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Development of fluorizoline analogues as prohibitin ligands that modulate C-RAF signaling, p21 expression and melanogenesis

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ABSTRACT

Fluorizoline is a cytotoxic trifluorothiazoline that targets the scaffold proteins prohibitins-1 and -2 (PHB1/2) to inhibit the kinase C-RAF and promote the expression of the cyclin-dependent kinase inhibitor p21 to induce cancer cell death. In melanocytes, fluorizoline also induces the synthesis of melanin. Herein we report the first structural requirement of fluorizoline analogues for these activities. We identified in particular some compounds that display enhanced anti-C-RAF and anti-MEK activities, and a higher cytotoxicity in HeLa cells compared to fluorizoline. These results provide a foundation for further optimization of PHB ligands for the treatment of cancers. We also discovered an analogue of fluorizoline that displays pharmacological effects opposed to those of fluorizoline and that can be used as a chemical tool to explore PHB signaling in cancers and other diseases.

Keywords: Cancer; MAP kinases; p21; melanogenesis; prohibitins; heterocycles.

1. Introduction

In 2014, Albericio, Gil, Lavilla and collaborators disclosed a series of trifluorothiazolines that are cytotoxic in cancer cells [1]. Fluorizoline (**1**, Figure 1) was found to be the most active compound of this series, with EC₅₀ in the low micromolar range in a wide array of cancer cells. Further studies showed that fluorizoline induces *in vitro* the death of chronic lymphocytic leukemia cells [2, 3], acute myeloid leukemia cells [4] and non-small cell lung cancer cells [5]. Using affinity chromatography, these compounds were found to target prohibitins-1 and -2 (PHB1/2), which regulate a multitude of signaling

pathways [6], including the activation of the kinase C-RAF (RAF1) by the protooncogene RAS [5]. PHB2 also down-regulates the cyclin-dependent kinase inhibitor p21^{Waf1/Cip} (p21), which is induced by depletion of γ -glutamylcyclotransferase (GGCT) leading to G0/G1 arrest, cellular senescence and growth inhibition in cancer cells [7].

Mechanistic investigations demonstrated that fluorizoline induces the death of cancer cells via their direct effect on PHBs through several pathways including an upregulation of the mitochondrial pro-apoptotic factors NOXA and BIM [8-10], an inhibition of C-RAF activation [5] and an upregulation of p21 (Figure 1) [11]. In addition to its anticancer effects, fluorizoline also induces the biosynthesis of melanin in melanocytes through [12]. Indeed, the binding of fluorizoline to PHBs triggers a cascade of events that includes the activation of the autophagy factor LC3-II from LC3-I, leading to an activation of the kinase ERK and MITF, the key transcription factor for melanocyte development and differentiation.

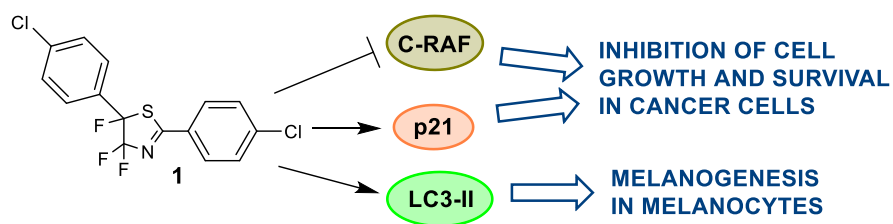


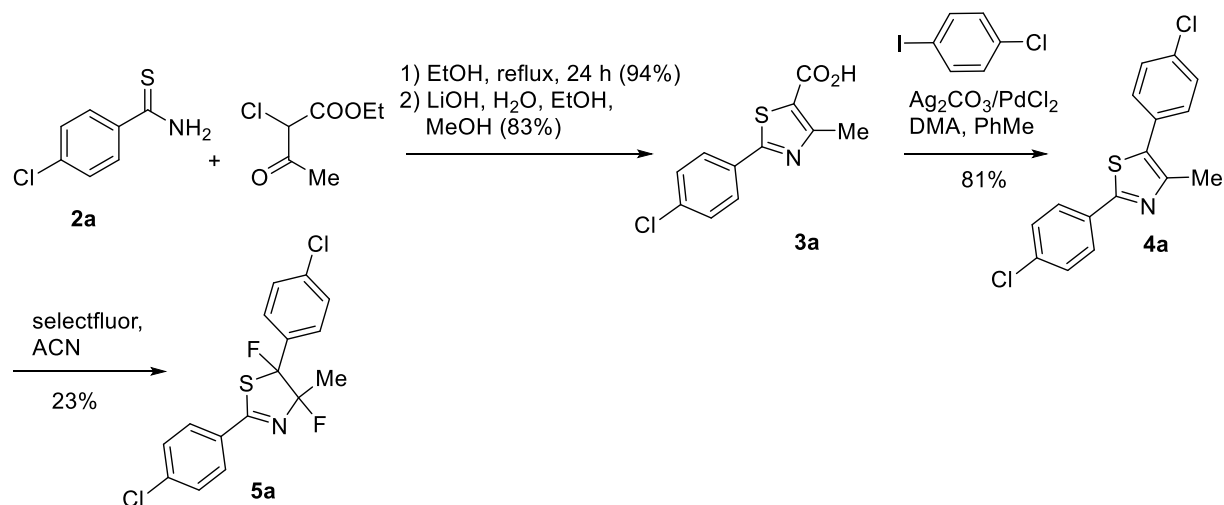
Figure 1. Mechanism of action of fluorizoline (**1**) in cancer cells and melanocytes. In cancer cells, fluorizoline upregulates the pro-apoptotic factors NOXA and BIM and inhibits the PHB-dependent activation of C-RAF. Fluorizoline also blocks the interaction of PHB2 with γ -glutamylcyclotransferase (GGCT) and its translocation to the nucleus to promote the expression of the cyclin-dependent kinase inhibitor p21. In melanocytes, fluorizoline activates the autophagy factor LC3-I into LC3-II to induce pigmentation of the cells.

