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## New anti-ovarian cancer quinolone derivatives acting by modulating microRNA processing machinery†

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MicroRNAs (miRNAs) play a crucial role in ovarian cancer (OC) pathogenesis and miRNA processing can be the object of pharmacological intervention. By exploiting our in-house quinolone library, we combined a cell-based screening with medicinal chemistry efforts, ultimately leading to derivative **33** with anti-OC activity against distinct cell lines ( $GI_{50}$  values 13.52–31.04  $\mu$ M) and  $CC_{50}$   $Wi-38$  = 142.9  $\mu$ M. Compound **33** retained anticancer activity against additional cancer cells and demonstrated a synergistic effect with cisplatin against cisplatin-resistant A2780 cells. Compound **33** bound TRBP by SPR ( $K_D$  = 4.09  $\mu$ M) and thermal shift assays and its activity was TRBP-dependent, leading to modulation of siRNA and miRNA maturation. Derivative **33** exhibited augmented potency against OC cells and a stronger binding affinity for TRBP compared to enoxacin, the sole quinolone identified as a modulator of miRNA maturation. Consequently, **33** represents a promising template for developing novel anti-OC agents with a distinctive mechanism of action.

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## Introduction

According to GLOBOCAN estimates, in 2020, ovarian cancer (OC) was diagnosed in 313 959 women worldwide, resulting in 207 252 deaths.<sup>1</sup> OC is the leading cause of death among women diagnosed with gynecologic cancers, making it the fifth most common cause of death in women.<sup>1–3</sup> Pathologically, most OCs (approximately 90%) originate from the epithelial surface, while a smaller part arises from germ

or stromal cells.<sup>4</sup> Malignant epithelial OCs can be classified into high-grade serous ovarian carcinoma (HGSC), endometrioid carcinoma, clear cell carcinoma, mucinous carcinoma, and low-grade serous ovarian carcinoma.<sup>5</sup> HGSC is considered to be the most lethal gynecological cancer<sup>6</sup> and is frequently characterized by p53 mutations (96% of cases), which lead to p53 loss of function and induce HGSC development.<sup>7</sup> Unfortunately, OCs are often associated with a poor prognosis due to diagnosis at advanced stages of disease and the development of chemotherapy resistance. The standard of care is cytoreductive surgery combined with adjuvant chemotherapy, but 70% of patients experience recurrence within 2 years of primary diagnosis, and the mortality rate reaches approximately 50% of women at five years.<sup>8,9</sup> The strategy of adjuvant chemotherapy is mainly defined for patients who respond to platinum-based treatments, while approaches for platinum-resistant patients still seem to be poorly defined.<sup>10</sup> Indeed, for platinum-refractory disease, doxorubicin, paclitaxel, gemcitabine and topotecan are used based on toxicity, treatment cost and accessibility; however, response rates range from 10% to 15% with an overall survival of about 1 year.<sup>11</sup> In addition, patients with platinum-sensitive OCs become platinum-resistant at advanced stages and acquire chemoresistance through

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various mechanisms.<sup>12</sup> Despite new treatments (*i.e.*, olaparib monotherapy or in combination with bevacizumab) recently approved as maintenance therapy for advanced OCs,<sup>13</sup> we are still far from satisfactory results and novel approaches are warranted.

In recent years, some studies have shown that microRNAs (miRNAs), small non-coding RNAs of approximately 19–22 nucleotides that are involved in fine-tuning gene expression at the post-transcriptional level, play diverse roles in OC by acting as tumor suppressors or proto-oncogenes and also by controlling drug resistance mechanisms.<sup>14</sup> Attempts to target miRNAs in cancer have been considered, with some small molecules acting as inhibitors of miRNA processing and function appearing to be more successful.<sup>15,16</sup> Nevertheless, analysis of Dicer expression in invasive epithelial OC specimens from 111 patients has shown that low Dicer expression is significantly associated with an advanced tumor stage.<sup>17</sup> Since Dicer is a key enzyme involved in the maturation of miRNAs,<sup>18</sup> its reduced expression results in low levels of miRNAs, suggesting that a strategy aimed at their enhancement may have anticancer effects. In 2008, enoxacin, a fluoroquinolone antibacterial drug, was reported as a small molecule enhancer of miRNA (SMER) processing and exhibited anticancer activity characterized by a broad spectrum of action and an innovative mechanism.<sup>19–21</sup> However, the SMER property appeared to be structure-dependent and not attributable to the whole class of quinolone molecules; in fact, within a set of approved quinolones and some synthesized enoxacin derivatives, none of the compounds showed activity except ciprofloxacin, which demonstrated to be only a very weak SMER.<sup>19</sup> Subsequently, enoxacin was reported to be able to inhibit several cancer cell lines with a dose that inhibits the growth of cells by 50% ( $GI_{50}$ ) of 125  $\mu$ M.<sup>20</sup> Several groups have confirmed that the anticancer activity of enoxacin was dependent on the modulation of miRNA processing by restoring miRNA expression to physiological levels.<sup>21–25</sup> Mechanistic studies have shown that enoxacin is able to enhance the transactivation response element RNA-binding protein-(TRBP)-mediated loading of pre-miRNAs on Dicer, by increasing the binding between pre-miRNAs and TRBP, thereby promoting Dicer cleavage at pre-miRNAs.<sup>19–21</sup> The inactivity of enoxacin as an anticancer agent in TRBP knock-out (KO) cells further confirmed that its anticancer effect is dependent on TRBP.<sup>19–21</sup>

Until 2021, enoxacin remained the only molecule acting as a SMER compound. Then, Fei Wang and colleagues reported the natural product gomisin M1 as an anti-hepatocellular carcinoma agent due to its ability to modulate miRNA processing, thus extending this peculiar mode of action beyond enoxacin and renewing interest in SMER molecules.<sup>26</sup>

To the best of our knowledge, the anticancer activity of enoxacin has never been evaluated against OC cells. Therefore, we were interested to evaluate whether enoxacin (Table 1 for the chemical structure) could retain its anticancer activity against the OC cell line SKOV-3, which

does not express p53 at protein and mRNA levels<sup>27</sup> and is resistant to tumor necrosis factor and other cytotoxic drugs (*i.e.*, diphtheria toxin, cisplatin, and adriamycin).<sup>28–30</sup> As expected, enoxacin exhibited a  $GI_{50}$  value of 125  $\mu$ M against the SKOV-3 cell line, thus extending its anticancer activity to OC. At this point, given the considerable number of quinolones present in our in-house library, previously synthesized as antibacterial, anti-mycobacterial, antiviral, and anticancer agents,<sup>31–40</sup> we selected ten of them (compounds **1–10** in Fig. 1) for testing against proliferating SKOV-3 cells. The number of quinolones was restricted due to the inability to perform a high-throughput screening (HTS) campaign and the initial prudence in testing a multitude of quinolones, given the findings of Shan and colleagues that unambiguously demonstrated that the RNAi-enhancing activity of enoxacin was structure-dependent and not universal across all classes of quinolones.<sup>19</sup> Therefore, the selection was primarily focused on 6-aminoquinolones (8 of the 10 selected), as they have been less extensively explored than 6-fluoro-quinolones. Consequently, it was deemed less probable that they were present in the library of quinolones tested by Shan *et al.*<sup>19</sup> Notably, compound **10** (rufloxacin), the sole 6-fluoroquinolone in our preliminary screening, was selected due to its status as an approved drug and extensive availability in our in-house library, having been identified by some of us in previous years.<sup>38</sup>

The encouraging outcomes of this preliminary assessment prompted us to embark on a preliminary medicinal chemistry campaign around the quinolone core, with the objective of developing compounds that exhibit anticancer activity against OC cell lines and act through the modulation of miRNA maturation. The findings of this study led to the identification of novel quinolones with promising anticancer activity, which act as SMER agents by binding to TRBP.

