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Dany Pechalrieu, Daniel Dauzonne, Paola Arimondo, Marie Lopez. Synthesis of novel 3-halo-3-nitroflavanones and their activities as DNA methyltransferase inhibitors in cancer cells. *European Journal of Medicinal Chemistry*, 2020, pp.111829. 10.1016/j.ejmech.2019.111829 . hal-02394113

HAL Id: hal-02394113

<https://hal.science/hal-02394113>

Submitted on 18 Dec 2020

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Synthesis of novel 3-halo-3-nitroflavanones and their activities as DNA methyltransferase inhibitors in cancer cells

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Highlights

- New 3-halo-3-nitroflavanones to be used as DNMT inhibitors in cancers were prepared using a new synthesis pathway.
- 3-Bromo-3-nitroflavanones show an increased stability compared to the chlorinated analogues.
- The new DNMT inhibitors identified show similar activity on purified DNMT and several of them exhibit an increased efficacy in cells.

Abstract

The implication of DNA methylation in cancer is today clearly established. Despite that nucleoside analogues are currently used for leukaemia treatment, their low stability in physiological conditions and their lack of selectivity arise the need for the identification of non-nucleoside DNA methyltransferase inhibitors. Here, we describe the synthesis and pharmacological characterisation of a novel class of DNA methyltransferase inhibitors: the 3-halo-3-nitroflavanones. We showed that 3-bromo-3-nitroflavanones **3b** and **4a** have a micromolar DNMT inhibition and an increased potency in a cell reporter model. Interestingly they are significantly more stable than the reference compounds and induce a low cytotoxicity,

supporting them as new candidates for the development of non-cytotoxic cell-reprogramming epi-drugs for anticancer treatment.

Keywords

Epigenetics; DNA methylation; flavanone; DNMT inhibitor; anticancer therapy.

1. Introduction

Epigenetic modifications play an essential role in the establishment and regulation of gene expression. While these changes do not modify the DNA sequence, they regulate gene expression by altering DNA methylation patterns of genes, chemical modifications of histones, expression profiles of RNAs, and nucleosome positioning [1]. Among these modifications, DNA methylation is one of the most studied together with histone acetylation. In cancers, hypermethylation of tumour suppressor gene (TSG) promoters is associated with TSG silencing, which participates to tumour formation, maintenance and progression [2]. DNA methylation is catalysed by DNA methyltransferases (DNMTs), comprising two families of active DNMTs, *i.e.* DNMT1 and DNMT3, mainly involved in maintenance and *de novo* DNA methylation, respectively [3,4]. The methyl group is added on position 5 of cytosine, mostly at CpG sites, by using *S*-adenosyl-L-methionine (SAM) as a cofactor. In recent years, a growing number of researches aimed at targeting epigenetic effectors to treat cancer. To date, only two nucleoside DNMT inhibitors (DNMTi), 5-azacytidine and 5-aza-2'-deoxycytidine, received FDA approval for the treatment of leukaemia [5]. Despite their high efficiency, their mechanism of action requires incorporation into DNA leading to undesired side effects, which, together with chemical instability, limits their use [6]. Due to these observations, recent research efforts have been focused on the identification of non-nucleoside DNMTi. Several DNMTi families were reported but to date none show enough potency to be considered as drug candidates [7,8]. Our group previously carried out a screen and identified 3-chloro-3-nitroflavanones as potent inhibitors of murine Dnmt3A/3L complex [9]. However, despite these promising results, these flavonoid compounds were not potent enough and were shown to be too unstable to be considered as suitable potential drug candidates, due to the possibility of undergoing a β -elimination with the loss of HCl to lead to the corresponding 3-nitroflavones. This encouraged us to pursue our research on new flavonoid DNMTi, making a

breakthrough in the design and synthesis to improve potency and compound stability. Here, we report the synthesis and biological evaluation of new flavonoid DNMTi. Reprogramming cells by restoring a normal DNA methylation pattern instead of killing them is the purpose of DNMTi. Therefore, stability and cell toxicity of the compounds was also measured to assure that an epigenetic effect is observed without the cytotoxicity associated with conventional chemotherapies.

2. Results and discussion

2.1. Chemical synthesis

Aiming at optimising the flavanone biological activity and chemical stability, and considering that 3-chloro-3-nitroflavanones are susceptible to HCl-elimination leading to corresponding inactive flavones, we modulated the substitution of position 3 of the 3-chloro-3-nitroflavanones. As we previously showed, the nitro group is crucial for inhibition activity [9,10], thus this group was maintained and we focused on the replacement of the chloride atom (Figure 1).

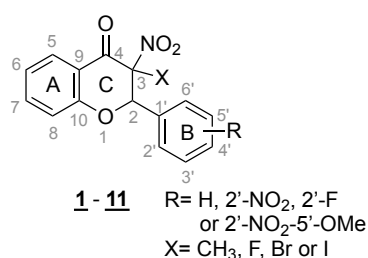
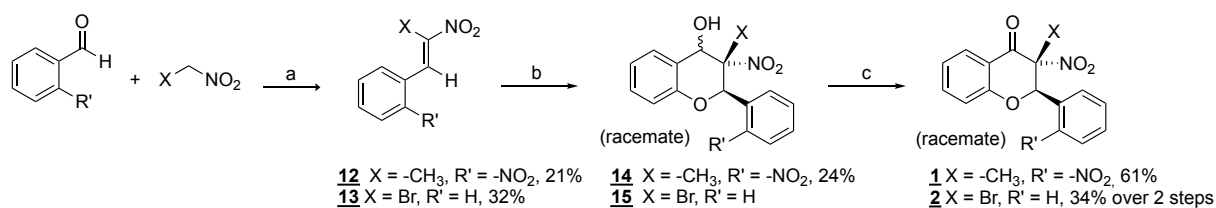


Figure 1. General structure of target 3-halo-3-nitroflavanones 1 - 11

In addition to a potential improvement of activity, we expected these new compounds to be more stable, limiting their ability to undergo β -elimination, which leads to inactive flavones. Such modification has never been reported before. Therefore, we first adapted the synthesis pathway described for the synthesis of 3-chloro-3-nitroflavanones [11,12] to the preparation of 3-methyl-3-nitro- (1) and 3-bromo-3-nitro- (2) substituted compounds (Scheme 1).



Scheme 1. Adapted pathway for the synthesis of 3-nitroflavanones **1** and **2**.

a) KF (0.2 equiv.), xylene, reflux, 7h; b) salicylaldehyde (5 equiv.), Et₃N (0.7 equiv.), THF, rt; c) PCC (2 equiv.), THF, rt, sonication.

The formation of nitrostyrenes (**12** and **13**) was carried out using commercially available nitroethane [13] or bromonitromethane, respectively. This condensation reaction leads exclusively to nitrostyrenes with the nitro group and the aromatic ring located on the opposite sides of the double bond (*Z*-2-bromo-2-nitrostyrene and *E*-2-methyl-2-nitrostyrene). For the formation of methylstyrene derivative **12**, the yield was quite low (21%) as the secondary carbon of nitroethane is much less electrophile compared to chloronitromethane or bromonitromethane. The structure of compound **12** was confirmed by crystal structure determination (Figure 2, Table SI-5).

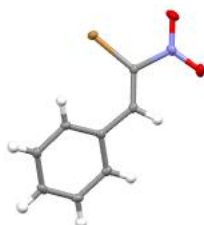
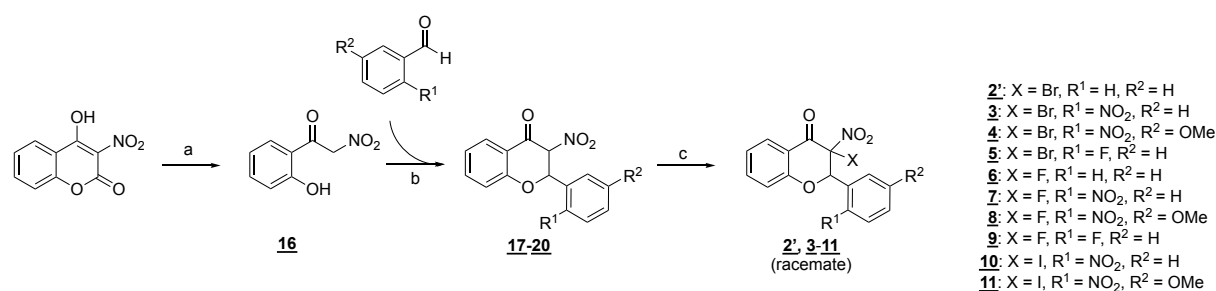


Figure 2. X-Ray crystal structure of compound **12.**

Then, cyclisation *via* a Henry-type reaction with an *ortho*-hydroxybenzaldehyde results in the corresponding flavan-4-ol **14** and **15**. In some cases cyclisation yields were relatively low due to the possible retrocyclisation as previously reported [14], occurring in basic conditions in presence of traces of water, which gives back the starting materials. Finally, oxidation with PCC using ultrasonic assistance, gave the expected flavanones **1** and **2**. These compounds are stable as long as stored dry or in an organic solvent (*e.g.* DMSO), exempt from any trace of water.

Although this synthesis pathway allowed access the target molecules, yields were quite low. Therefore, we next sought for a more versatile method in order to diversify the nature of the substituents on carbon-3 with better yields. Two publications from 1968 and 1981 [15,16] reported the synthesis of 3-bromo-3-nitroflavanones as intermediates for the synthesis of flavone analogues, by bromination of 3-nitroflavanones. We decided to adapt this strategy for the synthesis of our target compounds (Scheme 2).



Scheme 2. Synthesis pathway of 3-halo-3-nitroflavanones.

a) aqueous NaOH solution (5%, w/w, 8 equiv.), 60 °C, 2 h; b) benzaldehyde derivative (1.0 equiv.), Na₂CO₃ (1.5 equiv.) L-Proline (0.5 equiv.), EtOH, 40 °C, 5 h to o/n; c) NFSI, NBS or NIS (1.7 equiv.), Na₂CO₃ (1.3 equiv.), THF, rt, 1-5 h.

