Drug Discovery

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A Trifluorinated Thiazoline Scaffold Leading to Pro-apoptotic Agents Targeting Prohibitins**

Alba Pérez-Perarnau, Sara Preciado, Claudia Mariela Palmeri, Cristina Moncunill-Massaguer, Daniel Iglesias-Serret, Diana M. González-Gironès, Miriam Miguel, Satoki Karasawa, Satoshi Sakamoto, Ana M. Cosialls, Camila Rubio-Patiño, José Saura-Esteller, Rosario Ramón, Laia Caja, Isabel Fabregat, Gabriel Pons, Hiroshi Handa, Fernando Albericio, Joan Gil,* and Rodolfo Lavilla**

Abstract: *A new class of small molecules, with an unprecedented trifluorothiazoline scaffold, were synthesized and their pro-apoptotic activity was evaluated. With an EC_{50} in the low micromolar range, these compounds proved to be potent inducers of apoptosis in a broad spectrum of tumor cell lines, regardless of the functional status of p53. Fast structure–activity relationship studies allowed the preparation of the strongest apoptosis-inducing candidate. Using a high performance affinity purification approach, we identified prohibitins 1 and 2, key proteins involved in the maintenance of cell viability, as the targets for these compounds.*

Natural products provide an invaluable source of medicinal leads and thus have a significant impact on drug development.[1,2] During the last decades, several bioactive natural products containing oxazole and/or thiazole rings in their structures have been isolated.[3] Those heterocyclic moieties arise from enzymatic modifications (cylodehydrations) and are essential for bioactivity. However, synthetic access to these compounds is challenging: long synthetic sequences are required, they are non-parallelizable, and present great difficulties for the preparation of analogues, thus representing a major impediment for medicinal chemistry.

A new paradigm in scaffold design was provided by Hamilton and co-workers,[4] who reported an avenue for the development of new bioactive compounds based on the structural analogy of polyarylic compounds with α-helices. Furthermore, fluorinated compounds have unique biological and physicochemical properties which generate important chemical products and pharmaceuticals.[5–7] Although organofluorine compounds are scarce in natural products, 20–25% of drugs in the pipeline have at least one fluorine atom. Hence the introduction of fluorine in bioactive structures often leads to improved pharmacological properties. This observation therefore highlights the need for new chemical approaches to generate such compounds in an efficient manner. Thus, we reasoned that fluorinated diarylthiazoles could be a promising scaffold to mimic complex natural products displaying potent and selective bioactivity (Figure 1A).

A series of fluorinated thiazole derivatives containing distinct aryl groups was prepared through a synthetic sequence which comprises the arylation of the corresponding thiazole precursor by transition-metal couplings and the subsequent fluorination of the formed diarylthiazole **2** (Figure 1B). The Suzuki–Miyaura cross-coupling[8] is the best synthetic approach to obtain symmetric 2,5-diaryl thiazoles (Figure 1Ba). In contrast, the preparation of unsymmetrical

[*] Dr. A. Pérez-Perarnau,[+] Dr. C. M. Palmeri, C. Moncunill-Massaguer, Dr. D. Iglesias-Serret, Dr. D. M. González-Gironès, Dr. A. M. Cosialls, Dr. C. Rubio-Patiño, J. Saura-Esteller, Dr. I. Fabregat, Dr. G. Pons, Prof. J. Gil
Departament de Ciències Fisiològiques II, University of Barcelona
Institut d'Investigació Biomèdica de Bellvitge (IDIBELL)
L'Hospitalet de Llobregat (Spain)
E-mail: jgil@ub.edu

Dr. L. Caja, Dr. I. Fabregat
Institut d'Investigació Biomèdica de Bellvitge (IDIBELL)
L'Hospitalet de Llobregat (Spain)

Dr. S. Preciado,[+] Dr. M. Miguel, Dr. R. Ramón, Prof. R. Lavilla
Barcelona Science Park
Baldiri Reixac 10-12, 08028 Barcelona (Spain)
E-mail: rlavilla@pcb.ub.es

Prof. R. Lavilla
Laboratory Organic Chemistry
Faculty of Pharmacy, University of Barcelona (Spain)

Prof. F. Albericio
Department of Organic Chemistry
University of Barcelona (Spain)
and
Institute for Biomedical Research Barcelona and CIBER-BBN
Baldiri Reixac 10–12, 08028 Barcelona (Spain)
E-mail: albericio@irbbarcelona.org

Dr. S. Karasawa, Dr. S. Sakamoto, Prof. H. Handa
Tokyo Institute of Technology, Yokohama (Japan)

[+] These authors contributed equally to this work.