## Results and discussion

### Preliminary screening and design of new quinolones

Preliminary screening of in-house quinolones (compounds **1–10** – Fig. 1) against the SKOV-3 cell line revealed the amino-quinolone **5**, characterized by cyclopropyl, tetrahydroisoquinoline and methyl groups at C-1, C-7 and C-8 positions, respectively, as a promising anticancer compound with a  $GI_{50}$  of 38  $\mu$ M (about 3-fold lower than enoxacin). Amino-quinolone derivatives **1–4**, **6** and **7**, which differ from **5** mainly by the different substituent at the C-7 position, were significantly less active, suggesting that non-aromatic or more polar moieties than tetrahydroisoquinoline at this position are not well-tolerated. The amino-quinolone **8** and the naphthyridone derivative **9**, both sharing a thiazolyl-piperazine moiety at the C-7 position, also showed interesting  $GI_{50}$  values (50  $\mu$ M). Finally, the approved drug **10** exhibited poor anticancer activity ( $GI_{50}$  = 100  $\mu$ M), similar to the results obtained by Shan *et al.* who observed that ofloxacin (close analogue of **10**) did not show any significant RNAi enhancer effect.<sup>19</sup>



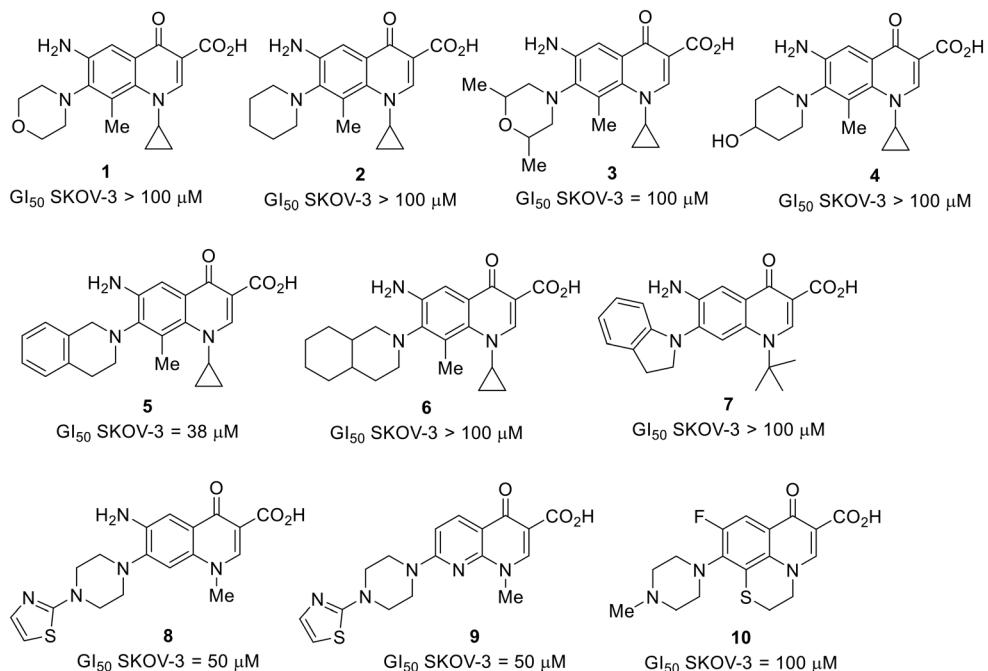


Fig. 1 Small set of selected in-house quinolones (1–10) and their antiproliferative activity against the SKOV-3 cell line expressed as GI<sub>50</sub> values.

Table 1 Antiproliferative effect of compounds against the SKOV-3 cell line and Wi-38 cells. Evaluation of GI<sub>50</sub> and CC<sub>50</sub> values

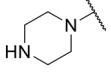
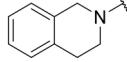
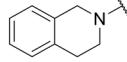
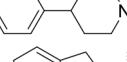
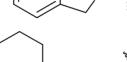
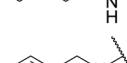
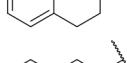
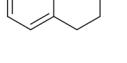
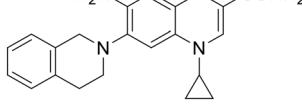
Compd.	X	R <sup>1</sup>	R <sup>6</sup>	R <sup>7</sup>	R <sup>8</sup>	GI <sub>50</sub> SKOV-3 <sup>a</sup> ( $\mu$ M)	CC <sub>50</sub> Wi-38 <sup>b</sup> ( $\mu$ M)
<b>Enoxacin</b>	N	-Et	-F		—	125	216 ± 123.1
<b>5</b>	C	-cPr	-NH <sub>2</sub>		-Me	37.71 ± 3.38	38.51 ± 28.97
<b>11</b>	C	-Et	-NH <sub>2</sub>		-Me	13.14 ± 1.29	53.50 ± 20.64
<b>12</b>	C	-cPr	-NH <sub>2</sub>		-Me	17.14 ± 2.61	52.96 ± 14.91
<b>13</b>	C	-cPr	-NH <sub>2</sub>		-Me	28.77 ± 4.11	NT <sup>c</sup>
<b>14</b>	C	-cPr	-NH <sub>2</sub>		-Me	102.9 ± 21.58	NT
<b>15</b>	C	-cPr	-NH <sub>2</sub>		-H	13.2 ± 1.13	36.27 ± 8.86
<b>16</b>	C	-Et	-NH <sub>2</sub>		-H	5.44 ± 0.13	8.19 ± 14.3
<b>17</b>						ND <sup>d</sup>	NT



Table 1 (continued)