During our medicinal chemistry program aiming at developing novel PHB ligands [5, 12-15], we pursued the exploration of the structural requirements of fluorizoline analogues for the inhibition of C-RAF, the activation of p21 in cancer cells, and the induction of melanogenesis in melanocytes.

2. Results and discussion

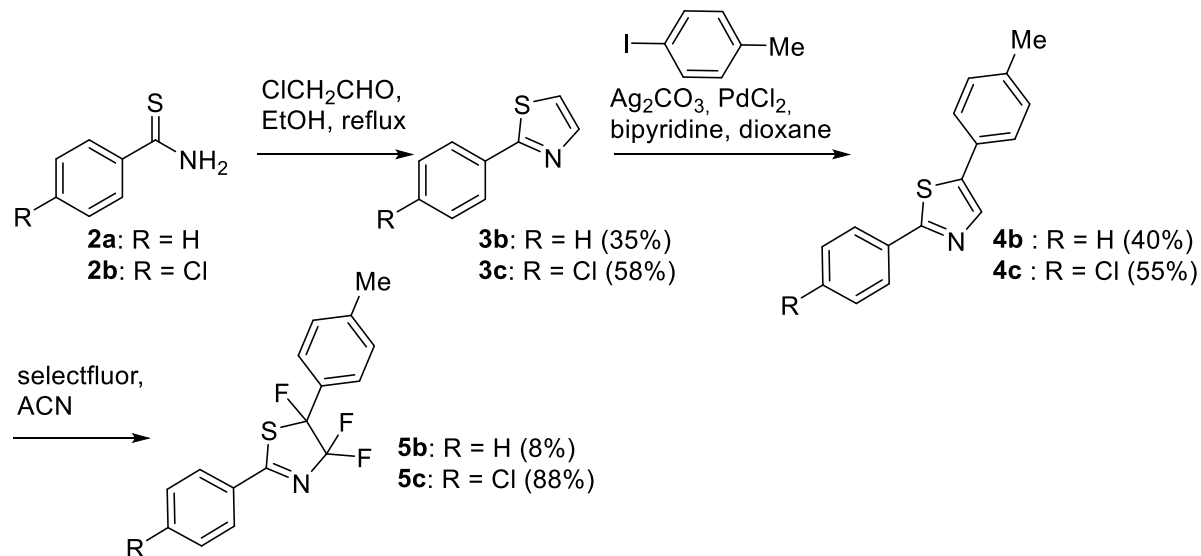
2.1. Synthesis

To explore the role of the two fluorine atoms in position 4, we replaced one of them by a methyl (Scheme 1). Decarboxylative arylation of thiazolecarboxylate **3a** [16, 17] conveniently afforded the 2,5-diarylthiazoles **4a**, which was treated by selectfluor to deliver the difluorinated compound **5a**. As far as we know, this compound is the first example of a 4,5-difluorothiazoline.



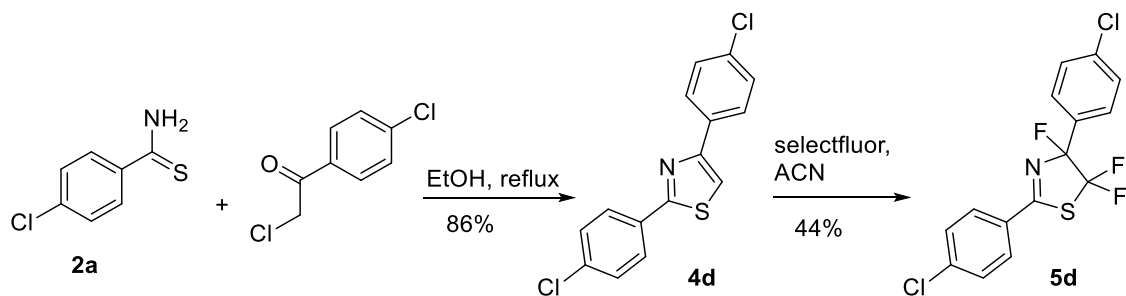
Scheme 1. Synthesis of trifluorothiazoline **5a**.