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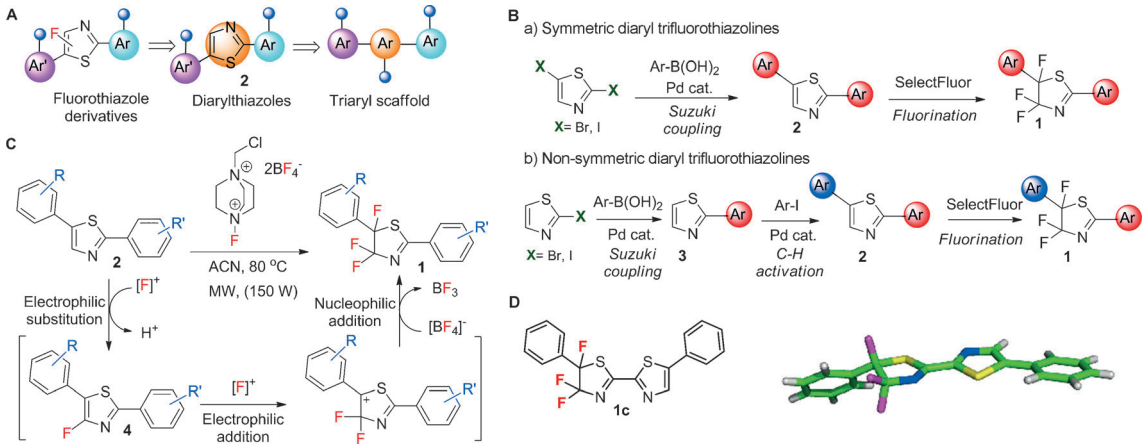

Figure 1. Structural and synthetic aspects of fluorinated thiazolines. A) Fluorinated diarylthiazoles as triaryl scaffolds. B) Synthetic access to trifluorinated thiazolines. C) Mechanistic hypothesis for the trifluorination reaction. D) Chemical and three-dimensional structure of **1c** from the X-ray diffraction of a monocrystal.[27] ACN = acetonitrile, MW = microwave.

derivatives was achieved by a sequential Suzuki coupling from 2-bromothiazole, followed by a second arylation step, by C–H activation[9] (Figure 1Bb). Related strategies were useful for specific compounds (see the Supporting Information). Both protocols are suitable for the introduction of a high degree of chemical diversity, thus allowing a wide variety of substituents on the phenyl rings. The diarylthiazoles **2** were fluorinated using Selectfluor in acetonitrile by means of conventional or microwave-assisted heating. Unexpectedly, this process afforded the trifluorinated compounds **1**.[10–12] This unprecedented transformation presumably takes place through a stepwise mechanism involving the initial electrophilic fluorination of the unsubstituted thiazole position followed by a second electrophilic process, thus yielding a fluorinated carbenium ion, which is then trapped by the BF_4^- anion and leads to the final trifluorothiazolines **1** (Figure 1C). The connectivity of the trifluorothiazoline moiety was elucidated through NMR studies. In addition, X-ray diffraction analysis of a single crystal of **1c** (Figure 1D) unequivocally confirmed the proposed structure.

Although the yields obtained were moderate (17–55%), it should be noted that three C–F bonds were formed in this single treatment. Interestingly, when using a stoichiometric amount of Selectfluor, monofluorinated thiazoles were obtained as the main products. Also, under standard reaction conditions, the electronically deactivated diarylthiazoles **2** only afforded the monofluorinated thiazoles **4**.