Compd.	X	R <sup>1</sup>	R <sup>6</sup>	R <sup>7</sup>	R <sup>8</sup>	GI <sub>50</sub> SKOV-3 <sup>a</sup> (μM)	CC <sub>50</sub> Wi-38 <sup>b</sup> (μM)
18	C	-cPr	-NH <sub>2</sub>		-H	152.3 ± 3.05	NT
19	C	-cPr	-NH <sub>2</sub>		-H	104.3 ± 2.03	NT
20	C	-cPr	-NH <sub>2</sub>		-H	65.42 ± 24.31	NT
21	C	-cPr	-NH <sub>2</sub>		-H	23.66 ± 1.02	103.4 ± 15.45
22	C	-cPr	-NH <sub>2</sub>		-H	18.52 ± 1.71	59.87 ± 5.95
23	C	-cPr	-NH <sub>2</sub>		-H	46.17 ± 8.38	NT
24	C	-cPr	-NH <sub>2</sub>		-H	9.19 ± 1.05	45 ± 13.37
25	C	-cPr	-NH <sub>2</sub>		-H	15.99 ± 2.94	79.74 ± 11.79
26	C	-cPr	-NH <sub>2</sub>		-H	33.93 ± 6.43	NT
27	N	-Et	-F		—	ND	NT
28	N	-cPr	-F		—	46.82 ± 7.44	NT
29	N	-Et	-F		—	16.18 ± 1.03	25.50 ± 11.5
30	N	-Et	-F		—	8.34 ± 1.28	4.65 ± 5.96
31	C	-Et	-F		-Me	14.89 ± 3.24	56.58 ± 37.36
32	C	-cPr	-F		-Me	25 ± 6.04	90.73 ± 30.86
33	C	-cPr	-F		-H	13.52 ± 1.45	142.9 ± 42.7

<sup>a</sup> GI<sub>50</sub>: 50% cell growth inhibition. <sup>b</sup> CC<sub>50</sub>: 50% cytotoxicity concentration. <sup>c</sup> NT: not tested; CC<sub>50</sub> was not determined for compounds showing GI<sub>50</sub> values ≥ 25 μM. <sup>d</sup> ND: activity not dose dependent.

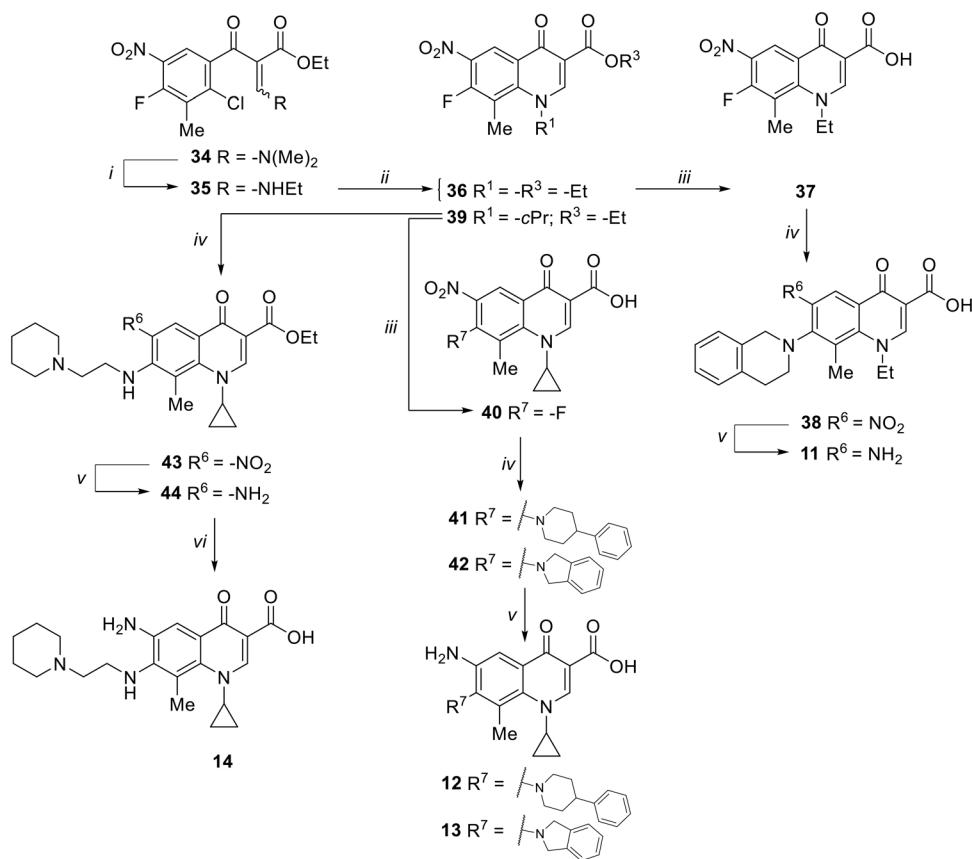
Since many quinolones have been reported in the literature as anticancer agents especially targeting topoisomerases, to exclude this already known mechanism, we evaluated the ability of the amino-quinolone 5 to target human topoisomerases (hTopI and hTopII). The results of cleavage assays conducted at 100  $\mu$ M (Fig. S1A†) indicated that compound 5 did not trap human topoisomerases. This observation led us to conclude that the anti-OC activity of compound 5 was likely mediated by a topoisomerase-independent mechanism of action.

Encouraged by this evidence, we considered the possibility of testing additional quinolones from our in-house library. However, we reasoned that the data from the preliminary screening strongly indicated that the tetrahydroisoquinoline, as a C-7 substituent of the 6-aminoquinolone scaffold, played a pivotal role in conferring the anticancer activity observed in compound 5. Consequently, we concluded that it was not advantageous to pursue the selection of additional 6-aminoquinolones from our in-house library, as they lacked sufficient structural similarity to facilitate the investigation of a structure–activity relationship (SAR) surrounding compound 5. Conversely, we decided to use derivative 5 as a starting hit compound and started the design of a series of chemical modifications (Table 1 for chemical structures) by replacing the

cyclopropyl at C-1 with an ethyl group (derivative 11) or the tetrahydroisoquinoline at the C-7 position with three different moieties (derivatives 12–14). Subsequently, removal of the C-8 methyl group of 5 and 11 led to the synthetically more accessible C-8 des-methyl analogues 15 and 16, respectively. After evaluating the role of the C-3 carboxyl function by designing the amide derivative 17, further efforts were focused on the C-7 substituent of 15, which was replaced with different groups, yielding derivatives 18–26. The resulting best C-7 substituents were then used to design close analogues of enoxacin (derivatives 27–30). Finally, since tetrahydroisoquinoline was found as the best moiety for the C-7 position, we planned the synthesis of fluoro-quinolones 31–33. Of note, compounds 32 and 33 were previously published by some of us as comparative analogues of antibacterial amino-quinolones.<sup>34</sup> However, both compounds were no longer available in our in-house library, thus we synthesized them using optimized procedures different from those previously reported.<sup>34</sup>

## Chemistry

Target compounds 11–14 were synthesized as reported in Scheme 1. Reaction of the acrylate intermediate 34 (ref. 33) with ethylamine in a mixture of Et<sub>2</sub>O and EtOH afforded



**Scheme 1** Reagents and conditions: i) ethylamine, Et<sub>2</sub>O/EtOH, rt, 15 min; ii) K<sub>2</sub>CO<sub>3</sub>, DMF, 100 °C, 1 h; iii) 6 N HCl, EtOH, reflux, 1 h; iv) amine, Et<sub>3</sub>N, dry DMF or DMSO, 80–100 °C, 1–2 h; v) RANEY®/Ni, H<sub>2</sub>, DMF, rt, 30 min–12 h; vi) 10% NaOH, MeOH, reflux, 1 h.