The synthesis of 4,4,5-trifluoro-2,5-diaryl-4,5-thiazolines **5b/c** began with the PdCl₂(bipy)-catalyzed C-H arylation of thiazoles **3b/c** to afford 2,5-diarylthiazoles **4b/c** following Itami's conditions (Scheme 2) [18]. Perfluorination of these adducts according to Lavilla's protocol gave access to trifluorothiazolines **5b/c**.



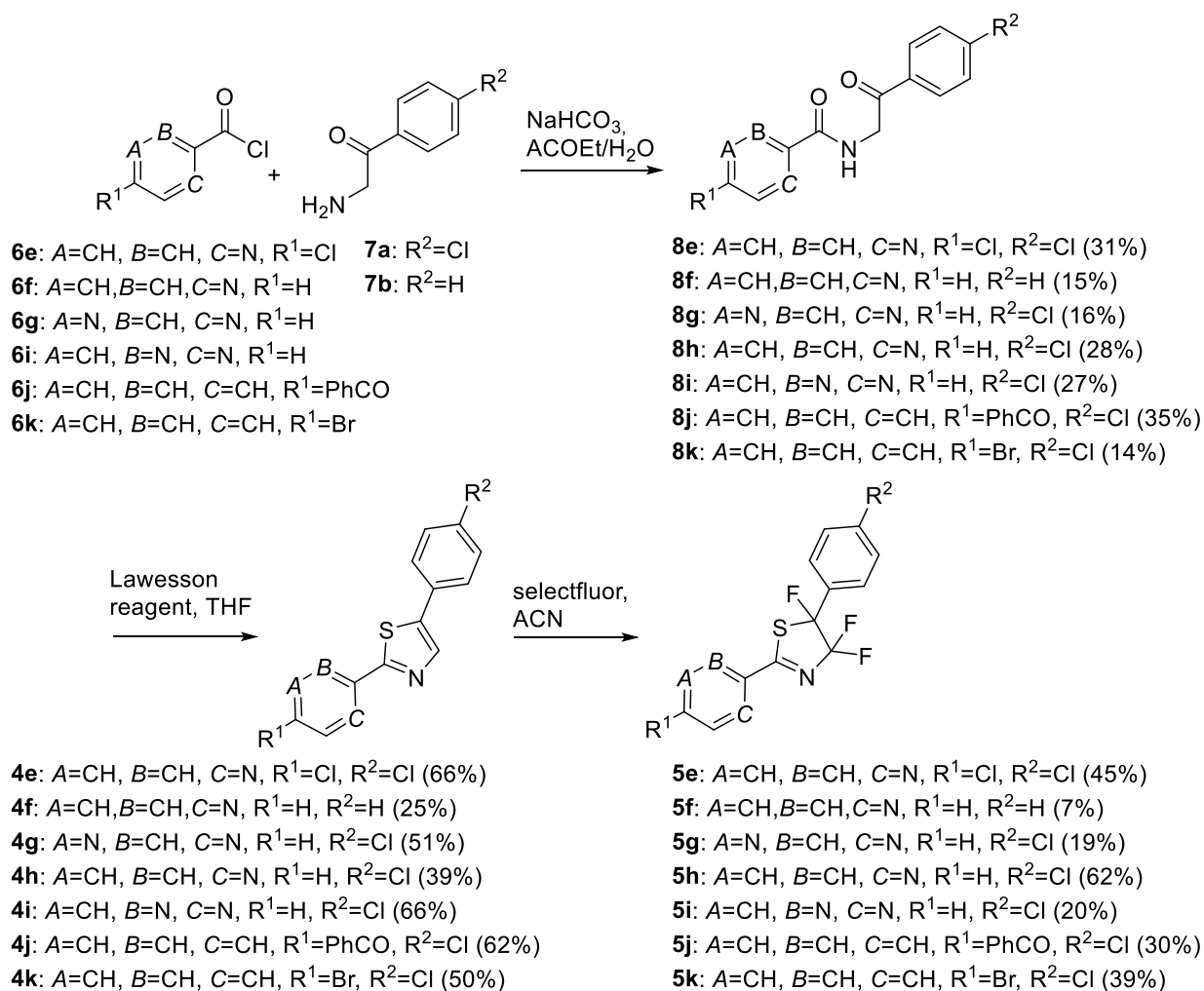
Scheme 2. Synthesis of trifluorothiazolines **5b/c**.

Next, to explore the importance of the orientation of the dipole S-C=N, we performed the first synthesis of a 4,5,5-trifluoro-4,5-thiazoline (Scheme 3).



Scheme 3. Synthesis of 4,5,5-trifluoro-4,5-thiazoline **5d**.

Trifluorothiazolines **5e-k** were prepared by acylation of 2-aminoacetophenones **7a/b** followed by ring closure of amides **8e-k** by using Lawesson reagent and a treatment with selectfluor [19] (Scheme 4).



Scheme 4. Synthesis of trifluorothiazolines **5e-k**.

