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ETP-46321, a dual p110α/δ class IA phosphoinositide 3-kinase inhibitor modulates T lymphocyte activation and collagen-induced arthritis

L. Aragoneses-Fenoll [a], M. Montes-Casado [a], G. Ojeda [a], Y.Y. Acosta [b], J. Herranz [b], S. Martínez [c], C. Blanco-Aparicio [c], G. Criado [d], J. Pastor [c], U. Dianzani [e], P. Portolés [a,*], J.M. Rojo [b,*]

[a] Unidad de Inmunología Celular, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain
[b] Departamento de Medicina Celular y Molecular, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain
[c] Experimental Therapeutics Programme, Spanish National Cancer Research Centre (CNIO), Spain
[d] Hospital 12 de Octubre, Instituto de Investigación Hospital 12 de Octubre (I+12), E-28041 Madrid, Spain
[e] Interdisciplinary Research Center of Autoimmune Diseases (IRCAD) and Department of Health Sciences, University of Piemonte Orientale (UPO), Novara, Italy

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ABSTRACT

Class IA phosphoinositide 3-kinases (PI3Ks) are essential to function of normal and tumor cells, and to modulate immune responses. T lymphocytes express high levels of p110α and p110δ class IA PI3K. Whereas the functioning of PI3K p110δ in immune and autoimmune reactions is well established, the role of p110α is less well understood.

Here, a novel dual p110α/δ inhibitor (ETP-46321) and highly specific p110α (A66) or p110δ (IC87114) inhibitors have been compared concerning T cell activation *in vitro*, as well as the effect on responses to protein antigen and collagen-induced arthritis *in vivo*.

In vitro activation of naive $CD4^+$ T lymphocytes by anti-CD3 and anti-CD28 was inhibited more effectively by the p110δ inhibitor than by the p110α inhibitor as measured by cytokine secretion (IL-2, IL-10, and IFN-γ), T-bet expression and NFAT activation. In activated $CD4^+$ T cells re-stimulated through CD3 and ICOS, IC87114 inhibited Akt and Erk activation, and the secretion of IL-2, IL-4, IL-17A, and IFN-γ better than A66. The p110α/δ inhibitor ETP-46321, or p110α plus p110δ inhibitors also inhibited IL-21 secretion by differentiated $CD4^+$ T follicular (Tfh) or IL-17-producing (Th17) helper cells. *In vivo*, therapeutic administration of ETP-46321 significantly inhibited responses to protein antigen as well as collagen-induced arthritis, as measured by antigen-specific antibody responses, secretion of IL-10, IL-17A or IFN-γ, or clinical symptoms.

Hence, p110α as well as p110δ Class IA PI3Ks are important to immune regulation; inhibition of both subunits may be an effective therapeutic approach in inflammatory autoimmune diseases like rheumatoid arthritis.

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1. Introduction

Class I phosphoinositide 3-kinases (PI3Ks) phosphorylate the 3 position of 4,5-biphosphate phosphoinositides located in cell membranes. This produces phosphatidylinositide 3,4,5-triphosphate (PIP_3) allowing the binding of proteins possessing pleckstrin homology (PH) domains. The recruited proteins are then activated to start metabolic cascades essential to different aspects of cell growth, proliferation, survival, differentiation, and migration [1–3]. Consequently, they are also essential to the development of normal and pathologic immune responses, including organ-specific or systemic autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus [4–7].

Class I PI3K are heterodimers formed by regulatory and catalytic subunits; recruitment of regulatory subunits serve to bring the catalytic subunits close to the membranes were both PI3K activators and substrates are located. The nature of regulatory subunits further differentiates class I PI3K into class IA and class IB:

Abbreviations: CIA, collagen induced arthritis; ConA, Concanavalin A; EAE, experimental autoimmune encephalomyelitis; KLH, keyhole limpet hemocyanin; PI3K, phosphoinositide 3-kinase; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

* Corresponding authors at: Unidad de Inmunología Celular, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. Pozuelo-Majadahonda, km 2, E-28220 Majadahonda, Madrid, Spain (P. Portolés). Centro de Investigaciones Biológicas, CSIC, Departamento de Medicina Celular y Molecular, Ramiro de Maeztu, 9, E-28040 Madrid, Spain (J.M. Rojo).

E-mail addresses: pportols@isciii.es (P. Portolés), jmrojo@cib.csic.es (J.M. Rojo).

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Class IA catalytic isoforms (p110α, p110β and p110δ) form heterodimers with the regulatory subunits p85α, p55α, p50α, p85β, and p55γ; activation is characteristically dependent on regulatory subunit recruitment to Tyr-phosphorylated Y-x-x-M sequence motifs. Class IB catalytic subunits (p110γ) bind regulatory subunits p101 and p84/p87; they are activated upon binding of their regulatory subunits to G-protein coupled receptors (GPCR).

Class I PI3K subunits have oncogenic potential; and particularly the p110α catalytic isoform is frequently mutated in different cancer cells [8]. Hence, PI3K inhibitors have been actively pursued as anti-cancer drugs. However, their impact on the immune system should be also carefully determined, in first place, because the importance of PI3K in the development of immune reactions confers an immunotherapeutic potential on PI3K inhibitors; secondly, because the overall impact of these inhibitors in tumor immunity needs to be determined [9].

Whereas the expression of p110α and p110β catalytic subunits is wide, the p110δ and p110γ polypeptides are mainly expressed in hematopoietic cells including T and B lymphocytes. Indeed, genetic and pharmacological data show that both the class IA p110δ and the class IB p110γ subunits are essential to adequate development, activation and differentiation of B and T lymphocytes, as well as interesting targets for therapy in immunopathology or cancer immunotherapy [5,7,10–12]. When T and B lymphocytes are considered, class IA p110δ catalytic subunits have specific functions in signaling through the antigen receptor, amplification of signals by B cell or T cell costimulatory molecules (i.e., CD19, CD28, ICOS (CD278, also called H4 [13])), through TNF family molecules, or cytokine receptors.