First, 2'-hydroxy-2-nitroacetophenone **16** was prepared from commercially available 4-hydroxy-3-nitrocoumarin by treatment with an aqueous sodium hydroxide solution. Then, cyclisation in basic conditions afforded 3-nitroflavanones **17-20**. Finally, halogenation with *N*-bromosuccinimide (NBS) afforded expected compounds **2'**, **3-5** in good to high yields. Noteworthy, contrarily to the first synthesis pathway, here both diastereoisomers can be obtained, since halogenation can occur on both sides of the molecules. For compounds **3** and **4** both diastereoisomers (**3a** (*anti*)-**3b** (*syn*) and **4a** (*anti*)-**4b** (*syn*)) were separated by flash chromatography, then were tested separately to compare their activity and stability. Compound **2'** was formed as a mixture of *syn/anti* diastereoisomers. A majority of the *syn*-diastereoisomer was obtained as confirmed by comparison of ¹H and ¹³C NMR signals with those of compound **2** obtained *via* the first synthesis pathway (Scheme 1) and thus *via* the *Z*-2-bromo-2-nitrostyrene intermediate. Noteworthy, H-2 signal is more deshielded for the *syn*-diastereoisomer ($\delta = 6.08$ ppm) than for the *anti*-analogue ($\delta = 5.75$ ppm) and C-2 signal shifted from $\delta = 82.9$ ppm for the *syn*-isomer to $\delta = 86.9$ ppm for the *anti*-analogue. For compound **5** only one diastereoisomer was formed and the relative stereochemistry *syn* was confirmed by crystal structure determination (Figure 3). In order to explore more halide

substitutions on 3-position of the flavanone, *N*-fluorobenzenesulfonimide (NFSI) was used instead of NBS to access fluoride-substituted compounds **6-9** [17]. Crystal structure determination and NMR studies showed that compounds **6** and **9** were obtained as *anti* isomer, compound **7** as *syn* isomer. Compound **8** was obtained as the *anti* diastereoisomer but with an unidentified impurity that could not be separated. 3-Iodo-3-nitroflavanones **10-11** were prepared similarly using *N*-iodosuccinimide. In this case, only *syn* diastereoisomers were obtained as determined by NMR spectra. ¹H NMR aromatic signals, especially H-6' and H-3' signals, of **10** are similar to those obtained for chlorine (**Flv69**) and bromine (**3b**) analogues and significantly different from the *anti*-derivative **3a** (spectra in supporting information). Similarly, the aromatic region of compound **11** matches the one of **Flv880** and **4b**, *syn*-conformation, and is different from compound **4a**, *anti*-conformation.

For compounds **3a**, **3b**, **5** and **9**, the structure was confirmed by crystal structures (Figure 3) with a good resolution (Tables SI-1 to SI-4).

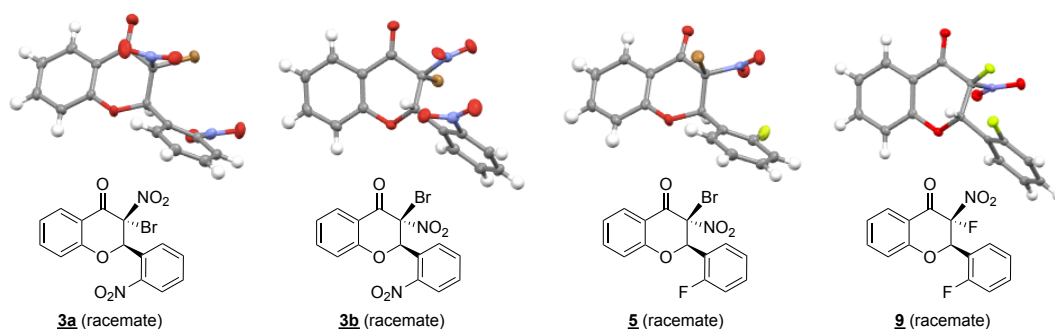


Figure 3. X-Ray crystal structures of compounds **3a, **3b**, **5**, **9**.**

These structure determinations provided the relative configuration of the compounds obtained as a racemic mixture.

Interestingly, crystal structures showed that the ring C always adopts a half-chair conformation, with the phenyl ring (ring B) in equatorial position.

Next, the stability of the synthesised compound was measured and compared to that of the previously prepared 3-chloro-3-nitroflavanones **Flv69** [9] and **Flv880** [10].

2.2 Chemical stability assays

The stability experiments were carried out in the assay buffer used for DNMT3A-c methylation tests and were followed by HPLC (Figure 4).

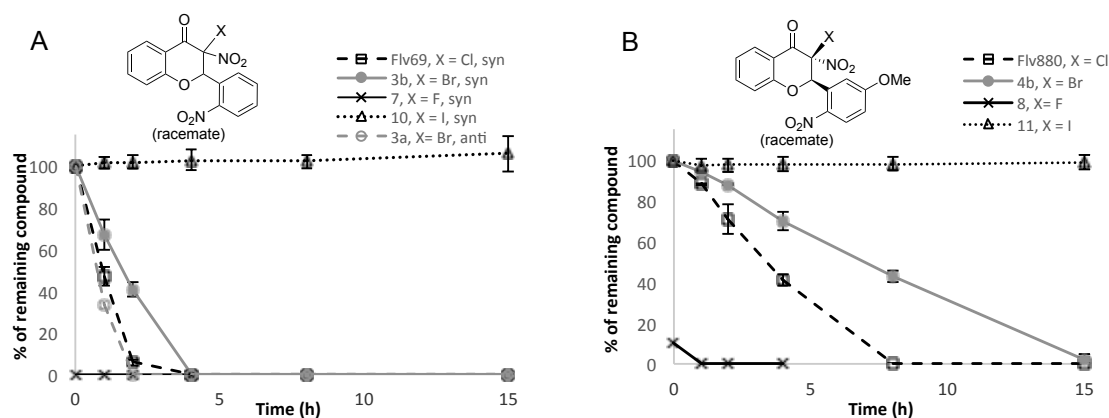


Figure 4. Stability of 3-halo-3-nitroflavanones in enzymatic buffer over 15 h.

Percentage of remaining compound is followed by HPLC analysis at 250 nm and the mean of at least 2 experiments is reported.

3-Fluoro-3-nitroflavanones **7-8** (Figure 4 green curves) and **6, 9** (data not shown) are totally instable in the enzymatic buffer, even at $t = 0$ min no remaining compound was detected by HPLC. Brominated derivative **3b** showed a marginal increase in stability with half-life time of 1.7 h (Figure 4A) when compared to the chlorinated compound **Flv69** presenting a half-life time of 1 h. More interestingly, for compounds bearing 2'-nitro-5'-methoxy substitution on cycle B, *i.e.* **Flv880** and **4b** (Figure 4B), the stability of brominated compound **4b** is highly increased and the half-time life is more than doubled going from 3.4 h for **Flv880** to 7 h for compound **4b**. In addition, the stability of both diastereoisomers **3a** and **3b** is compared in Figure 4A (grey circles). Whereas *syn* compound **3b** exhibits a half-life time of 1.7 h, this value drops down to 0.7 h for *anti* derivative **3a**. This observation is of high interest as it shows that *syn* derivative **3b**, with an antiperiplanar orientation of the C³-Br and C²-H bonds, is the most stable diastereoisomer (Figure 4). This presumes that, in the case of brominated derivatives, the instability is not due to a β -elimination as it was observed for the 3-chloro-3-nitroflavanones, since such a reaction would be favoured by an antiperiplanar configuration of the C³-Br and C²-H bonds. In such a hypothesis, compound **3b** should then be less stable than compound **3a**. Finally, both compounds containing an iodine atom **10** and **11** are extremely

more stable than their brominated and *a fortiori* chlorinated analogues with no degradation observed after 15 h.

Considering this stability results, only brominated and iodinated were further evaluated for their biological activities.

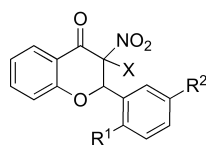
2.3 Biological results

This new library of 3-halo-3-nitroflavanones was first tested to evaluate the methylation inhibition activities using purified human DNMT3A-c. Second, a luciferase reexpression cellular model assay dependent on promoter DNA methylation was carried out with promising inhibitors. Finally, their cytotoxicity in different cancer cell lines was evaluated.

2.3.1 DNMT inhibition

The results of the inhibition of *h*DNMT3A-c by 3-nitroflavanones **1-5**, **10**, **11** and **17-19** are reported in Table 1 and compared to lead compounds **Flv69** and **Flv880**.

Table 1. *In vitro* inhibition activity of hDNMT3A-c of compounds 1-5, 10, 11, 17-19.



Cpd	X	R ¹	R ²	EC ₅₀ (μM)
Flv69	Cl	-NO ₂	H	4.4 ± 2.2
Flv880	Cl	-NO ₂	-OMe	4.4 ± 0.8
1	-CH ₃	-NO ₂	H	> 40
2	Br	H	H	6.7 ± 1.1
3a	Br	-NO ₂	H	5.1 ± 2.0
3b	Br	-NO ₂	H	3.2 ± 1.1
4a	Br	-NO ₂	-OMe	4.7 ± 1.6
4b	Br	-NO ₂	-OMe	5.3 ± 1.0
5	Br	F	H	5.7 ± 1.9
10	I	-NO ₂	H	2.8 ± 1.6
11	I	-NO ₂	-OMe	2.0 ± 1.4
17	H	H	H	inactive*
18	H	-NO ₂	H	inactive*
19	H	-NO ₂	-OMe	inactive*

EC₅₀ values were determined against the human DNMT3A-c in at least four independent experiments.

* 0% inhibition at 32 μM

Methyl substitution on 3-position led to totally inactive compound **1**, which illustrates the importance of the halide on this carbon. For all brominated compounds **2-5** the *in vitro* DNMT inhibition activity is similar ranging from EC₅₀ = 3.2 ± 1.1 (**3b**) to EC₅₀ = 6.7 ± 1.1 μM (**2**). These activities are slightly lower than for the chlorinated analogues **Flv69** and **Flv880**. Interestingly, compound **3**, for which both diastereoisomers (**3a** and **3b**) were obtained, showed similar inhibition activities with EC₅₀ = 5.1 ± 2.0 μM for **3a** and EC₅₀ = 3.2 ± 1.1 μM for **3b**. This similarity of diastereoisomers inhibition activity was also measured for diastereoisomers **4a** (EC₅₀ = 4.7 ± 1.6 μM) and **4b** (EC₅₀ = 5.3 ± 1.0 μM). This highlights the

fact that inhibition activity is independent from the relative stereochemistry of carbon-2 and -3 of these 3-halo-3-nitroflavanones. Regarding 3-iodo-3-nitroflavanones **10** and **11**, inhibition activity is slightly improved compared to 3-bromo- and 3-chloro-analogues with $EC_{50} = 2.8 \pm 1.6 \mu\text{M}$ and $2.0 \pm 1.6 \mu\text{M}$ for **10** and **11**, respectively.