2.2. Biological results

2.2.1. Inhibition of C-RAF

We previously showed that fluorizoline (**1**) and rocaglamide, a natural product, inhibit the activation of the kinases C-RAF and MEK1/2 [5]. Indeed, the interaction of C-RAF with PHBs at the plasma membrane is essential for C-RAF activation, and the binding of fluorizoline or rocaglamide to PHBs prevents this interaction, leading to an inhibition of the C-RAF-MEK-ERK pathway (Figure 2E). Here, we found that the replacement of a fluorine atom in position 4 by a methyl (**5a**), or the inversion of the dipole S-C=N (**5d**) promote the phosphorylation of C-RAF at Ser338, instead of inhibiting it (Figure 2A). Accordingly, **5d**, but not **5a**, enhanced the phosphorylation of MEK1.

The replacement of the 4-chlorophenyl by a 4-tolyl in position 5 (**5c**) increased the inhibition of C-RAF and MEK, and the deletion of the chlorine atom on the other aryl moiety (**5b**) abolished this activity. Interestingly the azaisostere **5e** displayed an enhanced anti-C-RAF (but not anti-MEK) activity compared to fluorizoline. The deletion of the two chlorine atoms on this azaisostere (**5f**) maintained the inhibition of C-RAF, which is surprising considering that the suppression of these two atoms in fluorizoline has been reported to be deleterious for cytotoxicity [1]. The reintroduction of a chlorine atom (**5h**) didn't modify the anti-C-RAF and anti-MEK activities. The replacement of the 2-pyridyl by a 2-pyrimidinyl (**5i**) was tolerated, but it was not the case for a pyrazinyl (**5g**), which was found inactive. The benzophenone derivative **5j** maintained an anti-C-RAF, suggesting that this compound could be used as a photoactivatable probe [20] to explore the binding domain of fluorizoline in PHBs. The replacement of the 2-chlorophenyl by a 2-bromophenyl (**5k**) was well tolerated for the inhibition of C-RAF.