Since one hallmark of cancer development and progression is the resistance of cancer cells to apoptosis,[13,14] we analyzed the biological activity of a primary series of fluorinated thiazoline compounds by means of a cell-based apoptosis screening. While the thiazolines **1a–f** (Figure 2A) demonstrated important pro-apoptotic activities in Jurkat and HeLa cells in a dose-dependent manner (Figure 2B), the related thiazoles **2a** and **4c** did not display significant cytotoxic effects (see Figure 2C and Figure S1). These results showed a relevant cytotoxicity associated with the novel scaffold, where small chemical modifications introduced along the series triggered relevant differences regarding the biological effects.

The induction of apoptosis is closely related to p53 activation.[15] This is a key protein in cancer treatment, since the *p*53 gene is mutated in roughly 50% of cancers, therefore leading to serious drug resistance. We next assessed the pro-apoptotic effects of **1a** and **1b** in an extended panel of cancer cell lines with defined mutational status, gene expression, and other molecular characteristics, including either wild-type or altered p53 pathway (http://p53.free.fr/). The EC_{50} was calculated at 24 hours after treatment for all these cell lines (see Figure S2). All 20 cell lines analyzed displayed substantial sensitivity to **1a** with an EC_{50} in the low micromolar range ($< 10\ \mu M$), independently of *p*53 status. In comparison, the **1b** analogue was at least twofold less active in the cell-based apoptosis assay, thus demonstrating the improvement in potency achieved in the SAR studies (see Figure 2 and Table S1). Furthermore, normal human T cell lymphocytes were found to be more resistant to **1a** than primary leukemic B cells from chronic lymphocytic leukemia (CLL) patients (Figure 2D).

Mitochondrial outer membrane permeabilization (MOMP) and release of mitochondrial intermembrane proteins, such as cytochrome *c*, are critical steps in the control of apoptosis.[13] We evaluated whether pro-apoptotic effects induced by fluorinated thiazolines occurred through cytochrome *c* release from mitochondria to the cytosol. To this end, Jurkat cells were treated with either **1a** or **2a**, and cytochrome *c* release was analyzed in cytosolic and mitochondrial extracts. The compound **1a** induced the release of cytochrome *c* from mitochondria to the cytosol while **2a** did not (Figure 2E).

As the trifluorinated thiazolines **1** are generated as racemic mixtures, we considered that their resolution and the subsequent study of the biological activity of each enantiomer would provide relevant information. In this context, we successfully separated the racemic mixture of **1b** by means of chiral-phase HPLC on a semipreparative scale and determined the structure and optical activity of both enantiomers (X-ray diffraction and polarimetric analyses; see Figure S3A). Interestingly, the eutomer (−)-**1b** induced a considerable loss of cell viability in HeLa cells, while its