In summary, first the substitution of the chlorine atom on 3-position of ring C of the 3-chloro-3-nitroflavanones does not improve the *in-vitro* DNMT inhibition activity, which is maintained at micromolar levels. Second, maintenance of micromolar activity was observed for all the 3-halo-3-nitroflavanones compared to the chlorinated reference, with slightly better activity for iodinated compounds. Importantly, when both diastereoisomers were obtained, they showed the same level of *in-vitro* DNMT inhibition activity.

2.3.2 Cellular activity in a reporter gene reexpression model

Next, cell activity was evaluated in KG-1 cells, stably transfected with the firefly luciferase reporter gene under the control of a methylated cytomegalovirus (CMV) promoter. DNMTi, decrease promoter methylation inducing an increase of the luciferase expression, which is measured by luminescence [18]. Results are reported in induction fold (IF) as a ratio of the luminescence level in the presence of the inhibitor to DMSO-treated cells. Thus, the more active is the inhibitor, the higher the IF (Figure 5).

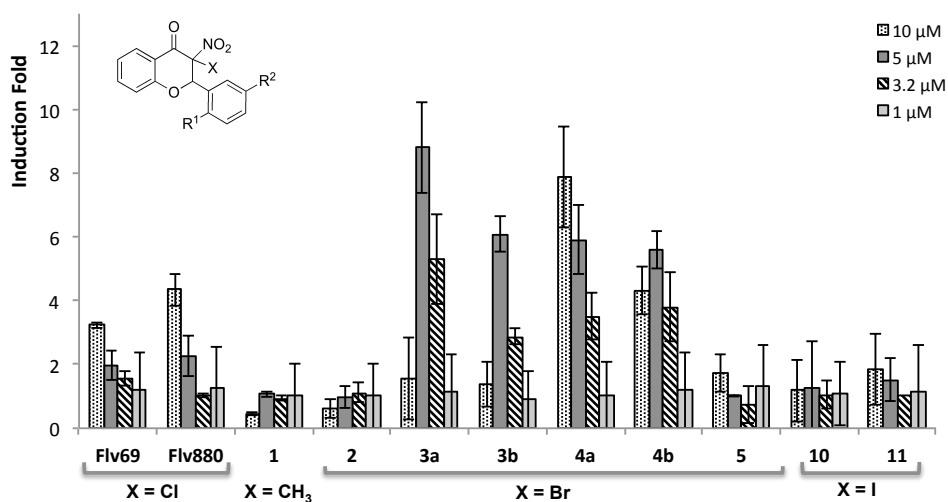


Figure 5. Luciferase reexpression level in KG-1 CMV-Luc reexpression model treated with 3-halo-3-nitroflavanones at 10, 5, 3.2, and 1 μM . Induction Fold (IF) values were determined in at least three independent experiments.

First, despite their inhibition activity against purified DNMT, 3-iodo-3-nitroflavanones **10** and **11** did not induce gene reexpression in cells with an IF ratio close to 1, which is neither dependent on concentration nor on the cycle-B substitution.

Regarding 3-bromo-3-nitroflavanones, the absence of substitution (**2**) and 2'-fluoro-substitution (**5**) on cycle B led to inactive compound with IF values similar to negative compound **1**. Noteworthy, compounds **2** and **5** are the only 3-bromo-3-nitroflavanones lacking the nitro group on ring B, which could make a significant difference in pharmacokinetic properties and therefore cell activity. Interestingly, all the other brominated derivatives (**3a-4b**) show a dose-dependent IF and are more potent compared to the chlorinated analogues **Flv69** and **Flv880**. More precisely, the brominated analogues **4a** and **4b** show a 2-fold higher reexpression level compared to the chlorinated compound **Flv880**. This increase in IF is observed for the whole range of tested concentrations (from 3.2 to 10 μM). Noteworthy, cytotoxicity may explain the lower IF at 10 μM for compound **4b**. The improvement of reexpression activity is even more significant comparing compound **Flv69** and its brominated analogues **3a** and **3b**. Whereas the compound cytotoxicity can explain the extremely low IF

(< 1) at 10 μ M, both compounds show a greatly improved activity with IF = 8.8 (**3a**) and 6.1 (**3b**) at 5 μ M compared to its chlorinated analogue **Flv69** (IF = 2.0 at 5 μ M). In summary, iodinated compounds **10-11**, despite good *in vitro* inhibition activities, did not show any activity in cells. Interestingly, brominated analogues (**3a-3b**), with an IF 3-fold higher than parent compounds **Flv69** and **Flv880**, are now the most promising flavonoid DNMTi.

2.3.3 Antiproliferative activity in cancer cell lines

In the field of epigenetic reprogramming, the Holy Grail is to identify compounds with inhibition activities on purified enzymes as well as capability of reinducing targeted gene expression in cellular models with no or weak cell cytotoxicity at the used concentrations. This strategy, limiting cytotoxic effects and favouring cell reprogramming through tumour suppressor genes reexpression, will be a real breakthrough in the development of anticancer therapies. This reprogramming will allow epi-drug to be used in combination as anticancer treatments, while minimizing the severe side effects observed with cytotoxic anticancer treatments. We showed in this work that compounds **3a-4b**, maintain *in vitro* inhibition activity and a higher capability to reinduce the expression of the luciferase gene in a cellular gene reporter model compared to their previously reported chlorinated analogues. We also demonstrated that, contrarily to fluorinated compounds **6-9**, brominated compounds **3a-4b** have a good stability in physiological conditions. Therefore, compounds **3a-4b** were evaluated for their cytotoxicity in colorectal cancer HCT116 and leukaemia KG1 cell lines after 72 h of daily treatment (Figure 6).

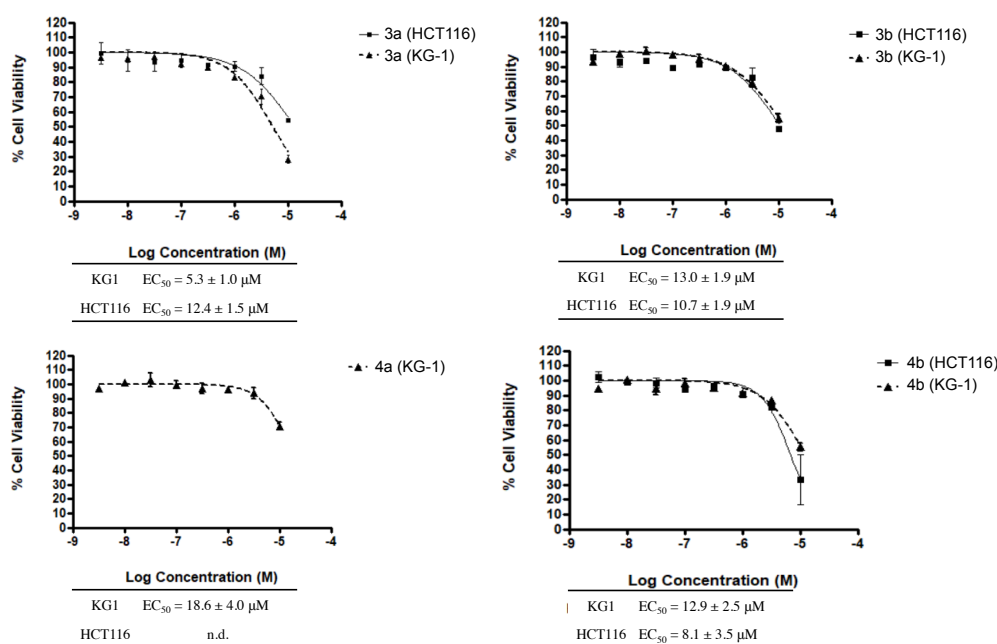


Figure 6. Cytotoxicity evaluation for compounds 3a, 3b, 4a and 4b.

Cells were treated daily for 72 h. Values were determined as a mean of two independent experiments.

Cell cytotoxicity ranges from micromolar to high micromolar with EC₅₀ values from 5.3 to 18.6 μM (Figure 6). Notably, cytotoxicity is similar for KG1 and HCT116 cell lines, respectively a leukaemia, haematological cancer cell lines, and a colorectal, *i.e.* solid tumour cancer cell line. For all compounds, the cytotoxicity value is higher than the *in vitro* EC₅₀ value on purified enzyme and higher than their active concentration in the reexpression cellular model. More interestingly, compounds 3b and 4a, exhibited lower cytotoxicity with EC₅₀ = 13.0 and 18.6 μM, respectively, in parallel with the lower *in vitro* EC₅₀ on the purified DNMT3A-c 3.2 and 4.7 μM, respectively. These compounds show the highest difference between the active concentrations and EC₅₀ cytotoxicity values. Although this difference is moderate it means that compounds exhibit DNMT inhibition activity and luciferase reexpression capability at concentrations at which they have limited cytotoxic effects. This is the first step toward the identification of low-cytotoxic cell-reprogramming epi-drugs.

3. Conclusion

In conclusion, we developed a new versatile synthesis pathway to provide access to 3-halo-3-nitroflavanones and prepared a library of compounds. The structure was confirmed by crystallography and several diastereoisomers could be isolated. We assessed the stability of the compounds and showed that, with half-life time up to 7 h, 3-bromo-3-nitroflavanones analogues were twice more stable than the chlorinated analogues. Regarding 3-iodo-3-nitroflavanones, they reveal a very high stability since they are totally stable up to 15 h. However, they unfortunately did not show any activity in cells. Four 3-bromo-3-nitroflavanones, **3a-4b**, exhibited the best profile as DNMTi for cell reprogramming that is to say they are active against the purified DNMT3A-c, induced gene expression in a cellular model much more efficiently than reference compounds and showed moderate cytotoxicity at active concentrations. This last point is crucial for epi-drugs and is a breakthrough in the way to think anticancer therapies as ideally, “epi-drugs” reprogram cell profile without being cytotoxic. Among these four 3-bromo-3-nitroflavanones, compounds **3b** and **4a** were identified as the most potent compounds and constitute new non-nucleoside DNMTi with good activity and stability that can now be considered as reference DNMTi for the future developments of new “epi-drugs” targeting DNA methylation.