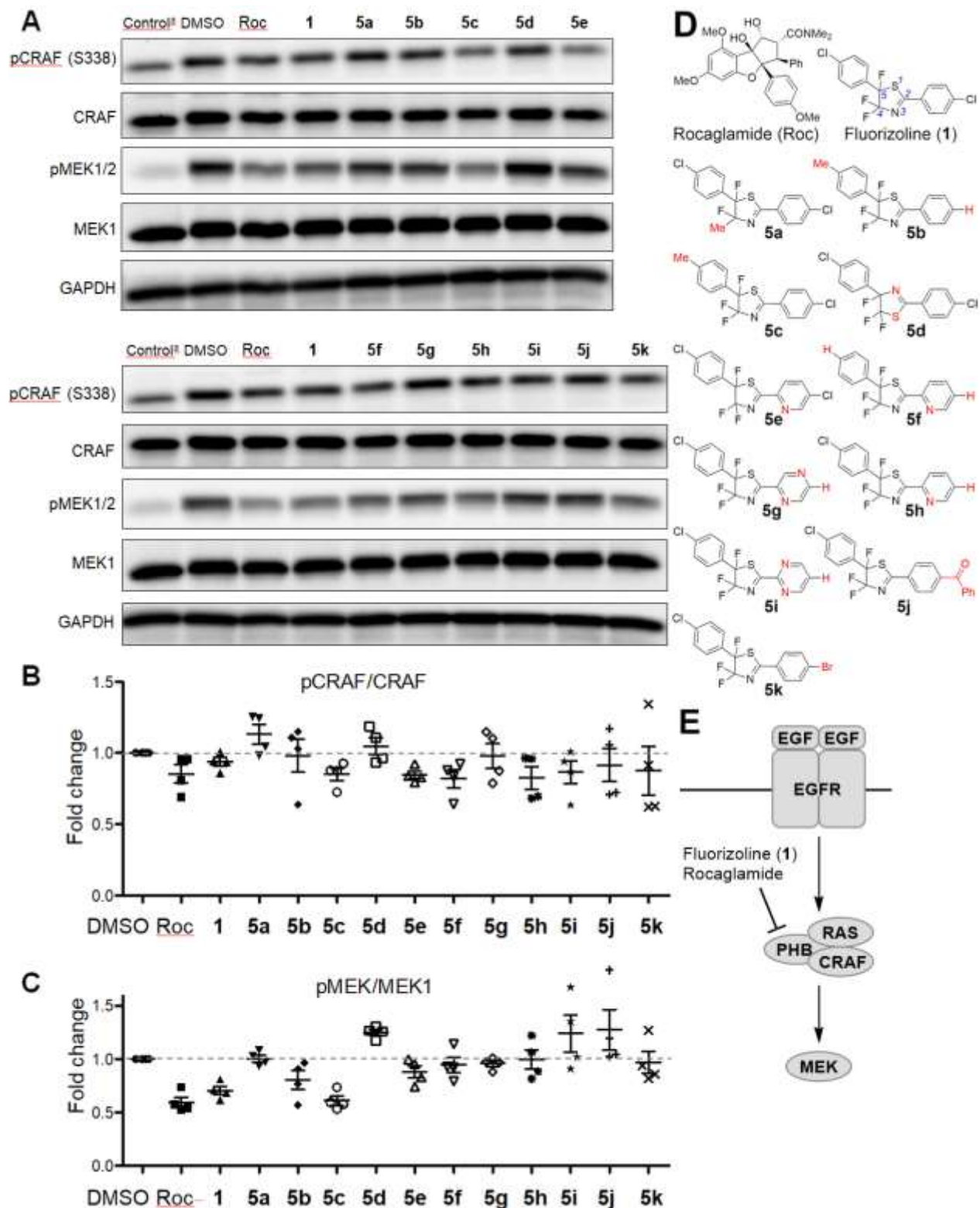


Figure 2. Effect of fluorizoline (1) and its analogues (5a-k) on RAS-MAPK activation. HeLa cells were treated with perfluorothiazolines (1, 5a-k, 10 μ M) for 1 h and stimulated with EGF for 30 min. The cells were collected and analyzed by western blot to monitor the phosphorylation of C-RAF and MEK1/2. ^a No

EGF was added to the cells. **A.** Representative Western blots. **B and C.** Dot plots show the relative values of phosphorylated MEK1 and C-RAF against MEK1/2 and C-RAF normalized to the control (DMSO) from 3 independent experiments. The bars represent mean \pm SEM. **D.** Structure of tested compounds. **E.** Representation of the EGFR/RAS/C-RAF/MEK pathway. Activation of EGFR by EGF leads to the phosphorylation and activation of the kinases C-RAF and MEK in a PHB-dependent manner.

2.2.2. Cytotoxicity in HeLa cells

Next, we performed MTT assays to determine whether these compounds inhibit the growth of HeLa cells (Figure 3). After a 24 h incubation, fluorizoline (**1**) and its analogues **5b**, **5c**, **5e** and **5k** (10 μ M) greatly decreased the numbers of viable cells in a dose-dependent manner, reflecting their anti-MEK activity.

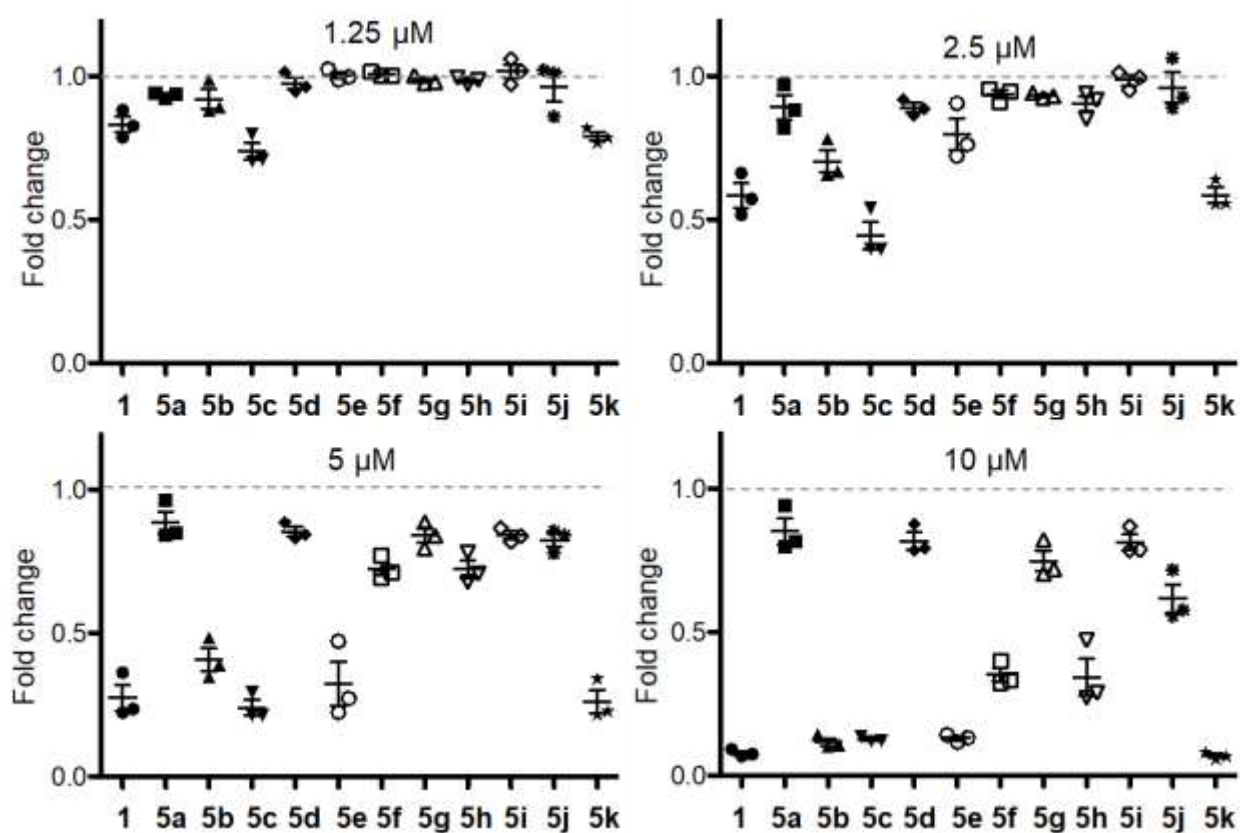


Figure 3. Cytotoxicity of fluorizoline (**1**) and its analogues (**5a-k**). HeLa cells were treated with perfluorothiazolines (**1**, **5a-k**) for 24 h and harvested for MTT assay. Dot plots show the relative values of compounds treated cells against DMSO treated cells from 3 independent experiments. The bars represent mean \pm SEM.

