, GPR34, GPR81, P2RY5, P2RY9, and P2Y10 receptors. The primary structures of the three target proteins were obtained from the UniProt database, and are identified, respectively, with the following codes: P32249 for EBI2, P25025 for CXCR2 and Q13304 for GPR17. For all these sequences a global alignment based on T-Coffee algorithm [8] has been produced and set as reference alignment for all the homology modeling procedures. The template for the modeling procedures of all the selected targets was the crystallographic structure of CXCR4 deposited in the RCSB PDB, and identified as 3ODU [9]. The recent NMR structure of the human C-X-C chemokine receptor type 1 (CXCR1) [10], which has a greater identity with respect to CXCR2 than CXCR4, is not a suitable modeling template, since its orthosteric ligand binding site is blocked by the extracellular loop 2 (EL2) (Fig. 1). The 'automatic disulfide bond detection' option was activated, but the presence and the geometry of the conserved cysteines were manually checked. For each receptor, ten models of the mainchain were built, and, for each one of these mainchains, one sidechain model was built using the unary quadratic optimization (UQO) procedure [11]. Each single model was submitted to a brief series of energy minimizations (EMs) meant only to relieve steric strain and scored according to the GB/IV scoring function [12]. The top scoring model for each receptor was further refined through an EM procedure to an RMS gradient value of 0.5 kcal/mol Å. In all the molecular mechanics procedures, the Amber12:EHT forcefield with the reaction field solvation model. The disulfide bonds not automatically detected by the modeling procedure were manually created through the MOE Builder module. The ELs were then submitted to EM runs, after fixing transmembrane helices and intracellular loops (ILs). Six EM runs, all down to an RMS gradient of 0.5 kcal/mol Å, were carried out while restraining the EL atoms with a quadratic force from 10^5 down to 10^{-1} kcal/mol Å^2. A further EM run was carried out without any restraint down to an RMS gradient of 0.5 kcal/mol Å.

The quality of the final model was carefully checked with the Geometry program of the MOE Protein module [13,14].

2.2. Binding site analysis

The binding sites for the three investigated receptors were identified through the Site Finder program of the MOE Compute module, which uses a geometric approach to calculate possible binding sites in a receptor starting from its 3D atomic coordinates.

2.3. Oxysterol database preparation

The molecular database file containing all the oxysterols reported in literature to bind class-A GPCRs was drawn through the MOE builder [3–5,15]. All the structures were energy minimized with the Amber12:

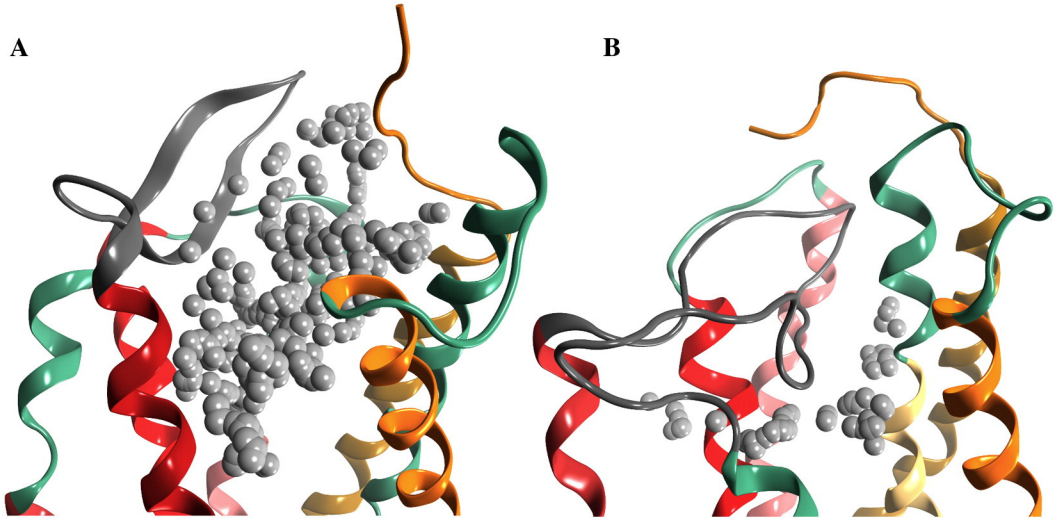

Fig. 1. Comparison between CXCR4 and CXCR1 extracellular loops 2. Alpha sphere highlights the ligand binding site of both receptors; secondary structures are rendered as ribbon and colored according GPCR characteristics. EL2 that overhang the binding site of CXCR4 (A) and occupies that of CXCR1 (B) is colored in silver.