4. Experimental section

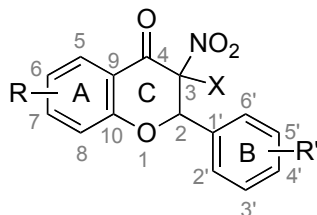
4.1 Chemistry

Material and methods

All starting materials and reagents were purchased from commercial suppliers, Sigma Aldrich, Alfa Aesar or TCI. Solution of Girard's reagent was prepared by dilution of Girard's reagent (3.9 g) in 30 ml of EtOH/AcOH/H₂O (8/1/1). All reactions were monitored by thin layer chromatography (TLC) and TLC plates were visualized with UV light. Flash silica chromatographies were performed on a Puriflash 430 apparatus (Interchim) using 30- μ m spherical silica columns. ¹H NMR at 400 MHz and ¹³C NMR at 100 MHz were acquired on a Bruker Avance 400 instrument. ¹H NMR at 500 MHz and ¹³C NMR at 125 MHz were

acquired on a Bruker Avance II instrument equipped with ^{13}C cryoprobe. For ^1H and ^{13}C NMR acquired in CDCl_3 chemical shifts (δ) are reported in ppm relative to the solvent residual peak: proton ($\delta = 7.26$ ppm) and carbon ($\delta = 77.2$ ppm). ^1H NMR and ^{13}C NMR spectras were assigned using 2D NMR experiments, such as, ^1H - ^1H gCOSY, ^1H - ^{13}C HSQC and ^1H - ^{13}C HMBC experiments. Multiplicity is indicated as follows: s (singlet); d (doublet); t (triplet); q (quartet); m (multiplet); dd (doublet of doublets); td (triplet of doublets); qd (quartet of doublets); ddd (doublet of doublets of doublets); tdd (triplet of doublets of doublets); br (broad). Coupling constants (J) are reported in Hertz (Hz). High and low resolution electrospray ionization mass spectra were acquired using electrospray as the ionization technique in positive ion and/or negative ion modes as stated. All MS analysis samples were prepared as solutions in MeOH. Compound purity was verified to be higher than 95% using reversed-phase HPLC on an X-terra C18 MS column (3.9 100 mm; Waters) with a linear gradient 0 to 95% CH_3CN in H_2O , eluents containing 0.01% formic acid. The X-ray crystallographic structural determinations were performed at the Service de Diffraction des Rayons X, Laboratoire de Chimie de Coordination, Toulouse, France.

Numbering of flavonoid compounds will be done as below



Chemical synthesis

***syn*-3-Chloro-2,3-dihydro-3-nitro-2-(2'-nitrophenyl)-4*H*-1-benzopyran-4-one (Flv69)**

The **Flv69** was obtained in 3 steps following a previously described procedure [11,12]. ^1H NMR (CDCl_3 , 500 MHz): δ (ppm) = 8.11 (dd, $J = 1.4, 7.8$ Hz, 1H, H-3'); 7.87 (ddd, $J = 0.4, 1.7, 8.0$ Hz, 1H, H-5); 7.93 (dd, $J = 1.3, 8.1$ Hz, 1H, H-6'); 7.76 (td, $J = 1.4, 7.6$ Hz, 1H, H-4'); 7.71 (tdd, $J = 1.2, 1.7, 7.8$ Hz, 1H, H-7); 7.66 (td, $J = 1.5, 7.7$ Hz, 1H, H-5'); 7.29 (tdd, $J = 0.6, 1.0, 7.6$ Hz, 1H, H-6); 7.26 (s, 1H, H-2); 7.17 (qd, $J = 0.4, 1.0, 8.4$ Hz, 1H, H-8). ^{13}C NMR (CDCl_3 , 125 MHz): δ (ppm) = 177.9 (C=O); 160.1 (C-9); 149.4 (C-2'); 138.5 (C-7); 132.9 (C-4'); 131.7 (C-5'); 130.6 (C-3'); 129.6 (C-5); 125.4 (C-6'); 124.3 (C-6); 124.3 (C-1'); 118.5 (C-8); 116.9 (C-10); 100.9 (C-3); 76.1 (C-2).

***syn*-3-Chloro-2,3-dihydro-2-(5'-methoxy-2'-nitrophenyl)-3-nitro-4*H*-1-benzopyran-4-one (Flv880)**

The **Flv880** was obtained in 3 steps according to a previously described procedure [10]. **¹H NMR** (CDCl₃, 500 MHz): δ (ppm) = 8.07 (dd, J = 1.7, 8.0 Hz, 1H, H-5); 7.87 (d, J = 9.2 Hz, 1H, H-3'); 7.71 (td, J = 1.8, 7.9 Hz, 1H, H-7); 7.58 (d, J = 2.7 Hz, 1H, H-6'); 7.42 (s, 1H, H-2); 7.28 (td, J = 0.9, 7.7 Hz, 1H, H-6); 7.17 (m, 1H, H-8); 7.06 (dd, J = 2.8, 9.1 Hz, 1H, H-4'). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 177.9 (C=O); 162.7 (C-5'); 160.1 (C-9); 142.0 (C-2'); 138.3 (C-7); 129.7 (C-5); 128.2 (C-3'); 126.9 (C-1'); 124.3 (C-6); 118.5 (C-8); 117.0 (C-10); 116.6 (C-6'); 115.3 (C-4'); 101.0 (C-3); 76.0 (C-2); 56.3 (OCH₃).

2,3-Dihydro-3-methyl-2-(2'-nitrophenyl)-3-nitro-4H-1-benzopyran-4-one (1)

The compound **14** (46 mg, 0.14 mmol, 1.0 equiv.) was solubilised in anhydrous THF (2 ml). After addition of pyridinium chlorochromate (151 mg, 0.70 mmol, 5.0 equiv.), the round-bottom flask was equipped with a condenser and a drying tube and the mixture was submitted to sonication at 40 °C for 5 h. The reaction mixture was filtered, the precipitate was rinsed with CH₂Cl₂ and the filtrate was concentrated under vacuum. The residue was purified by flash chromatography using a linear gradient (cyclohexane/EtOAc; 100/0 to 50/50) to give the title compound **1** (28 mg, 0.08 mmol, 61% yield) as a white solid. R_f = 0.21 (cyclohexane/EtOAc 3:1). **ESI-MS**: m/z = 351 [M+Na]⁺. **¹H NMR** (CDCl₃, 500 MHz): δ (ppm) = 8.02 (dd, J = 1.7, 8.0 Hz, 1H, H-5); 7.87 (dd, J = 1.3, 8.2 Hz, 1H, H-3'); 7.74 (dd, J = 1.4, 7.9 Hz, 1H, H-6'); 7.68 (td, J = 1.3, 7.5 Hz, 1H, H-5'); 7.64 (m, 1H, H-7); 7.60 (td, J = 1.4, 7.8 Hz, 1H, H-4'); 7.22 (td, J = 0.9, 7.6 Hz, 1H, H-6); 7.11 (dd, J = 0.6, 8.5 Hz, 1H, H-8); 7.05 (s, 1H, H-2); 1.78 (s, 3H, CH₃). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 184.9 (C=O); 160.1 (C-9); 149.8 (C-2'); 137.8 (C-7); 132.6 (C-5'); 131.0 (C-4'); 129.4 (C-6'); 128.9 (C-5); 126.2 (C-1'); 125.5 (C-3'); 123.8 (C-6); 118.4 (C-10); 118.3 (C-8); 93.4 (C-3); 77.0 (C-2); 15.3 (CH₃). **HRMS**: Calcd for C₁₆H₁₂N₂O₆Na 351.0588, Found 351.0581.

syn-3-Bromo-2,3-dihydro-2-phenyl-3-nitro-4H-1-benzopyran-4-one (2)

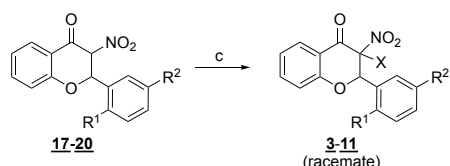
The title compound **2** was synthesised following the procedure described for the synthesis of compound **1**, starting from compound **15**. After purification by flash chromatography, the title compound **2** (26 mg, 0.07 mmol, 34% over 2 steps) was obtained exclusively as the *syn* diastereoisomer as white solid. R_f = 0.55 (cyclohexane/EtOAc 3:2). **ESI-MS**: m/z = 370 [M+Na]⁺. **¹H NMR** (CDCl₃, 500 MHz): δ (ppm) = 8.08 (dd, J = 1.7, 8.0 Hz, 1H, H-5); 7.69 (td, J = 1.9, 7.7 Hz, 1H, H-7); 7.52 (m, 2H, H-2', H6'); 7.47 (m, 1H, H-4'); 7.46 (m, 2H, H-3', H-5'); 7.25 (m, 1H, H-6); 7.20 (br d, J = 8.4 Hz, 1H, H-8); 6.08 (s, 1H, H-2). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 179.6 (C=O); 160.1 (C-9); 138.2 (C-7); 131.2 (C-1'); 130.6

(C-4'); 129.4 (C-5); 128.8 (2C) (C-3', C-5'); 128.0 (2C) (C-2', C-6'); 123.8 (C-6); 118.6 (C-8); 116.9 (C-10); 99.7 (C-3); 82.9 (C-2); 15.3 (CH₃). **HRMS**: Calcd for C₁₅H₁₀BrNO₄Na 369.9685, Found 369.9718.