We recently demonstrated that fluorizoline upregulates the cyclin-dependent kinase inhibitors p21 in several human cancer cell lines, including MCF7 breast cancer cells [11]. In line with these studies, we found that two compounds substituted in position 2 by a 2-pyrazinyl (**5g**) or a 4-bromophenyl (**5k**) were more potent than fluorizoline to lower the viability of MCF7 cells and increase the expression of p21.

Importantly, as previously, **5d** displayed an opposite pattern of pharmacological activity: it promoted the proliferation of MCF7 cells and downregulated the expression of p21.

Next, we performed knockdown of p21 in MCF7 cells, which significantly rescued the inhibition of cell proliferation induced by **5b** and **5g** for 48 h (Figure 4C). The efficient knockdown of p21 was verified by Western blot analysis (Figure 4D). These results, similar with those already observed with fluorizoline [7], confirm that the inhibition of MCF7 cells proliferation is highly dependent upon the induction of p21.

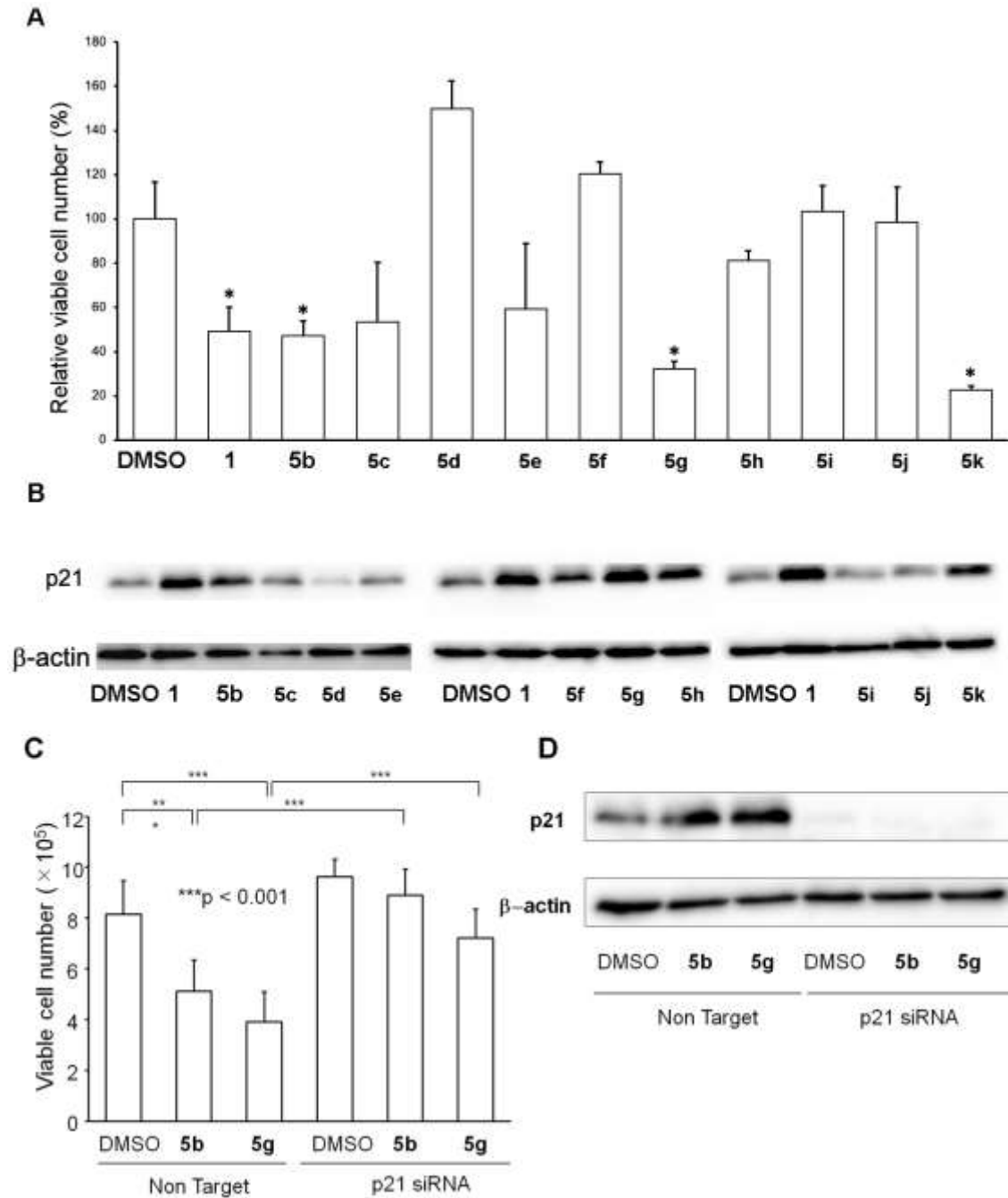


Figure 4. Inhibition of MCF7 proliferation and induction of p21 expression by fluorizoline (**1**) and its analogues **5b-5k** at 1 μ M concentration. **A.** The number of trypan blue-negative viable cells is shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, T test vs DMSO). **B.** Expression of p21 analyzed by Western blot. MCF7 cells were treated with perfluorothiazolines (**1**, **5a-k**, 1 μ M) for 72 h. The cells were counted with a trypan blue staining and p21 expression was analyzed by Western blot analysis. **C and D.** p21 knockdown restores cell proliferation suppressed by **5b** and **5g**. MCF7 cells were transfected with siRNA targeting p21 or non-target control siRNA and treated with 1 μ M of **5b** and **5g** for 48 h. **C.** The cells were counted with a trypan blue staining. (* $P < 0.001$ by 2-way repeated-measures ANOVA) **D.** The expression levels of p21 protein were analyzed by Western blot analysis. β -actin is shown as a loading control.