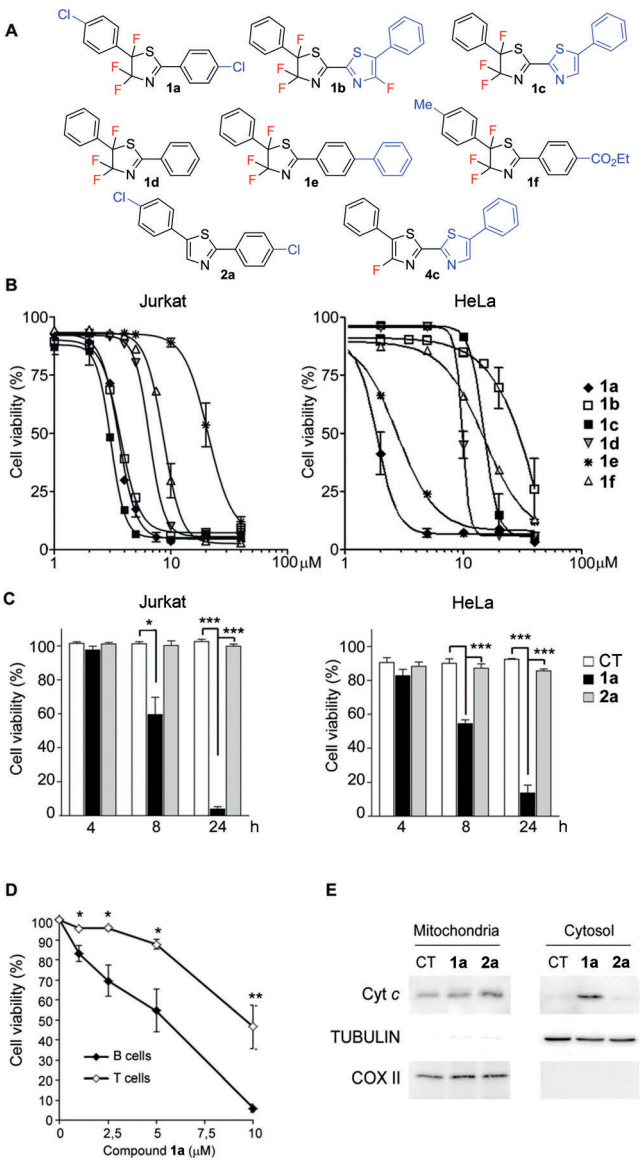

Figure 2. Effects of fluorinated thiazolines on cancer cells viability. A) Chemical structures of thiazoline-derived compounds. B) Jurkat and HeLa cells were exposed to DMSO or to a range of doses of the indicated compounds and cell viability was assessed after 24 h. C) Cell lines were treated with DMSO (CT) or a single dose of 10 µM **1a** or **2a** for different time periods. D) B and T lymphocytes from CLL patients ($n=4$) were incubated with a range of doses of **1a** for 24 h and cell viability was assessed. E) Thiazoline-induced apoptosis occurs through cytocrhrome *c* release. Jurkat cells were treated for 8 h with either **1a** or **2a** and cytochrome *c* release was analyzed. Viability data represent the percentage of non-apoptotic cells (Annexin V negative) versus DMSO-treated controls, shown as mean values ± SEM of at least three independent experiments. Differences between paired values are statistically significant as determined by Student's t-test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ **1a**-treated cells versus DMSO- or **2a**-treated cells (in C) or B lymphocytes versus T lymphocytes (in D).

distomer (+)-**1b** was considerably less potent. These results were confirmed in the mantle cell lymphoma Z-138 cell line (see Figure S3B). Although not exclusively, the fluorescence emitted by **1b** co-localized with mitochondria stained with MitoTracker. Cells treated with the vehicle for 4 hours and cells treated with racemic **1b** or (+)-**1b** presented filamentous mitochondrial structures. Only those cells incubated with (−)-**1b** showed extensive disruption of the mitochondrial network (see Figure S3C).

To study the mechanism by which fluorinated thiazolines induced apoptosis, we sought to identify potential target proteins by performing an affinity-based proteomic screening using polymer-coated magnetic nanobeads (FG beads).[16,17] The pro-apoptotic fluorinated thiazoline **1a** was selected for pull-down experiments. Thus, to ensure its immobilization onto the beads, we synthesized a trifluorothiazoline derivative with a carboxylic acid attached to the aryl ring linked to the thiazole 2-position (**1g**; Figure 3A). This derivative was suitably conjugated with the amino-functionalized FG beads through an amide bond.

In this context, we prepared the compound **1h**, the amide derivative of the carboxylic acid **1g**, which mimics the probe and therefore served to demonstrate its suitability. We first tested the target-binding capacity of **1g** and **1h** in a cell viability assay, and compared it to that of the primary hit compound **1a**. While **1g** failed to induce apoptosis in Jurkat and in HeLa cells, probably because of limited cell permeability, **1h** retained the pro-apoptotic activity of **1a** (see Figure S4). This result indicates that target proteins likely interact with **1h** in a similar way to that with the parent thiazoline **1a**.