***anti/syn*-3-Bromo-2,3-dihydro-2-phenyl-3-nitro-4*H*-1-benzopyran-4-one (**2'**)**

The title compound **2'** was synthesised from the compound **17** (54 mg) following the *general procedure 1*. The title compound **2'** (65 mg) was obtained as a mixture of *syn/anti* diastereoisomers (75/25) as a white solid with 93% yield. *R_f* = 0.40 (EtOAc/cyclohexane 1:5). **ESI-MS**: *m/z* = 370 [M+Na]⁺. **¹H NMR** (CDCl₃, 500 MHz): δ (ppm) = 8.10, 8.08 (2 x dd, *J* = 1.7, 7.9 Hz, 1H, H-5); 7.69, 7.67 (2 x td, *J* = 1.8, 7.7 Hz, 1H, H-7); 7.53-7.51 (m, 2H, H-2', H-6'); 7.47-7.46 (m, 1H, H-4'); 7.45-7.43 (m, 2H, H-3', H-5'); 7.27, 7.25 (2 x br dd, 1H, H-6); 7.19, 7.16 (2 x br d, *J* = 6.5 Hz, 1H, H-8); 6.08 (s, 0.75H, H-2_{*syn*}); 5.75 (s, 0.25H, H-2_{*anti*}). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 179.6, 178.2 (C=O); 161.0, 160.2 (C-9); 138.1, 138.0 (C-7); 131.6, 131.2 (C-1'); 130.7, 130.7 (C-4'); 129.5, 129.4 (C-5); 128.8, 128.7 (C-3', C-5'); 128.2, 128.0 (C-2', C-6'); 124.0, 123.8 (C-6); 118.7, 118.7 (C-8); 116.9 (C-10); 99.7, 97.8 (C-3); 86.9 (C-2_{*anti*}); 82.3 (C-2_{*syn*}). **HRMS**: Calcd for C₁₅H₁₀BrNO₄Na 369.9685, Found 369.9711.

General procedure 1*- Synthesis of 3-halo-3-nitro-flavanones **3-11*



The appropriate 3-nitroflavanone **17** to **20** (1.0 equiv.) was solubilised in anhydrous THF under Ar. The appropriate halogenating agent, *N*-fluorobenzenesulfonimide, *N*-bromosuccinimide or *N*-iodosuccinimide, (1.7 equiv.) and Na₂CO₃ (1.3 equiv.) were added. The reaction mixture was stirred at rt until disappearance of starting material, typically 2-5 h. After addition of CH₂Cl₂ and H₂O, the aqueous layer was acidified to pH 6 using a 10% citric acid solution and was extracted with CH₂Cl₂ (x3). The combined organic layers were dried over sodium sulfate, filtered and concentrated under vacuum. Purification by flash chromatography using a linear gradient (cyclohexane/EtOAc 100:0 to 85:15) afforded the final 3-halo-3-nitroflavanones (**3-11**).

***anti*-3-Bromo-2,3-dihydro-3-nitro-2-(2'-nitrophenyl)-4*H*-1-benzopyran-4-one (**3a**)** and ***syn*-3-Bromo-2,3-dihydro-3-nitro-2-(2'-nitrophenyl)-4*H*-1-benzopyran-4-one (**3b**)**

The title compounds **3a** and **3b** were synthesised simultaneously from the 3-nitroflavanone **18** (54 mg) following the *general procedure 1*. The expected 3-bromo-3-nitroflavanones **3a** (41 mg) and **3b** (207 mg) were separated by flash chromatography and obtained as white solids with an overall yield of 75%. $R_f = 0.44$ (**3b**), 0.50 (**3a**) (cyclohexane/EtOAc 5:1). **ESI-MS**: $m/z = 415$ $[M+Na]^+$.

Compound 3a

1H NMR ($CDCl_3$, 500 MHz): δ (ppm) = 8.11 (dd, $J = 1.7, 8.0$ Hz, 1H, H-5); 8.06 (dd, $J = 1.2, 8.2$ Hz, 1H, H-3'); 7.71 (td, $J = 1.8, 8.0$ Hz, 1H, H-7); 7.67 (td, $J = 1.9, 7.6$ Hz, 1H, H-4'); 7.76-7.72 (m, 2H, H-5', H-6'); 7.30 (td, $J = 1.0, 7.5$ Hz, 1H, H-6); 7.18 (dd, $J = 0.6, 8.6$ Hz, 1H, H-8); 7.06 (s, 1H, H-2). **^{13}C NMR** ($CDCl_3$, 125 MHz): δ (ppm) = 177.1 (C=O); 160.6 (C-9); 149.0 (C-2'); 138.0 (C-7); 133.7 (C-5'); 131.4 (C-4'); 129.7 (C-5); 128.9 (C-6'); 126.4 (C-1'); 125.3 (C-3'); 124.4 (C-6); 119.1 (C-10); 118.6 (C-8); 97.1 (C-3); 79.8 (C-2). **HRMS**: Calcd for $C_{15}H_9BrN_2O_6Na$ 414.9536, Found 414.9560.

Compound 3b

1H NMR ($CDCl_3$, 500 MHz): δ (ppm) = 8.15 (dd, $J = 0.9, 8.1$ Hz, 1H, H-3'); 8.07 (dd, $J = 1.6, 7.9$ Hz, 1H, H-5); 7.91 (dd, $J = 1.3, 8.1$ Hz, 1H, H-6'); 7.75 (td, $J = 1.3, 7.8$ Hz, 1H, H-4'); 7.70 (td, $J = 1.7, 7.8$ Hz, 1H, H-7); 7.66 (td, $J = 1.3, 7.9$ Hz, 1H, H-5'); 7.28 (td, $J = 0.8, 7.6$ Hz, 1H, H-6); 7.16 (br d, $J = 8.3$ Hz, 1H, H-8); 7.00 (s, 1H, H-2). **^{13}C NMR** ($CDCl_3$, 125 MHz): δ (ppm) = 178.3 (C=O); 159.8 (C-9); 149.5 (C-2'); 138.3 (C-7); 133.7 (C-4'); 131.6 (C-5'); 130.2 (C-3'); 129.6 (C-5); 125.4 (C-6'); 124.1 (C-1'); 124.3 (C-6); 118.6 (C-8); 116.9 (C-10); 97.1 (C-3); 76.2 (C-2). **HRMS**: Calcd for $C_{15}H_9BrN_2O_6Na$ 414.9536, Found 414.9552.

***anti*-3-Bromo-2,3-dihydro-2-(5'-methoxy-2'-nitrophenyl)-3-nitro-4H-1-benzopyran-4-one (4a)** and ***syn*-3-Bromo-2,3-dihydro-2-(5'-methoxy-2'-nitrophenyl)-3-nitro-4H-1-benzopyran-4-one (4b)**

The title compounds **4a** and **4b** were synthesised simultaneously from the 3-nitroflavanone **19** (141 mg) following the *general procedure 1*. The expected 3-bromo-3-nitroflavanones **4a** (15 mg) and **4b** (98 mg) were separated by flash chromatography and obtained as white solids with a global yield of 66%. $R_f = 0.35$ (**4b**), 0.44 (**4a**) (cyclohexane/EtOAc 5:1). **ESI-MS**: $m/z = 445$ $[M+Na]^+$.

Compound 4a

¹H NMR (CDCl₃, 500 MHz): δ (ppm) = 8.15 (d, J = 9.2 Hz, 1H, H-3'); 8.12 (dd, J = 1.6, 7.9 Hz, 1H, H-5); 7.71 (td, J = 1.9, 7.9 Hz, 1H, H-7); 7.30 (td, J = 0.8, 7.6 Hz, 1H, H-6); 7.25 (s, 1H, H-2); 7.19 (dd, J = 0.8, 8.4 Hz, 1H, H-8); 7.17 (d, J = 2.7 Hz, 1H, H-6'); 7.07 (dd, J = 2.7, 9.2 Hz, 1H, H-4'); 3.91 (s, 1H, OCH₃). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 177.2 (C=O); 163.4 (C-5'); 160.7 (C-9); 141.8 (C-2'); 138.0 (C-7); 129.7 (C-5); 129.1 (C-1'); 128.2 (C-3'); 124.4 (C-6); 119.1 (C-10); 118.6 (C-8); 115.8 (C-4'); 114.1 (C-6'); 97.2 (C-3); 79.7 (C-2); 56.4 (OCH₃). **HRMS**: Calcd for C₁₆H₁₁BrN₂O₇Na 444.9622, Found 444.9641.

Compound 4b

¹H NMR (CDCl₃, 500 MHz): δ (ppm) = 8.08 (dd, J = 1.7, 8.0 Hz, 1H, H-5); 8.01 (d, J = 9.2 Hz, 1H, H-3'); 7.70 (td, J = 1.7, 7.8 Hz, 1H, H-7); 7.64 (d, J = 2.8 Hz, 1H, H-6'); 7.28 (td, J = 7.7 Hz, 1H, H-6); 7.17 (dd, J = 0.9, 8.4 Hz, 1H, H-8); 7.16 (s, 1H, H-2); 7.06 (dd, J = 2.7, 9.1 Hz, 1H, H-4'); 3.96 (s, 1H, OCH₃). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 178.4 (C=O); 162.6 (C-5'); 159.9 (C-9); 142.1 (C-2'); 138.2 (C-7); 129.7 (C-5); 128.2 (C-3'); 127.5 (C-1'); 124.3 (C-6); 118.5 (C-8); 117.0 (C-10); 116.4 (C-6'); 115.2 (C-4'); 97.5 (C-3); 76.1 (C-2); 56.4 (OCH₃). **HRMS**: Calcd for C₁₆H₁₁BrN₂O₇Na 444.9644, Found 444.9641.

***syn*-3-Bromo-2,3-dihydro-2-(2'-fluorophenyl)-3-nitro-4*H*-1-benzopyran-4-one (5)**

The title compound **5** was synthesised from the 3-nitroflavanone **20** (54 mg) following the *general procedure 1*. The expected 3-bromo-3-nitroflavanone **5** (32 mg) was obtained as a white solid with 46% yield. R_f = 0.51 (cyclohexane/EtOAc 6:4). **¹H NMR** (CDCl₃, 500 MHz): δ (ppm) = 8.10 (dd, J = 1.7, 7.9 Hz, 1H, H-5); 7.94 (td, J = 1.7, 7.4 Hz, 1H, H-6'); 7.68 (td, J = 1.8, 7.8 Hz, 1H, H-7); 7.47 (m, 1H, H-4'); 7.31 (td, J = 1.0, 7.7 Hz, 1H, H-5'); 7.26 (td, J = 0.8, 7.6 Hz, 1H, H-6); 7.16 (dd, J = 0.6, 8.4 Hz, 1H, H-8); 7.94 (td, J = 1.1, 9.4 Hz, 1H, H-3'); 6.41 (s, 1H, H-2). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 179.1 (C=O); 160.1 (C-9); 160.0 (d, J = 252.2 Hz, C-2'); 138.1 (C-7); 132.5 (d, J = 9.8 Hz, C-4'); 130.5 (d, J = 2.0 Hz, C-6'); 129.5 (C-5); 124.4 (d, J = 3.7 Hz, C-5'); 124.1 (C-6); 119.2 (d, J = 12.1 Hz, C-1'); 118.6 (C-8); 117.1 (C-10); 116.0 (d, J = 19.9 Hz, C-3'); 98.3 (C-3); 76.7 (d, J = 2.8 Hz, C-2). **HRMS**: Calcd for C₁₅H₉BrFNO₄Na 387.9591, Found 387.9637. Structure was confirmed by X-ray crystal structure.