2.2.3. Stimulation of cell pigmentation

We previously found that fluorizoline (1 and 10 μ M) significantly promotes the pigmentation of HBL human melanoma cells by measuring the extent of pigment formation [21]. In this assay, the only analog that exhibited significantly superior effects to fluorizoline was **5b**, substituted with a phenyl and a tolyl group at the 2 and 5 positions (Figure 5). The analogues **5e**, **5i** and **5j**, substituted by a phenyl group and a tolyl group in position 2 by a 5-chloro-2-pyridinyl, 2-pyrimidinyl, and 4-benzophenone, exhibited an activity similar to that of fluorizoline. The other compounds displayed a reduced activity. In contrast to the previous assays, **5d** behaved similarly to the other analogues: it slightly stimulated pigmentation instead of inhibiting melanogenesis as expected.

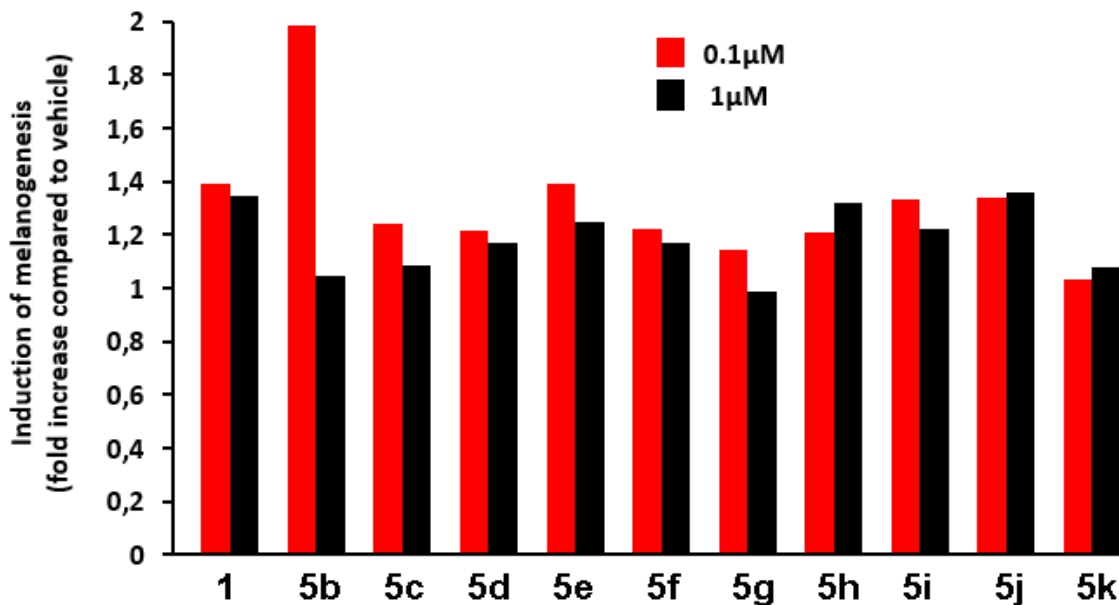


Figure 5. Effect of fluorizoline analogs on cell pigmentation. HBL human melanoma cells were treated for ten days with fluorizoline (**1**) and its analogs (0.1 and 1 μ M). All experiments were undertaken independently in duplicate. The melanin content was expressed as relative increase compared to vehicle-treated HBL cells.

3. Conclusion

While the structure-activity relationships (SAR) of several classes of PHB ligands are well documented [6], no advance on the SAR of trifluorothiazolines has been reported since their seminal disclosure in 2014 [1]. Therein, we identified in particular **5c** that displays an enhanced anti-MEK activity and a higher cytotoxicity in HeLa cells compared to fluorizoline, paving the road for the development of new drugs against cancers and RASopathies [2-5, 8, 22].