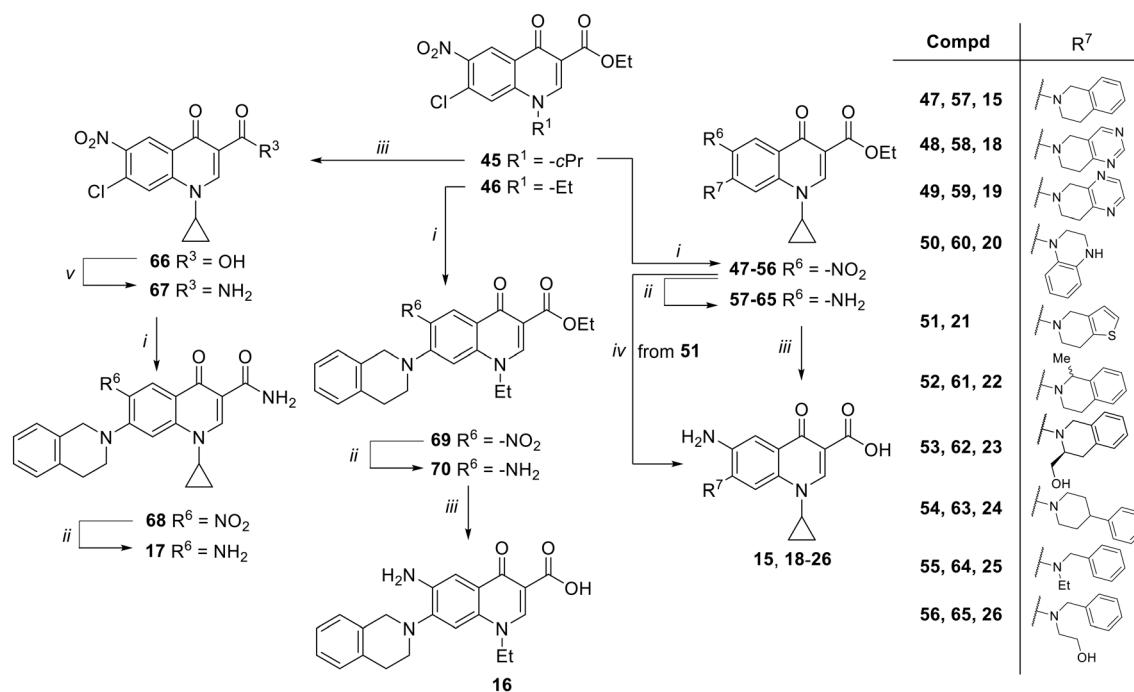
derivative **35**, which was cyclized in dry DMF in the presence of  $K_2CO_3$  to give the quinolone intermediate **36**. Subsequent hydrolysis of **36** with a mixture of 6 N HCl and EtOH afforded the quinolone acid derivative **37**, which was reacted with tetrahydroisoquinoline in dry DMF in the presence of  $Et_3N$  as a base to give compound **38**. Under  $H_2$  flow, RANEY®/Ni catalyzed reduction in DMF afforded the target compound **11**. Similarly, acid hydrolysis of quinolone ester **39** (ref. 33) afforded the corresponding acid **40**,<sup>33</sup> which was reacted with 4-phenylpiperidine or isoindoline in dry DMSO in the presence of  $Et_3N$  as a base to afford C-7 substituted quinolone derivatives **41** and **42**. Subsequently, nitro group reduction afforded the target compounds **12** and **13**. On the other hand, starting from the ester intermediate **39** (ref. 33) and exploiting the good nucleophilic property of 2-amino-ethylpiperidine, the nitro derivative **43** was first obtained and then reduced to **44** and hydrolyzed under basic conditions to give the target compound **14**.

Target compounds **15–26** were synthesized as reported in Scheme 2. Reaction of nitro-quinolone derivative **45** (ref. 31) with various amines in dry DMF or DMSO in the presence of  $Et_3N$  as a base afforded C-7 substituted quinolone analogues **47–56**. Subsequent hydrogenation under a catalytic amount of RANEY®/Ni of **47–50** and **52–56** afforded amino derivatives **57–65**, which were then hydrolyzed to target compounds **15**, **18–20** and **22–26**. On the other hand, derivative **21** was obtained directly from the corresponding nitro ester derivative **51** by reaction with

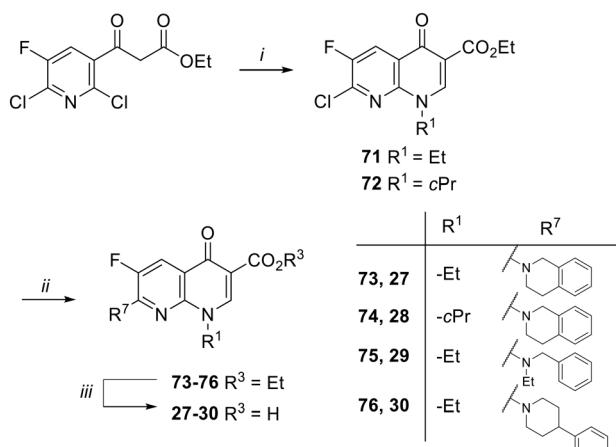
$SnCl_2$  in EtOH at reflux. In parallel, the ester derivative **45** was also converted to the corresponding acid to give intermediate **66**, which was reacted with  $SOCl_2$  and then treated with 7 N  $NH_3$  in MeOH and dry DMF to give the amide intermediate **67**. Nucleophilic substitution of **67** with 1,2,3,4-tetrahydroisoquinoline in dry DMSO in the presence of  $Et_3N$  gave the C-7 substituted intermediate **68**, which was subjected to RANEY®/Ni catalyzed reduction of the nitro group to give the target compound **17**. Starting from derivative **46**,<sup>37</sup> nucleophilic substitution with 1,2,3,4-tetrahydroisoquinoline in dry DMSO using  $Et_3N$  as a base afforded derivative **69**, which was first reduced to amino derivative **70** and then hydrolyzed under basic conditions to give target compound **16**.

Target compounds **27–30** were synthesized as reported in Scheme 3. Starting from commercially available ethyl 3-(2,6-dichloro-5-fluoropyridin-3-yl)-3-oxopropanoate, naphthyridone derivatives **71** and **72** were synthesized by a one-pot procedure involving the initial reaction with DMF-DMA, a catalytic amount of AcOH and dry toluene, followed by the addition of alkylamines (ethylamine or cyclopropyl amine) and then tetrabutylammonium hydroxide. The reaction of **71** and **72** with different amines in dry DMF and in the presence of  $Et_3N$  afforded the ester derivatives **73–76**, which were hydrolyzed to give the target compounds **27–30**.