***anti*-3-Fluoro-2,3-dihydro-2-phenyl-3-nitro-4*H*-1-benzopyran-4-one (6)**

The title compound **6** was synthesised from the 3-nitroflavanone **17** (64 mg) following the *general procedure 1*. The expected 3-fluoro-3-nitroflavanone **6** (46 mg) was obtained as a white solid with 67% yield. R_f = 0.50 (cyclohexane/EtOAc 3:2). **ESI-MS**: m/z = 310

[M+Na]⁺. ¹H NMR (CDCl₃, 500 MHz): δ (ppm) = 8.06 (dd, *J* = 1.7, 7.9 Hz, 1H, H-5); 7.73 (td, *J* = 1.8, 7.9 Hz, 1H, H-7); 7.49-7.46 (m, 5H, H-2', H-3', H-4', H-5', H-6'); 7.27 (td, *J* = 1.1, 7.6 Hz, 1H, H-6); 7.23 (dd, *J* = 0.7, 8.5 Hz, 1H, H-8); 5.67 (d, *J* = 6.0 Hz, 1H, H-2). ¹³C NMR (CDCl₃, 125 MHz): δ (ppm) = 178.7 (d, *J* = 20.8 Hz, C=O); 161.3 (C-9); 138.6 (C-7); 130.7 (C-4'); 130.1 (d, *J* = 1.1 Hz, C-1'); 129.2 (2C, C-3', C-5'); 128.6 (d, *J* = 1.0 Hz, C-5); 127.0 (2C, C-2', C6'); 123.9 (C-6); 119.3 (d, *J* = 1.5 Hz, C-10); 119.0 (C-8); 107.5 (d, *J* = 247.6 Hz, C-3); 81.5 (d, *J* = 26.3 Hz, C-2). HRMS: Calcd for C₁₅H₁₀FNO₄Na 310.0486, Found 310.0498.

***syn*-3-Fluoro-2,3-dihydro-3-nitro-2-(2'-nitrophenyl)-4*H*-1-benzopyran-4-one (7)**

The title compound **7** was prepared from the 3-nitroflavanone **18** (110 mg) following the *general procedure 1*. The expected 3-fluoro-3-nitroflavanone **7** (73 mg) was obtained as a white solid with 63% yield. *R_f* = 0.54 (cyclohexane/EtOAc 1:1). ESI-MS: *m/z* = 355 [M+Na]⁺. ¹H NMR (CDCl₃, 500 MHz): δ (ppm) = 8.13 (dd, *J* = 1.2, 8.3 Hz, 1H, H-3'); 8.09 (dd, *J* = 1.6, 7.9 Hz, 1H, H-5); 7.83 (dd, *J* = 1.2, 8.0 Hz, 1H, H-6'); 7.75 (m, 2H, H-7, H-5'); 7.68 (td, *J* = 1.3, 8.0 Hz, 1H, H-4'); 7.31 (br t, *J* = 7.6 Hz, 1H, H-6); 7.24 (br d, *J* = 8.3 Hz, 1H, H-8); 7.03 (d, *J* = 2.9 Hz, 1H, H-2). ¹³C NMR (CDCl₃, 125 MHz): δ (ppm) = 177.8 (d, *J* = 20.3 Hz, C=O); 160.8 (C-9); 148.2 (C-2'), 138.6 (C-7); 134.3 (C-5'); 131.5 (C-4); 128.8 (d, *J* = 1.2 Hz, C-5); 128.2 (C-6'); 125.8 (C-3'); 124.8 (d, *J* = 1.3 Hz, C-1'); 124.3 (C-6); 119.5 (d, *J* = 1.3 Hz, C-10); 118.7 (C-8); 108.0 (d, *J* = 252.2 Hz, C-3); 75.3 (d, *J* = 23.4 Hz, C-2). HRMS: Calcd for C₁₅H₉FN₂O₆Na 355.0337, Found 355.0350.

***anti*-3-Fluoro-2,3-dihydro-2-(5'-methoxy-2'-nitrophenyl)-3-nitro-4*H*-1-benzopyran-4-one (8)**

The title compound **8** was synthesised from the nitroflavanone **19** (131 mg) following the *general procedure 1*. The expected 3-fluoro-3-nitroflavanone **8** was obtained with 53% purity. *R_f* = 0.55 (cyclohexane/EtOAc 1:1). ESI-MS: *m/z* = 385 [M+Na]⁺. ¹H NMR (CDCl₃, 500 MHz): δ (ppm) = 8.21 (d, *J* = 9.1 Hz, 1H, H-3'); 8.08 (dd, *J* = 1.6, 8.1 Hz, 1H, H-5); 7.75 (td, *J* = 1.5, 7.7 Hz, 1H, H-7); 7.30 (br t, *J* = 7.7 Hz, 1H, H-6); 7.25 (d, *J* = 2.8 Hz, 1H, H-6'); 7.24 (d, *J* = 8.7 Hz, 1H, H-8), 7.21 (d, *J* = 2.4 Hz, 1H, H-2); 7.06 (dd, *J* = 2.6, 6.4 Hz, 1H, H-4'); 3.94 (s, 3H, OCH₃). ¹³C NMR (CDCl₃, 125 MHz): δ (ppm) = 177.8 (d, *J* = 20.1 Hz, C=O); 163.8 (C-5'); 160.6 (C-9); 140.7 (C-2'); 138.4 (C-7); 128.6 (d, *J* = 1.0 Hz, C-5); 128.6 (C-3'), 127.6 (d, *J* = 1.3 Hz, C-1'); 124.1 (C-6); 119.5 (d, *J* = 1.3 Hz, C-10); 118.8 (C-8); 115.2 (C-

4'), 113.5 (C-6'); 108.2 (d, $J = 253$ Hz, C-3); 75.2 (d, $J = 26$ Hz, C-2); 56.2 (OCH₃). **HRMS**: Calcd for C₁₆H₁₁FN₂O₇Na 385.0443, Found 385.0486.

***anti*-3-Fluoro-2,3-dihydro-2-(2'-fluorophenyl)-3-nitro-4*H*-1-benzopyran-4-one (9)**

The title compound **9** was synthesised from the 3-nitroflavanone **20** (54 mg) following the *general procedure 1*. The expected 3-fluoro-3-nitroflavanone **9** (44 mg) was obtained as a white solid with 77% yield. $R_f = 0.52$ (cyclohexane/EtOAc 6:4). **ESI-MS**: $m/z = 328$ [M+Na]⁺. **¹H NMR** (CDCl₃, 500 MHz): δ (ppm) = 8.08 (dd, $J = 1.7, 7.9$ Hz, 1H, H-5); 7.72 (td, $J = 1.7, 7.8$ Hz, 1H, H-7); 7.53-7.46 (m, 2H, H-4', H-6'); 7.29-7.24 (m, 2H, H-6, H-5'); 7.22-7.17 (m, 2H, H-8, H-3'); 6.08 (d, $J = 4.0$ Hz, 1H, H-2). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 178.5 (d, $J = 20.0$ Hz, C=O); 161.3 (C-9); 160.3 (d, $J = 251.4$ Hz, C-2'); 138.6 (C-7); 132.5 (d, $J = 8.6$ Hz, C-4'); 128.7 (d, $J = 1.1$ Hz, C-5); 127.7 (d, $J = 2.3$ Hz, C-6'); 125.2 (d, $J = 3.6$ Hz, C-5'); 124.0 (C-6); 119.5 (d, $J = 1.4$ Hz, C-1'); 119.0 (C-8); 118.0 (dd, $J = 1.1, 12.7$ Hz, C-10); 116.1 (d, $J = 21.3$ Hz, C-3'); 107.8 (d, $J = 249.8$ Hz, C-3); 75.1 (dd, $J = 3.4, 28.8$ Hz, C-2). **HRMS**: Calcd for C₁₅H₉F₂NO₄Na 328.0392, Found 328.0417. Structure was confirmed by X-ray crystal structure.

***syn*-3-Iodo-2,3-dihydro-3-nitro-2-(2'-nitrophenyl)-4*H*-1-benzopyran-4-one (10)**

The title compound **10** was synthesised from the 3-nitroflavanone **18** (105 mg) following the *general procedure 1*. The expected 3-iodo-3-nitroflavanone **10** (83 mg) was obtained as a white solid with 56% yield. $R_f = 0.52$ (cyclohexane/EtOAc 6:4). **¹H NMR** (CDCl₃, 500 MHz): δ (ppm) = 8.12 (br d, $J = 7.9$ Hz, 1H, H-6'); 8.07 (dd, $J = 1.6, 7.9$ Hz, 1H, H-5); 7.91 (dd, $J = 1.0, 8.1$ Hz, 1H, H-3'); 7.75 (br t, $J = 7.9$ Hz, 1H, H-5'); 7.71-7.64 (m, 2H, H-7, H-4'); 7.28 (br t, $J = 7.3$ Hz, 1H, H-6); 7.13 (d, $J = 8.3$ Hz, 1H, H-8); 6.14 (s, 1H, H-2). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 180.2 (C=O); 159.6 (C-9); 149.7 (C-2'); 138.1 (C-7); 132.5 (C-5'); 131.4 (C-4'); 129.7 (C-5); 129.5 (C-6'); 125.9 (C-1'); 125.5 (C-3'); 124.3 (C-6); 118.2 (C-8); 116.8 (C-10); 86.1 (C-3); 77.1 (C-2).