We also uncovered that the isomer **5d**, characterized by an inverted S-C=N dipole, stimulates the activation of C-RAF and MEK instead of inhibiting them. As far as we know, this is the first PHB ligand that induces C-RAF activation. Such a reversal of activity is not unprecedented. Indeed, in our previous work on triazines, we developed PHB ligands that promote melanogenesis and other ones that inhibit it [21]. Paradoxically activating C-RAF signaling has been shown to induce the death of the Merkel cell carcinoma (MCC) cell line UISO [23], suggesting that C-RAF activators, such as **5d**, hold some therapeutic potential to treat this highly aggressive type of cancer. In a more general manner, PHBs represent therapeutic targets for an ever-increasing number of diseases [6], and in this context, the discovery of new classes of PHB ligands may provide the means to use targeted approaches for disorders involving PHBs.

4. Experimental part

4.1. Chemistry

Detailed synthetic methods can be found in SI Materials and Methods.

4.2. Biology

4.2.1. Cells

HeLa S3 (DSMZ) was authenticated by Eurofin genomics and cultured in DMEM (10 % heat inactivated FBS). HeLa cells were seeded at 2×10^5 cells/ml in 12 well cell culture plate and cultured for 1 day. HeLa cells were starved in the serum free medium with compounds for 1h followed by the stimulation with EGF (100 ng/ml) for 30 min. After stimulation, 150 μ l of Laemmli buffer was added to the well and the sample were used for SDS-PAGE.

MCF7 human breast cancer cells were obtained from RIKEN BioResource Center (Tsukuba, Japan) and cultured in DMEM supplemented with 10% fetal bovine serum (HyClone, South Logan, UT, USA) and 1% penicillin and streptomycin. Cells were maintained in 5% CO₂ at 37°C.

MCF7 cells were seeded at 5×10^4 cells/well in 6 well cell culture plates and cultured for 1 day. Then, cells were treated with perfluorothiazolines (1, 5a-k, 1 μ M) at 1 μ M for 72h. A trypan blue dye exclusion test was performed using 0.4% trypan blue solution (Wako Pure Chemical Industries, Osaka, Japan) and a Countess II automated cell counter (Thermo Fisher Scientific, Waltham, MA, USA).

HBL melanoma cells (LOCE-MM001) were established in the LOCE (Laboratory of Oncology and Experimental Surgery) at the Institut J. Bordet-Université Libre de Bruxelles. HBL cells were grown in HAM's F10 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), and with L-glutamine, penicillin, and streptomycin at standard concentrations (Thermo Fisher Scientific) [24, 25].

4.2.2. SDS-PAGE and Western blotting

The samples were subjected to 10% SDS-PAGE followed by Western blotting. After transfer, the membrane was incubated with 3% BSA/TBST (20mM Tris-HCl, pH 7.5, 150mM NaCl, 0.05% Tween-20) for 1 h at room temperature followed by the incubation with primary antibody for overnight at 4°C. After the overnight incubation, the membrane was washed with TBST 5 times and incubated with HRP-conjugated secondary antibody in TBST for 1 h at room temperature. After the secondary antibody treatment, the membrane was washed and the signal was detected by chemiluminescence substrate (Millipore) and Chemi Doc touch (Bio-Rad). A list of antibodies is provided in SI Materials and Methods.

4.2.3. MTT assay

HeLa cells were seeded in 96 well plates at a concentration of 1×10^5 cells/ml. 100 μ l cell suspension was added into 96 well cell culture plates with compounds (1.25-10 μ M). 10 μ l of MTT solution was added to the wells after 24 h and incubated for 4 h. After incubation with MTT, 100 μ l solubilisation buffer was added and incubated overnight. MTT was measured at OD 570 nm.

4.2.4. Transfection of siRNA targeting p21

Synthesized siRNAs were purchased from RNAi (Tokyo, Japan) or Gene Design (Osaka, Japan). The siRNA sequences were as follows: non-targeting, 5'-GUACCGCAGUCAUUCGUAUC -3' (sense) and 5'-UACGAAUGACGUGCGGUACGU -3' (anti-sense); p21, 5'- CUGUACUGUUCUGUGUCUU -3' (sense) and 5'-AAGACACAGAACAGUACAG -3' (anti-sense). Transient transfection of siRNA targeting p21 was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, MCF7 cells were seeded in 6-well plates (5×10^4 cells / well), cultured for 24 h, and transfected with siRNAs at a concentration of 10 nM. At 24 h post-transfection, the cells were treated with **5b** 1 μ M or **5g** 1 μ M for 72 h, and then collected and analyzed with Western blot and a trypan blue dye exclusion test was performed using 0.4% trypan blue solution (Wako Pure Chemical Industries, Osaka, Japan) and a Countess II automated cell counter (Thermo Fisher Scientific, Waltham, MA, USA).

4.2.5. Analysis of cell visible pigmentation

A macroscopic evaluation of cell pigmentation revealed that eumelanogenesis was stimulated by fluorizoline analogues after ten days of treatment of HBL cells. Pellets consisting of cell debris and dark pigment were washed twice with PBS and were centrifuged again at 1200 g. The intensity of cell pigmentation was assessed using ImageJ.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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