Among Class IA PI3Ks, T lymphocytes express high levels of p110α and p110δ catalytic subunits and comparatively low levels of p110β [14]. Intriguingly, p110α binds better than p110δ to PI3K regulatory subunits, and consequently it is more efficiently recruited to costimulatory molecules like CD28 and ICOS [14], suggesting that p110α might play a relevant role in T lymphocyte function.

However, the data on the role of p110α in lymphocyte function are scarce, in part because of the embryonic lethality of p110α-deficient mice, in part because of the relative non-specificity of some p110α inhibitors [15,16]. Still, specific silencing [14] or the use of highly specific inhibitors of p110α like A66 [17] show a clear, if minor, role of p110α on B cell and T cell activation. Here, the effect of ETP-46321, a dual inhibitor of the p110α and p110δ PI3K isoforms [18,19] has been compared to the effect of highly specific p110δ- or p110α-specific inhibitors (IC87114 and A66, respectively). We show that ETP-46321 shares the characteristics of both inhibitors, and is a strong inhibitor of lymphocyte proliferation and the secretion of cytokines essential to antibody or inflammatory responses.

Rheumatoid arthritis (RA) is a systemic, inflammatory, autoimmune disease characterized by uncontrolled inflammation of the joints and the presence of autoantibodies directed against multiple autoantigens. Its precise etiology is unknown, yet T lymphocytes intervene in the pathogenic process, as shown by the presence of T cells in the inflammatory infiltrate of affected joints and the strong association of the disease with molecules involved in T cell activation [20]. Furthermore, blockade of CD28-dependent T-cell costimulation by abatacept (CTLA-4Ig) has provided a successful therapy for RA [21,22].

Its relatively high incidence among the general population (approximately 1% worldwide) makes RA an important target for drug discovery. The animal model of collagen-induced arthritis (CIA) reproduces many features of human RA and is widely used to assess the effects of potential novel therapies [23].

The fact that p110δ PI3K contributes significantly to antigen activation of antibody producing B lymphocytes, but both the p110α and p110δ PI3K isoforms participate in B lymphocyte development in the bone marrow and in B cell survival in the periphery [24] adds interest to the study of dual p110α and p110δ PI3K inhibitors in RA.

Our data indicate that therapeutic administration of ETP-46321 can be successfully used *in vivo* to inhibit secretion of antigen-specific antibodies and effector cytokines in response to protein antigen as well as in mice undergoing collagen-induced arthritis. Thus, PI3K inhibitors like ETP-46321 can be candidate drugs to treat abnormal adaptive immune responses, including autoimmune diseases where $CD4^+$ T lymphocytes and antibody responses have a prime role.

2. Materials and methods

2.1. Mice

C57BL/6 mice aged 8–16 weeks were used throughout this study. They were bred in the animal care facility of the Centro de Investigaciones Biológicas under specific pathogen-free conditions. For the collagen-induced arthritis experiments, female DBA/1O-laHsd mice were purchased from Harlan Laboratories (Horst, The Netherlands). They were housed in the animal facility of the Centro Nacional de Microbiología, Instituto de Salud Carlos III, in seal-safe cages under air flow supply. All the experimental procedures were performed according to established institutional and national guidelines.

2.2. Inhibitors

The PI3Kα/δ inhibitor ETP-46321 was synthesized by the Experimental Therapeutics Programme, Spanish National Cancer Research Centre (CNIO), as described in [18,19]. PI3Kα inhibitor A66 was from Selleck Chemicals (Houston, Texas); PI3Kδ inhibitor IC87114 was from Symansis Pty. (Timaru, New Zealand); LY 294002 was from Sigma–Aldrich (Saint Louis, Missouri). Some characteristics of these inhibitors are summarized in Table 1.

2.3. T lymphocyte isolation and activation

To obtain naive $CD4^+$ T lymphocytes ($CD4^+CD62L^+$ T cells), spleens were passed through a 70 μm mesh. After centrifugation, red blood cells were lysed and the cells washed in culture medium (Click's medium supplemented with 10% heat inactivated FCS). Then, naive $CD4^+$ T cells were isolated using Miltenyi $CD4^+CD62L^+$ T cell isolation kit II for mouse cells (Ref. 130-093-227, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' instructions. The isolated cells were routinely >97% $CD4^+$, >95% $CD62L^+$. Cells (10^6) were cultured in 1 ml culture medium in 24-well culture plates (Costar) pre-coated with anti-CD3 antibody (YCD3-1 [30], 5 μg ml^{-1}). Where indicated, anti-CD28 (H57, 2.5 μg ml^{-1}, eBioscience, San Diego, California), DMSO or inhibitors dissolved in DMSO (1 μl per culture) were added. At 24 h, the cultures were resuspended, centrifuged and the supernatants assayed for cytokine content.

Table 1
IC50 values (nM) of PI3K inhibitors for catalytic activity of different PI3K isoforms.

	p110α	p110β	p110δ	p110γ	mTORC1/C2
A66 (α)	32.0	20,000.0	18,050.0	18,810.0	
IC87114 (δ)	>100,000.0	1820.0	70.0	1240.0	
ETP-46321 (α,δ)	2.4	549.0	14.0	153.0	>5000.0
LY294002 (Broad)	700.0	306.0	1330.0	7260.0	8910.0

Data compiled from references [17–19,25,26–29].

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