Next, to identify the **1a**-target proteins, we conjugated **1g** to amino-functionalized FG beads (Figure 3B). To purify **1g**-interacting proteins, unconjugated and **1g**-conjugated FG beads were exposed to whole HeLa cell lysates. After extensive washing, proteins bound to the surface-immobilized compound were eluted under harsh conditions, separated by SDS-PAGE, and visualized by silver staining (see Figure S5A). The pattern of bands obtained was further confirmed in Jurkat cell lysates (Figure S5B). For selectivity profiling against potential off-targets, we followed a drug competition strategy. Increasing concentrations of active **1a** or an excess of the inactive analogue **2a** were incubated with protein lysates from HeLa cells before the pull-down assay was carried out. The yields of two proteins present in the standard purification were significantly reduced or even absent in the **1a**-competition sample, thus suggesting potential specific targets. Remarkably, the free compound **2a**, which showed no pro-apoptotic activity, was unable to impair the interaction of these potential target proteins with the **1g**-conjugated FG beads (Figure 3C). Additional competition affinity purification was carried out with the active thiazoline **1b**, and confirmed the same putative target proteins. We next obtained these candidate protein bands and identified them as prohibitin 1 (PHB1) and prohibitin 2 (PHB2) by means of tandem mass spectrometry (MS/MS). The target identity was confirmed by SDS-PAGE immunoblotting using specific antibodies, where the disappearance of the target protein bands in the competition sample with the unmodified compound **1a** was visible. Notably, target proteins remained bound to the immobilized probe at the lower concentrations of the free active competitor, thus revealing a dose-dependent interaction. Furthermore, the inactive probe **2a** was unable to