The synthesis of target compounds **31–33** is reported in Scheme 4. Although the synthetic procedure of analogue **32** has been reported previously,<sup>34</sup> we encountered some problems related to the formation and handling of the

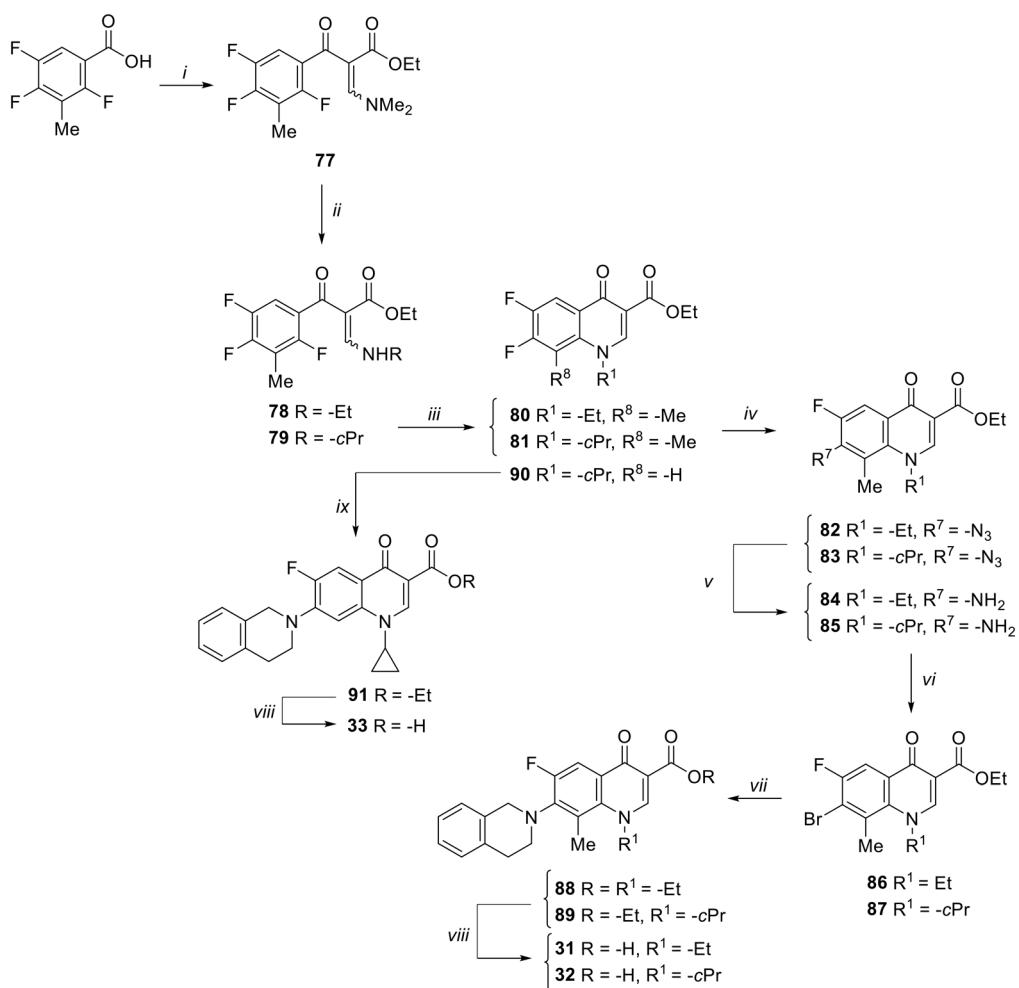


**Scheme 2** Reagents and conditions: i) amine,  $Et_3N$ , dry DMF or DMSO, 80–110 °C, 90 min–48 h; ii) RANEY®/Ni,  $H_2$ , DMF, rt, 1–6 h; iii) 6 N HCl, EtOH, reflux, 9 h (for **15**) or 10% NaOH, MeOH, reflux, 30 min–4 h; iv)  $SnCl_2$ , EtOH, reflux, 2 h; v)  $SOCl_2$ , reflux, 2 h; then, 7 N  $NH_3$  in MeOH, dry DMF, rt, 3 h.



**Scheme 3** Reagents and conditions: i) DMF–DMA, AcOH, dry toluene, rt, 2 h; then, ethylamine or cyclopropylamine, rt, 30 min; then, 1.5 M  $\text{Bu}_4\text{NOH}$ , rt, 5 min; ii) amine,  $\text{Et}_3\text{N}$ , dry DMF, 90 °C, 2–3 h; iii) 6 N HCl, EtOH, reflux, 4–16 h or 10% NaOH, MeOH, reflux, 1 h (for 29).

difluoroborate complex envisaged in the published procedure;<sup>34</sup> therefore, a different chemical synthesis was carried out to obtain both C-8 methyl derivatives 31 and 32. The new procedure involved the chlorination of commercial 2,4,5-trifluoro-3-methylbenzoic acid by oxalyl chloride, catalyzed by dry DMF, in dry  $\text{CH}_2\text{Cl}_2$  to give the corresponding acyl chloride, which was immediately reacted with ethyl 3-(*N,N*-dimethylamino)acrylate in dry toluene to give intermediate 77. Reaction of 77 with ethylamine or cyclopropylamine in a mixture of  $\text{Et}_2\text{O}$  and EtOH afforded derivatives 78 and 79, respectively. Cyclization of 78 and 79 was performed with NaH in dry DMF at 0 °C to give 6-fluoro quinolone intermediates 80 and 81,<sup>41</sup> respectively. Since various attempts to introduce the tetrahydroisoquinoline moiety at the C-7 position of the 6-fluoro-8-methyl quinolone by nucleophilic substitution were unsatisfactory, we explored a different procedure by modifying a previously reported chemical synthesis.<sup>42</sup> Accordingly, derivatives 80 and 81 were reacted



**Scheme 4** Reagents and conditions: i)  $\text{C}_2\text{O}_2\text{Cl}_2$ , cat. dry DMF, dry  $\text{CH}_2\text{Cl}_2$ , rt, 4 h; then, ethyl 3-(*N,N*-dimethylamino)acrylate,  $\text{Et}_3\text{N}$ , dry toluene, 90 °C, 4 h; ii) ethylamine or cyclopropylamine,  $\text{Et}_2\text{O}$ , EtOH, rt, 1 h; iii) NaH, dry DMF, 0 °C, 3 h; iv)  $\text{NaN}_3$ , dry DMF, 90 °C, 24 h; v) Pd/C,  $\text{H}_2$ , DMF, rt, 3 h; vi)  $\text{CuBr}_2$ , 5% HBr, 2 M  $\text{NaNO}_2$ , 0 °C to rt, 30 min; vii) 1,2,3,4-tetrahydroisoquinoline,  $\text{Pd}_2(\text{dba})_3$ , BINAP,  $\text{Cs}_2\text{CO}_3$ , dry toluene, reflux, 13 h; viii) 6 N HCl, EtOH, reflux, 1–4 h; ix) 1,2,3,4-tetrahydroisoquinoline,  $\text{Et}_3\text{N}$ , dry DMSO, 100 °C, 4 h.



with  $\text{NaN}_3$  in dry DMF to give derivatives **82** and **83**, which were then reduced by  $\text{H}_2$  flow with a catalytic amount of Pd/C to give amino derivatives **84** and **85**. Subsequently, a Sandmeyer reaction allowed the synthesis of bromo analogues **86** and **87**, which were reacted with tetrahydroisoquinoline in a Buchwald–Hartwig reaction to give C-7 functionalized quinolone ester derivatives **88** and **89**. Finally, acid hydrolysis afforded the target compounds **31** and **32**.