***syn*-3-Iodo-2,3-dihydro-2-(5'-methoxy-2'-nitrophenyl)-3-nitro-4*H*-1-benzopyran-4-one (11)**

The title compound **11** was synthesised from the 3-nitroflavanone **19** (72 mg) following the *general procedure 1*. The expected 3-iodo-3-nitroflavanone **11** (24 mg) was obtained as a white solid with 24% yield. $R_f = 0.57$ (cyclohexane/EtOAc 1:2). **ESI-MS**: $m/z = 493$ [M+Na]⁺. **¹H NMR** (CDCl₃, 500 MHz): δ (ppm) = 8.09 (dd, $J = 1.4, 8.0$ Hz, 1H, H-5); 8.00 (d, $J = 9.2$ Hz, 1H, H-3'); 7.67 (td, $J = 1.8, 7.8$ Hz, 1H, H-7); 7.65 (d, $J = 2.6$ Hz, 1H, H-6');

7.29 (m, 1H, H-6); 7.15 (br d, $J = 8.3$ Hz, 1H, H-8); 7.06 (dd, $J = 2.7, 9.1$ Hz, 1H, H-4'); 6.27 (s, 1H, H-2); 3.98 (OCH₃). ¹³C NMR (CDCl₃, 125 MHz): δ (ppm) = 180.3 (C=O); 162.4 (C-5'); 159.7 (C-9); 142.4 (C-2'); 138.0 (C-7); 129.7 (C-5); 128.5 (C-1'); 128.3 (C-3'); 124.3 (C-6); 118.3 (C-8); 116.8 (C-10); 115.8 (C-6'); 115.1 (C-4'); 86.8 (C-3); 77.4 (C-2), 56.4 (OCH₃).

(E)-1-(2-methyl-2-nitroethenyl)-2-nitro-benzene (12)

The 2-nitrobenzaldehyde (1.1 g, 6.95 mmol, 1.0 equiv.) was solubilised in toluene (10 ml) in a round-bottom flask equipped with a Dean-Stark apparatus. Dimethylamine hydrochloride (5.4 g, 66.3 mmol, 9.5 equiv.), potassium fluoride (1.1 g, 18.0 mmol, 2.6 equiv.) and nitroethane (1.5 ml, 29.9 mmol, 1.5 equiv.) were added and the mixture was refluxed for 10 h. The reaction mixture was diluted in CH₂Cl₂ and washed with water (x2). The aqueous layers were backextracted with CH₂Cl₂ (x2). The combined organic layers were dried over sodium sulfate and concentrated under vacuum. The residue was purified by flash chromatography using a linear gradient (cyclohexane/EtOAc; 100/0 to 50/50) to give compound **12** (303.8 mg, 1.46 mmol, 21% yield) as slightly yellow oil. $R_f = 0.25$ (cyclohexane/EtOAc 5:2). ¹H NMR (CDCl₃, 500 MHz): δ (ppm) = 8.35 (s, 1H, CH=C); 8.26 (d, $J = 8.0$ Hz, 1H, H-3), 7.75 (t, $J = 7.5$ Hz, 1H, H-5), 7.63 (t, $J = 7.5$ Hz, 1H, H-4), 7.39 (d, $J = 8.0$ Hz, 1H, H-6); 2.25 (s, 3H, CH₃). ¹³C NMR (CDCl₃, 125 MHz): δ (ppm) = 148.6, 147.5 (2C-NO₂); 134.0 (C-5); 131.0 (CH=C); 130.9 (C-6); 130.3 (C-4); 128.7 (C-1); 125.4 (C-3); 13.7 (CH₃). *E*-stereochemistry was confirmed by ¹H-¹H-ROESY and ¹H-¹H-NOESY experiments.

(Z)-1-(2-bromo-2-nitroethenyl)-benzene (13)

The benzaldehyde (1 ml, 9.89 mmol, 1.0 equiv.) was diluted in toluene (25 ml) containing 4Å activated molecular sieves. Bromonitromethane (0.7 ml, 10.4 mmol, 1.04 equiv.) and piperidine (0.06 ml, 0.61 mmol, 0.06 equiv.) were added and the mixture was refluxed for 6 h. The reaction mixture was filtered and filtrate was concentrated under vacuum. The residue was purified by flash chromatography using a linear gradient (cyclohexane/EtOAc 100:0 to 50:50) to give compound **13** (714 mg, 3.13 mmol, 32% yield) as a bright orange oil, which tends to crystallize. $R_f = 0.54$ (cyclohexane/EtOAc 3:1). ¹H NMR (CDCl₃, 500 MHz): δ (ppm) = 8.65 (s, 1H, CH=C); 7.89 (m, 2H) (H-2, H-6), 7.53-7.48 (m, 3H, H-3, H-4, H-5). ¹³C NMR (CDCl₃, 125 MHz): δ (ppm) = 136.6 (CH=C); 132.1 (C-4); 131.1 (2C, C-2, C-6); 130.5 (C-1); 129.1 (2C, C-3, C-5); 128.3 (CH=C). Structure was confirmed by crystal structure (Supporting Information)

3,4-Dihydro-4-hydroxy-3-methyl-3-nitro-2-(2'-nitrophenyl)-2H-1-benzopyran (14)

The compound **12** (206 mg, 1.0 mmol, 1.0 equiv.) was put under argon in a round-bottom flask dried under the flame and was solubilised in anhydrous THF (2 ml). After addition of the salicylaldehyde (0.6 ml, 5.6 mmol, 5.6 equiv.) and the Et₃N (0.1 ml, 0.72 mmol, 0.7 equiv.), reaction was stirred overnight at room temperature under Ar. After neutralization with acetic acid (0.15 ml), Girard's reagent solution was added and the reaction mixture was stirred for 2 h at rt before extraction with CH₂Cl₂. The organic layer was washed with H₂O and aqueous layer was then backextracted with CH₂Cl₂ (x2). The combined organic layers were dried with sodium sulfate, filtered and concentrated under vacuum. The crude mixture was purified by flash chromatography using a linear gradient (cyclohexane/EtOAc; 100/0 to 50/50) to give compound **14** (80 mg, 0.24 mmol, 24% yield) as slightly yellow oil. $R_f = 0.35$ (cyclohexane/EtOAc 5:2). **ESI-MS**: $m/z = 353$ [M+Na]⁺. **¹H NMR** (CDCl₃, 500 MHz) (*N.B.*: all NMR signals are doubled due to the presence of both diastereoisomers; only the major one is assigned here): δ (ppm) = 8.03 (dd, $J = 1.3, 8.2$ Hz, 1H, H-3'); 7.97 (dd, $J = 1.2, 7.9$ Hz, 1H, H-6'); 7.78 (td, $J = 1.3, 7.6$ Hz, 1H, H-5'); 7.63 (m, 1H, H-5); 7.61 (m, 1H, H-4'); 7.29 (m, 1H, H-7); 7.13 (td, $J = 1.6, 7.6$ Hz, 1H, H-6); 6.93 (dd, $J = 0.9, 8.2$ Hz, 1H, H-8); 6.32 (s, 1H, H-2) 6.02 (s, 1H, H-4); 2.89 (br s, 1H, OH); 1.62 (s, 3H, CH₃).

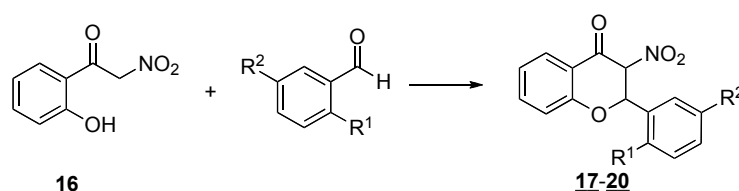
3-Bromo-3,4-dihydro-4-hydroxy-3-nitro-2-phenyl-2H-1-benzopyran (15)

The title compound **15** was synthesised following the procedure described for the synthesis of compound **14** starting from compound **13** (49 mg, 0.22 mmol, 1.0 equiv.), using salicylaldehyde (0.12 ml, 1.13 mmol, 5.1 equiv.) and Et₃N (15 μ l, 0.11 mmol, 0.5 equiv.). After purification by flash chromatography using a linear gradient (cyclohexane/EtOAc 100:0 to 80:20), the title compound **15** was obtained as a mixture of both diastereoisomers used as such for the synthesis of compound **2** (Method 1). $R_f = 0.29$ (EtOAc/cyclohexane 1:5). **¹H NMR** (CDCl₃, 500 MHz) (*N.B.*: all NMR signals are doubled due to the presence of both distereoisomers. Only the major one is assigned here): δ (ppm) 7.58 (m, 1H, H-5); 7.49 (m, 2H, H-2', H-6'); 7.42 (m, 3H, H-3', H-4', H-5'); 7.35 (m, 1H, H-7); 7.14 (td, $J = 1.1, 7.7$ Hz, 1H, H-6); 7.02 (dd, $J = 1.1, 8.2$ Hz, 1H, H-8); 5.90 (s, 1H, H-4); 5.65 (s, 1H, H-2); 2.59 (br s, 1H, OH). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 152.1 (C-9); 132.0 (C-1'); 130.6 (C-7); 130.4 (C-4'); 128.6 (2C) (C-3', C-5'); 128.0 (2C) (C-2', C-6'); 127.0 (C-5); 122.9 (C-6); 122.8 (C-10); 116.9 (C-8); 108.8 (C-3); 80.5 (C-2); 74.3 (C-4).

1-(2-hydroxyphenyl)-2-nitroethanone (16)

The 4-hydroxy-3-nitrocoumarine (3.12 g, 15.1 mmol, 1 equiv.) was solubilised in NaOH solution (5%, w/v in H₂O, 100 ml). The reaction mixture was stirred for 2 h at 60 °C and turned from yellow to bright dark orange. The mixture was cooled down in ice and acidified by slow addition of acetic acid (30 ml). The precipitate was filtered to give the expected compound **16** (2.35 g 13.0 mmol, 86% yield) as an off-white solid. $R_f = 0.31$ (cyclohexane/EtOAc 1:1). **ESI-MS**: $m/z = 204 [M+Na]^+$, $180 [M-H]^-$. **¹H NMR** (CDCl₃, 500 MHz): δ (ppm) = 11.22 (s, 1H, OH); 7.60 (td, $J = 1.5, 7.9$ Hz, 1H, H-4'); 7.44 (dd, $J = 1.5, 8.1$ Hz, 1H, H-6'); 7.06 (dd, $J = 0.8, 8.7$ Hz, 1H, H-3'); 6.98 (td, $J = 0.8, 7.6$ Hz, 1H, H-5'); 5.91 (s, 2H, CH₂). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 190.8 (C=O); 162.9 (C-OH); 138.5 (C-4'); 128.8 (C-6'); 120.1 (C-5'); 119.4 (C-3'); 117.1 (C-1'); 80.7 (CH₂).