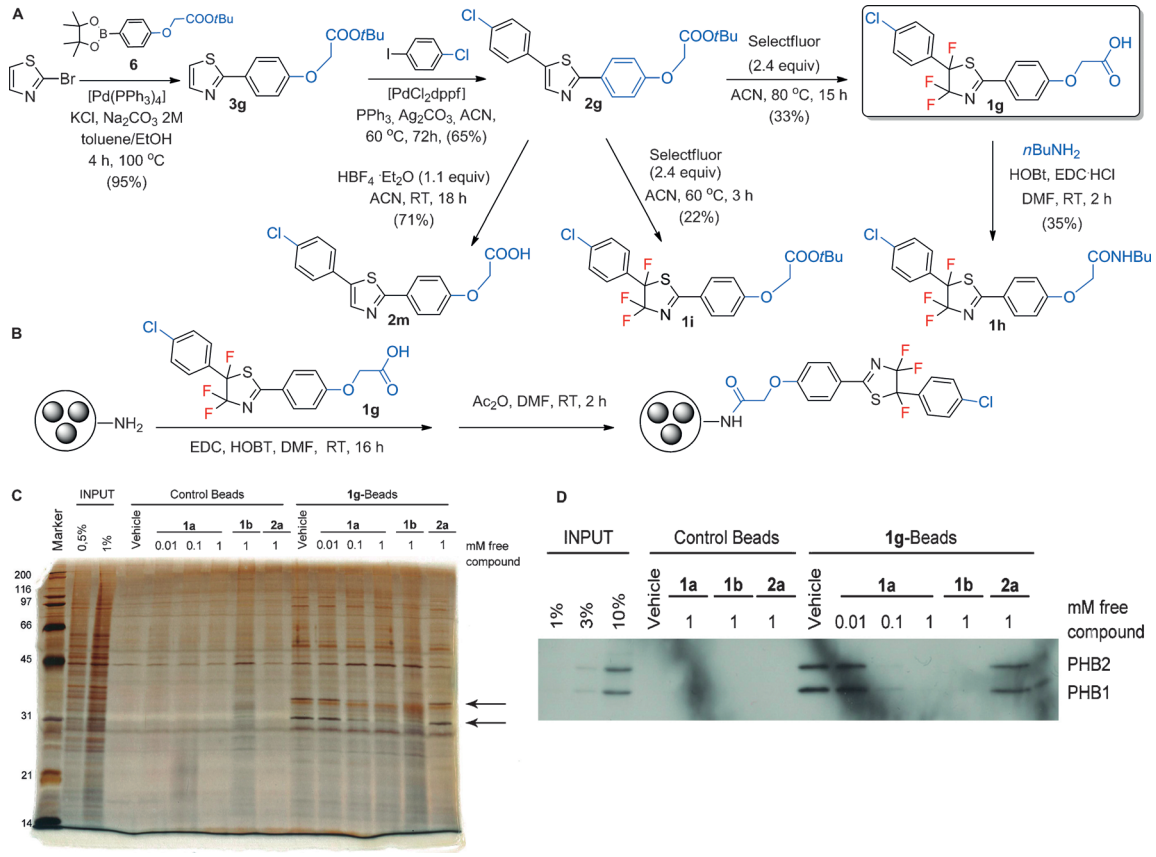

Figure 3. Identification of prohibitins as the main targets for pro-apoptotic fluorinated thiazolines. A) Synthesis of the trifluorinated precursor **1g**, and the positive control **1h** for the affinity probes. B) Scheme of drug immobilization of **1g** onto the magnetic nanobeads. C) Drug competition binding assay and purification of **1g**-interacting proteins. 200 μg of HeLa cell lysate was pre-treated with either 5% DMSO (Vehicle), different concentrations of free active compound **1a** (0.01, 0.1, 1 mM), or the highest concentration of free **1b** or free inactive compound **2a** (1 mM). Afterwards control beads (non-conjugated) or **1g**-beads (1 mM **1g**-conjugated beads) were incubated with pre-treated HeLa cell lysates. Arrows indicate the **1g**-interacting proteins selectively kept by **1a.** D) Target identity validation by Western blot analysis using specific antibodies. DMF = *N*,*N*-dimethylformamide, dppf = 1,1′-bis(diphenylphosphino)ferrocene, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, HOBt = hydroxybenzotriazole.

impair the binding of prohibitins to the immobilized compound (Figure 3D).

To further test whether prohibitins were bona fide targets for fluorinated thiazolines, and given that PHB1 and PHB2 are closely related proteins which interact in vivo to form complexes,[21,22] we expressed isolated GST-tagged recombinant prohibitins in *Escherichia coli*. Protein samples were obtained and subjected to SDS-PAGE to examine their binding affinity to **1g**. To exclude that this binding was caused by the reporter, we expressed GST protein and used it as a control. GST-PHB1 and GST-PHB2 bound to the **1g**-conjugated FG beads (see Figure S6). This binding could not be ascribed to any complex formation between PHB1 and PHB2. Taken together, our results support the notion that **1a** engaged both intended targets, thereby revealing a direct physical interaction.

Prohibitins are specifically required in proliferating cells, thus indicating a potential role in cancer progression.[18] The major contribution of prohibitins to the preservation of mitochondrial integrity[19] and the prevention of apoptosis[20] and senescence[21] might explain their overexpression in several types of tumor cells. Prohibitins are invariant components of the mitochondrial structure. However, recent discoveries have emphasized their role in other cellular compartments, thus showing that these proteins contribute to the regulation of several signaling pathways.[22] Prohibitins have recently emerged as key elements in the maintenance of cellular quiescence and in determining whether cells should proliferate or enter programmed cell death.[23] There are records of natural products (aurilide[24] and rocaglamides[25]) which bind to prohibitins.[26] However, the complexity of such compounds and synthetic/availability issues compromise their use in biological studies and in therapeutics. In contrast, our new chemotype is free from these problems and can be readily used in biological and medicinal chemistry programs. Finally, these fluorinated thiazolines can be considered useful probes to further study the functions of prohibitins. In conclusion, our work describes the successful preparation of a family of synthetic, easily accessible fluorinated thiazolines featuring an innovative scaffold. Their p53-independent pro-apoptotic activity relies on targeting prohibitins and may represent a promising new therapeutic strategy for cancer treatment and drug discovery.

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Communications

Drug Discovery

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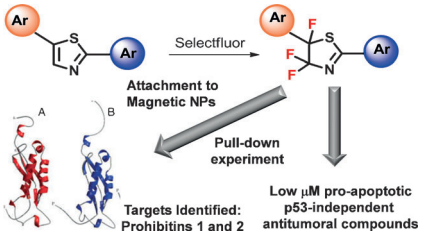

Not one, not two, but three: A novel fluorination of thiazoles selectively yields a trifluorinated heterocyclic scaffold. Robust chemistry leads, in short sequences, to a family of potent p53-independent pro-apoptotic antitumor compounds. Pull-down experiments show that these derivatives are selective ligands of prohibitins. NP = nanoparticle.