On the other hand, the synthesis of C-8 des-methyl quinolone **33** was performed by a standard procedure starting with the nucleophilic substitution of derivative **90**,<sup>43</sup> with the 1,2,3,4-tetrahydroisoquinoline to give intermediate **91**. Subsequent acid hydrolysis afforded the target compound **33**.

### Biological results

All the compounds were first tested for their ability to inhibit SKOV-3 proliferation at scalar concentrations, which allowed the determination of  $\text{GI}_{50}$  values (Table 1). In parallel, for compounds with  $\text{GI}_{50} \leq 25 \mu\text{M}$ ,  $\text{CC}_{50}$  values were determined in Wi-38 cells to observe any toxic effect against non-tumor cells (Table 1) and to evaluate any potential specific effect against tumor cells.

Replacement of the cyclopropyl ring of **5** with an ethyl group resulted in **11** that showed an increase in antiproliferative activity ( $\text{GI}_{50} 13.14 \mu\text{M}$  vs. SKOV-3) coupled with a  $\text{CC}_{50}$  value of  $53.50 \mu\text{M}$  on Wi-38 cells, being approximately 4-fold more toxic against tumor cells. Notably, cytotoxicity experiments of the first hit compound **5**, performed for comparison, showed a poor selectivity towards OC cell lines with respect to Wi-38 cells, with a  $\text{CC}_{50}$  value comparable to its  $\text{GI}_{50}$ .

Similar to **11**, good results were obtained when the tetrahydroisoquinoline of **5** was replaced by a phenylpiperidine moiety (compound **12**) giving a  $\text{GI}_{50}$  of  $17.14 \mu\text{M}$  against SKOV-3 and a  $\text{CC}_{50}$  in Wi-38 of  $52.96 \mu\text{M}$ . On the other hand, when isindoline and the more polar aminoethyl-piperidine moiety were used to replace the tetrahydroisoquinoline of **5**, we obtained compounds **13** and **14**, which showed a comparable or worse profile than compound **5**. Removal of the C-8 methyl group of **5** led to the more potent compound **15** ( $37.71 \mu\text{M}$  vs.  $13.2 \mu\text{M}$ ), which achieved a modest selective anticancer effect. On the other hand, derivative **16**, the C-8 des-methyl analogue of **11**, showed an improvement in anticancer potency but also an increase in non-tumor cell toxicity compared to **11**. Due to synthetic accessibility, the removal of the C-8 methyl group from the amino-quinolone scaffold led to an improvement in terms of yield and reaction time. Therefore, we decided to focus our efforts on **15** and evaluate the role of the carboxyl function by replacing it with an amide group (derivative **17**), which was completely inactive. We then replaced the tetrahydroisoquinoline of **15** with more polar bicyclic heterocycles, thus obtaining

compounds **18–20**, which were less active, suggesting that the benzene ring of the tetrahydroisoquinoline seemed to be important to retain a potent anticancer effect, while the presence of a free NH moiety was detrimental. On the other hand, the bioisosteric replacement of the benzene ring of the tetrahydroisoquinoline of **15** with a thiophene moiety yielded the tetrahydrothienopyridine derivative **21**, which retained a  $\text{GI}_{50}$  comparable to that of **15**, while exhibiting a significantly higher  $\text{CC}_{50}$  value ( $103.4 \mu\text{M}$  vs.  $36.27 \mu\text{M}$ ), thus highlighting a good propensity to act preferentially on tumor cells. Modifications of the aliphatic part of the tetrahydroisoquinoline by introducing a methyl at the C-1' position (compound **22**) or a methyl alcohol at C-3' (compound **23**) did not lead to improvements in terms of anti-proliferative activity. Interestingly, good results were obtained when less rigid moieties were introduced at the quinolone C-7 position to replace the classical tetrahydroisoquinoline. Indeed, **24** and **25** showed comparable  $\text{GI}_{50}$  values to **15** coupled with favorable  $\text{CC}_{50}$ s. However, when we tried to introduce a free alcoholic group to **25** in order to improve the polarity, we obtained compound **26**, which showed a 2-fold increase in  $\text{GI}_{50}$  ( $33.93 \mu\text{M}$  vs.  $15.99 \mu\text{M}$ ). At this point, we considered introducing the best C-7 substituents identified so far for the quinolone scaffold in place of the piperazine ring of the naphthyridone nucleus of enoxacin. However, compounds **27–30** were less active than their respective quinolone analogues and in some cases very toxic when tested in Wi-38 cells (compounds **29** and **30**). Ultimately, given the favorable outcomes observed with tetrahydroisoquinoline in the C-7 position when integrating the SAR data from amino-quinolone and naphthyridone derivatives, our research shifted its focus to fluoroquinolones. To this end, we synthesized three distinct molecules incorporating this moiety (compounds **31–33**). Interestingly, compound **31**, the close analogue of **11**, showed a similar activity to **11** both in terms of anti-proliferative activity and cytotoxicity on non-tumor cells, suggesting a similar behavior for compounds possessing NH<sub>2</sub> and F groups at the C-6 position. On the other hand, the replacement of the amino group of **5** with a fluorine yielded **32**, which exhibited a  $\text{GI}_{50}$  of  $25 \mu\text{M}$  and a  $\text{CC}_{50}$  of  $90.73 \mu\text{M}$ , making it significantly less toxic to non-tumor cells than its amino analogue. Finally, the best results were obtained when the amino group of **15** was replaced by a fluorine to yield **33**, which showed a comparable  $\text{GI}_{50}$  to **15** ( $13.52 \mu\text{M}$ ), but a significant reduction in non-tumor cell

**Table 2** Antiproliferative effect reported as  $\text{GI}_{50}$  against different OC cell lines

Compd.	OC cell lines	
	$\text{GI}_{50}$ OVCA433 ( $\mu\text{M}$ )	$\text{GI}_{50}$ A2780 ( $\mu\text{M}$ )
<b>33</b>	$31.04 \pm 6.75$	$18.13 \pm 8.25$
<b>Enoxacin</b>	$117.80 \pm 8.89$	$142.33 \pm 8.73$



toxicity ( $CC_{50}$  on Wi-38 = 142.9  $\mu$ M). Compound 33 thus emerged as a promising fluoroquinolone worthy of further biological profiling.