General procedure 2 – Synthesis of 3-nitroflavanones 17-20



The compound **16** (1.0 equiv.) was solubilised in absolute ethanol. The appropriate benzaldehyde (1.0 equiv.), D-proline (0.5 equiv.) and Na₂CO₃ (1.5 equiv.) were added to the mixture and the reaction was stirred overnight at rt. After dilution in CH₂Cl₂, compound was extracted from organic layer with H₂O. Aqueous layer was acidified to pH 2-3 using HCl 1M and extracted with CH₂Cl₂ (x3). The combined organic layers were dried over Na₂SO₄, filtered and concentrated under vacuum. The crude mixture was purified by flash chromatography using a linear gradient (CH₂Cl₂/MeOH 100:0 to 70:30) to give expected 3-nitroflavanones **17-20**.

trans-2,3-Dihydro-2-phenyl-3-nitro-4H-1-benzopyran-4-one (17)

The title compound **17** was synthesised from the compound **16** (299 mg, 1.65 mmol) and benzaldehyde (0.17 ml, 1.68 mmol), following the *general procedure 2*. The title compound **17** (301 mg, 1.12 mmol) was obtained after flash chromatography as a white solid with 68% yield. $R_f = 0.14$ (EtOAc/cyclohexane 6:4). **ESI-MS**: $m/z = 292 [M+Na]^+$. **¹H NMR** (CDCl₃, 500 MHz): δ (ppm) = 8.01 (dd, $J = 1.7, 8.0$ Hz, 1H, H-5); 7.63 (td, $J = 1.8, 7.8$ Hz, 1H, H-7); 7.53 (m, 2H, H-2', H-6'); 7.48-7.45 (m, 3H, H-3', H-4', H-5'); 7.19 (td, $J = 0.7, 7.6$ Hz, 1H, H-6); 7.10 (br d, $J = 8.5$ Hz, 1H, H-8); 5.89 (d, $J = 11.9$ Hz, 1H, H-2); 5.80 (d, $J = 11.8$ Hz, 1H, H-3); 3.98 (OCH₃). **¹³C NMR** (CDCl₃, 125 MHz): δ (ppm) = 181.6 (C=O); 161.0 (C-9);

138.0 (C-7); 133.7 (C-1'); 130.6 (C-4'); 129.4 (2C, C-3', C-5'); 128.3 (C-5); 127.6 (2C, C-2', C-6'); 123.3 (C-6); 119.0 (C-10); 118.5 (C-8); 91.7 (C-3); 81.3 (C-2).

2,3-Dihydro-3-nitro-2-(2'-nitrophenyl)-4H-1-benzopyran-4-one (18)

The title compound **18** was synthesised from the compound **16** (301 mg, 1.66 mmol) and 2-nitrobenzaldehyde (255 mg, 1.69 mmol), following the *general procedure 2*. The title 3-nitroflavanone **18** (354 mg, 1.13 mmol) was obtained after flash chromatography with 68% yield as a mixture of diastereoisomers and used as a mixture for the following step. $R_f = 0.05$ (EtOAc/cyclohexane 6:4). **ESI-MS**: $m/z = 313 [M-H]^-$, $337 [M+Na]^+$.

2,3-Dihydro-2-(5-methoxy-2-nitrophenyl)-3-nitro-4H-1-benzopyran-4-one (19)

The title compound **19** was synthesised from compound **16** (185 mg, 1.02 mmol) and 5-methoxy-2-nitrobenzaldehyde (187 mg, 1.03 mmol), following the *general procedure 1*. The title 3-nitroflavanone **19** (167 mg, 0.49 mmol) was obtained after flash chromatography with 48% yield as a mixture of diastereoisomers and used as a mixture for the following step. $R_f = 0.20$ (CH₂Cl₂/MeOH 95:5). **ESI-MS**: $m/z = 367 [M+Na]^+$.

2,3-Dihydro-2-(2'-fluorophenyl)-3-nitro-4H-1-benzopyran-4-one (20)

The title compound **20** was synthesised from compound **16** (178 mg, 0.98 mmol) and 2-fluorobenzaldehyde (185 μ l, 1.76 mmol), following the *general procedure 1*. The title compound **20** (125 mg, 0.44 mmol) was obtained after flash chromatography with 44% yield as a mixture of diastereoisomers and used as a mixture for the following step. $R_f = 0.31$ (CH₂Cl₂/MeOH 95:5).

Stability measurement

The compound stability was measured by HPLC analysis using an X-terra column (100 x 4.6 mm, 5 μ m) with 1 ml/min flow and the following gradient: H₂O/acetonitrile 95:5 for 2 min then up to 0:100 in 10 min and maintained at 0:100 for 2 min with H₂O and acetonitrile containing 0.1% formic acid. The tested compound was first injected in solution in 100% DMSO (10 mM concentration) to check its purity. Then 50 μ l of a solution of 1 mM of tested compound was prepared by dilution in DNMT3A-c enzyme buffer (Hepes 20 mM pH 7.2, KCl 50 mM, EDTA 1 mM final concentration). The percentages of remaining compound were determined with the area of the corresponding HPLC peak. The mean of at least two experiments and the standard error is reported.

4.2 Biology

DNMT3A-c expression and purification

The human catalytic DNMT3A (DNMT3A-c) was expressed in *Escherichia coli* Rosetta-gami™ 2(DE3) pLysS competent cells (Novagen) carrying the plasmid pET28a-DNMT3A(624-912) containing a 6-His tag used by Gros *et al.* [19]. Briefly, after bacterial transformation and preculture, cells were grown in lysogeny broth (Lennox) supplemented with 25 µg/mL of kanamycin and 20 µg/mL of chloramphenicol at 37 °C to OD_{600 nm} ≈ 0.5. Then, culture was cooled down to 20 °C and induced overnight with 1 mM isopropyl β-D-1-thiogalactopyranoside or cooled down to 34 °C and induced during 4 hours. Protein purification was carried out at 4 °C based on Gowher *et al* protocol [20]. Polyhistidine-tagged proteins were purified by affinity chromatography, on Ni-NTA agarose resin (Qiagen). Coomassie-stained SDS-PAGE gel was used to evaluate the purity more than 90% and the DNMT3A-c concentration was determined by Bradford assay or by using NanoDrop 2000 spectrophotometer (Thermo Scientific) with an estimated extinction coefficient.

DNMT inhibition assay

Compound activities were determined with a fluorescence-based assay [9]. In brief, a double-strand DNA, comprising a unique CpG site overlaying an endonuclease restriction site for methylation-sensitive enzyme was used. This oligonucleotide comprises a 6-FAM at one end and biotin on the other end allowing immobilization into a 384-well plate (Perkin Elmer) pre-coated with avidin. Compounds to be evaluated and SAM as methyl donor were added followed by DNMT3A-c to start the methylation reaction, which was prolonged 1 h at 37 °C. After several washing, with PBS tween (0.05 %) containing NaCl (0.5 M) and PBS tween (0.05 %). restriction step was performed with HpyCH4IV (New England, BioLabs) to hand on only the specific fluorescence signal. Fluorescence was quantified on an EnVision 2103 Multilabel Reader (Perkin Elmer). Inhibition percentages are defined as $100 \times (100 - (X_{\text{meth}} - X_{\text{restri}}) / (X_{\text{DNA}} - X_{\text{restri}}))$, where X_{meth} , X_{restri} and X_{DNA} are respectively the fluorescence signals of the compound methylation, restriction and DNA controls. These percentages for a concentration range were then used to calculate the half maximal effective concentration (EC₅₀) (average of at least 2 experiments) for the compound of interest by using the nonlinear regression with sigmoidal dose–response (variable slope) tool of GraphPad Prism software.

Luciferase expression measurement in cmv-Luc KG1 cell model [10,18]

KG-1 cells, stably transfected with the firefly luciferase (luc+ from pGL3 by Promega) reporter gene under the control of the cytomegalovirus (CMV) promoter (from pEGFP-N1 by Clontech Laboratories Inc.) partially methylated (50 %), was seeded at 20 000 cells per well in a 96-well plate. After 24 h incubation in the presence of the test compound or solvent (DMSO), the induction of the promoter was measured by quantification of luciferase using the One-Glo assay (Promega) according to the manufacturer's protocol. The luminescence was measured on an EnVision multilabel plate reader (PerkinElmer), and the data are expressed as the fold induction as compared with the DMSO control. The mean of three experiments and the standard error is reported.

Antiproliferative activity

For antiproliferative activity, cells were seeded (2×10^4 cells/ml for KG-1 in RPMI-1640 medium supplemented with 15 % foetal calf serum (Lonza, France) and 1.5×10^4 cells/ml for HCT116 in MEM medium containing HEPES, L-glutamine and supplemented with 10% foetal calf serum (Lonza), at 37 °C under 5 % CO₂) at day 0 in 96-well plates. Cells were treated with tested compound solutions at a dose range from 3.2×10^{-9} M to 1×10^{-5} M. Treatment was repeated on days 2 and 3, and, on day 4, the antiproliferative activity of compounds was measured using the ATP quantification method "ATPlite One Step Luminescence Assay System" (PerkinElmer). Raw data were analysed with Prism 4.03 to generate EC₅₀ values corresponding to the compound concentration required to cause a 50% decrease cell viability as compared with untreated controls. The values presented are the mean of at least two independent experiments run in duplicates.

Supplementary Information

Crystallographical data, ¹H and ¹³C NMR spectra of compound **Flv69**, **Flv880**, **1-13**, **16** and **17** are available in supplementary information.

Declaration of interest

The authors do not have any conflicts of interest to declare.

Acknowledgements

We thank Dr Laure Vendier (LCC, UPR 8241 CNRS, Toulouse) for crystallography structure determinations and Dr Jacques Fahy for helpful scientific discussion about synthesis pathways. This work was supported to PBA and ML PlanCancer2014 (N°EPIG201401) and to DP by Pierre Fabre and the Centre National de la Recherche Scientifique (CNRS).

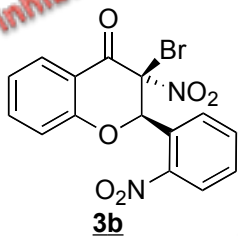
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Graphical abstract

Non-nucleoside
DNMT inhibitors



- DNMT3A-c inhibition ✓