### Proliferation inhibitory effect of compound 33 against different OC cell lines

Based on the promising results of compound 33 regarding its anti-proliferative activity against SKOV-3 cell lines, coupled with a marked tendency to act preferentially on tumor cells, we decided to investigate whether its activity could be extended to two additional OC cell lines: OVCA433 and A2780 (Table 2).

Notably, 33 exhibited anti-proliferative activity against both OC cell lines, although it showed a slight increase in  $GI_{50}$  values. However, in both cases, 33 was significantly more potent than enoxacin, highlighting its potential as a “broad-spectrum” anti-OC agent.

### Investigation on the mode of action of compound 33

As previously evaluated for compound 5, we assessed whether our best compound 33 acted by inhibiting or trapping hTopI and hTopII. As expected, 33 showed no inhibition or trapping activity of human topoisomerases

when tested at 100  $\mu$ M (Fig. S1B†), thus ruling out that the toxicity observed in SKOV-3 was due to topoisomerase inhibition/trapping.

Subsequently, to evaluate whether compound 33 acted through an enoxacin-like mechanism, we performed SPR experiments on TRBP for compound 33 and the inactive amide derivative 17 as a negative control (Fig. 2 – enoxacin as a positive control). Interestingly, 33 bound TRBP with a  $K_D$  value of 4.09  $\mu$ M, whereas derivative 17 showed a very high  $K_D$  value of 1.116 mM. It is also noteworthy that compound 33 exhibited a binding affinity approximately 40-fold stronger for TRBP than enoxacin.

A cellular thermal shift (Fig. 3A and B) further confirmed the involvement of TRBP in the mechanism of action of compound 33. In fact, treatment of TRBP with 50  $\mu$ M of 33 resulted in a significant thermal shift when protein destabilization was compared with DMSO treatment (Fig. 3A). In addition, 33 was able to induce destabilization of TRBP in a concentration-dependent manner (Fig. 3B). Higher melting values are typical in thermal shift assays when a ligand binds to a protein. However, the destabilization of TRBP after treatment with compound 33 is similar to what has been observed with other TRBP binders, such as the SMER gomisin M1 (ref. 26) and the

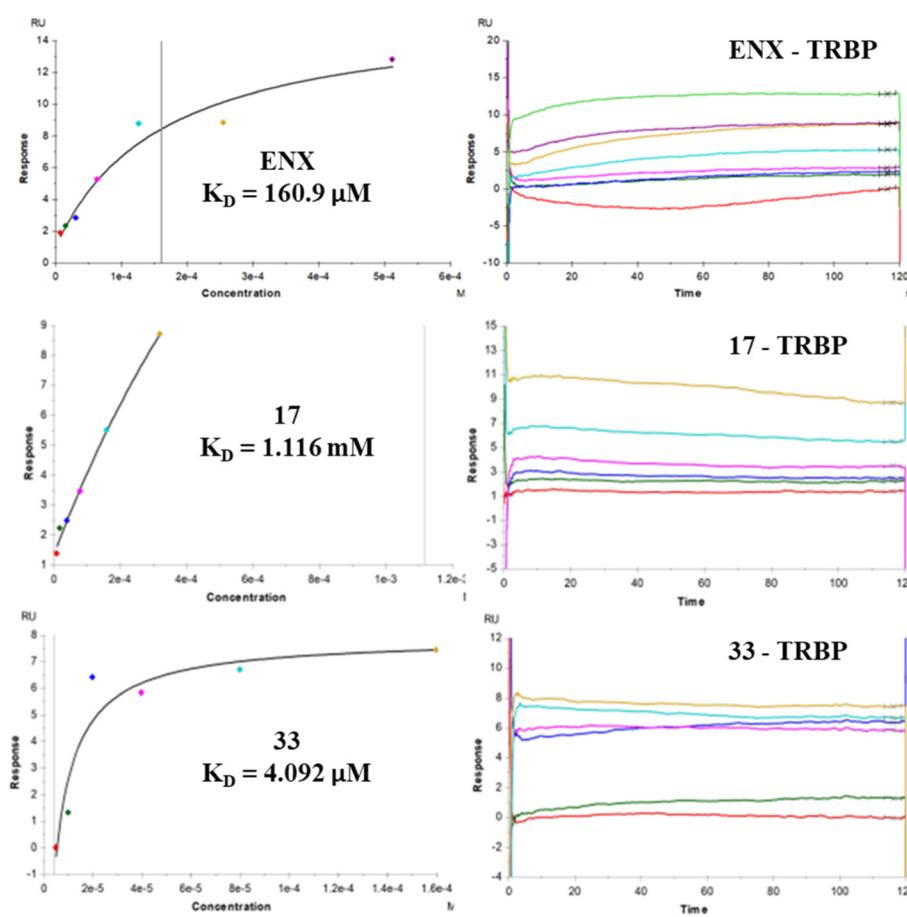
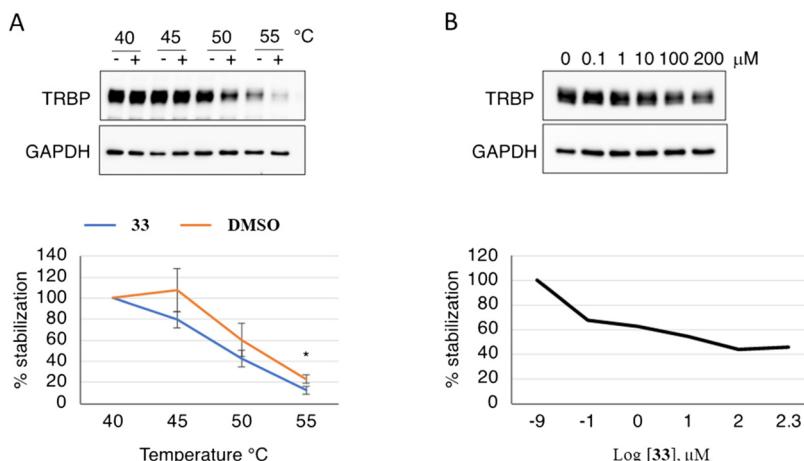


Fig. 2 SPR analyses of enoxacin (ENX), 17 and 33 towards TRBP.





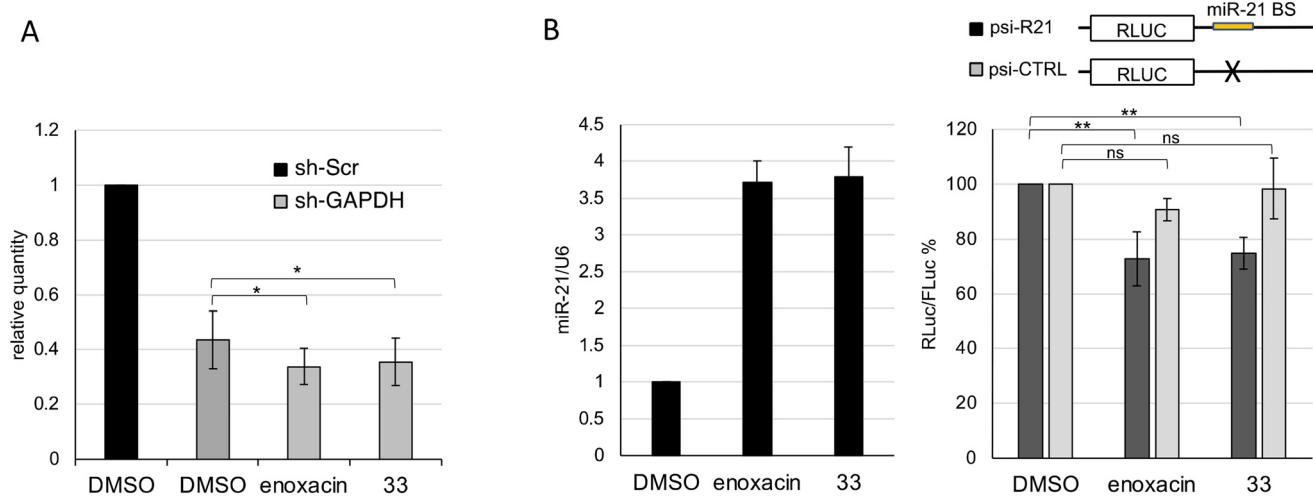
**Fig. 3** Cellular thermal shift assays of TRBP in the presence of **33**. A) Representative Western blot analysis showing the destabilization effect of **33** on TRBP at different temperatures. Graph showing the quantified levels of TRBP over GAPDH from three independent experiments. B) Western blot analysis showing the concentration dependency of the destabilization effect of **33** on TRBP at 55 °C. Graph showing the quantified levels of TRBP over GAPDH. Error bars represent SE. *p*-Value was calculated using paired two-tailed Student's *t*-test (\* *p* < 0.05).

sulfonamide derivative CIB-3b, which was previously reported to inhibit miR-21 maturation.<sup>44</sup>

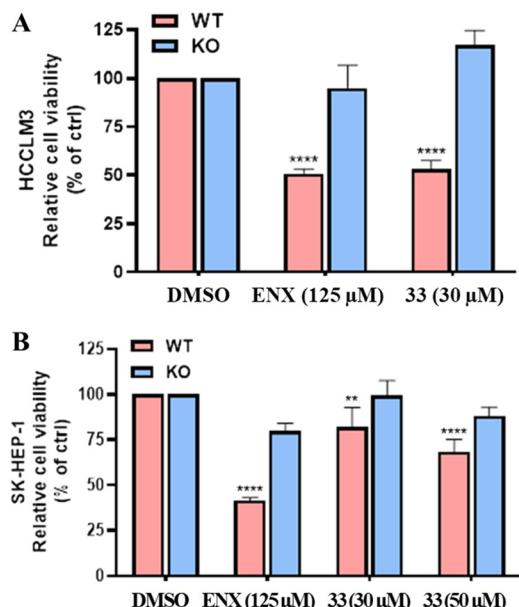
To further confirm the role of TRBP in the mechanism of action of **33**, we also examined its ability to promote shRNA to siRNA maturation (RNAi enhancing activity) (Fig. 4A). Notably, compound **33** at 50 μM exhibited a modest but significant RNAi enhancing effect by decreasing the amount of GAPDH mRNA, indirectly indicating an increase in siRNA maturation compared to DMSO. This effect was comparable to that of enoxacin tested at 50 μM. We also tested the ability of **33** to promote the maturation of endogenous miRNAs using a luciferase reporter construct carrying the

miR-21-5p binding site downstream of Renilla cDNA (psi-R21). As shown in Fig. 4B, **33** produced significant positive effects on miR-21-5p biogenesis and this caused a decrease in Renilla luciferase activity. Notably, this effect parallels that obtained with the positive control enoxacin. The effect is specific since no significant variation was observed when using a control construct, psi-CTRL, which lacks the miR-21-5p binding site.

To confirm that the anticancer activity of **33** was directly dependent on TRBP, we evaluated its anti-proliferative effect on HCCLM3 and SK-Hep-1 wild-type (WT) and KO cells for TRBP (enoxacin was used as a control – Fig. 5). Notably, after



**Fig. 4** Compound **33** promotes siRNA/miRNA maturation. A) RNAi enhancing effect of compound **33** and enoxacin at 50 μM. Values are shown as relative quantity with respect to sh-Scr/DMSO treated cells set to a value of 1. ATP5O is used as a reference gene. B) Left graph: Upregulation of miR-21 in HeLa cells upon 6 hours of treatment with **33** and enoxacin at 50 μM. Values are shown as relative quantity with respect to DMSO treated cells set to a value of 1. U6 is used as a reference gene. Upper: Schematic representation of the psi-R21 and psi-CTRL constructs. "miR-21 BS" depicts the binding site of the miRNA. Lower graph: miRNA enhancing effect of compound **33** and enoxacin at 50 μM. Values are shown as the ratio between Renilla (RLuc) and firefly (FLuc) luciferase signals. Error bars represent SE of at least three independent experiments. *p*-Values were calculated using paired two-tailed Student's *t*-test (\* *p* < 0.05; \*\* *p* < 0.01; ns: not significant).



**Fig. 5** Antiproliferative effect after 48 hours of enoxacin at 125  $\mu$ M and compound 33 A) against WT and KO TRBP HCCLM3 cell lines (30  $\mu$ M) and B) against WT and KO TRBP SK-Hep-1 cell lines (30 and 50  $\mu$ M). T-Test results (\*\*  $P \leq 0.01$ , \*\*\*\*  $P \leq 0.0001$ ).

48 hours, 33 at 30  $\mu$ M showed no antiproliferative effect on both KO TRBP cells, while it significantly reduced (about 50%) the viability of WT HCCLM3 cells. On the other hand, the anti-proliferative effect of 33 at 30  $\mu$ M on WT TRBP SK-Hep-1 cells was less evident and thus, it was also tested at 50  $\mu$ M on both KO and WT cells. Of note, 33 at 50  $\mu$ M significantly reduced the viability of WT SK-Hep-1 cells while not affecting KO TRBP SK-Hep-1 cells. Taken together, these data confirmed that 33 exerts its anti-proliferative activity by acting on TRBP and thus likely exhibits behavior similar to that of enoxacin. Furthermore, it can be stated that compound 33 retained anticancer activity against cancer cell lines other than those of ovarian origin, including HCCLM3 (hepatocellular carcinoma) and SK-Hep-1 (liver adenocarcinoma).

As previously stated in the introduction, this ability was observed for enoxacin as well, which retained anticancer activity against a wide panel of cancer cells with  $GI_{50}$  values of approximately 125  $\mu$ M.<sup>20</sup> This valuable broad-spectrum anticancer activity is to be attributed to its distinctive mechanism of action, which enables it to re-regulate dysregulated miRNAs in cancer cells. As one of the objectives of the study was to identify novel quinolones with an enoxacin-like mechanism, further investigation was conducted to ascertain whether compound 33 could exert any antiproliferative effect against three additional cancer cell lines: MCF-7 (breast cancer), A549 (lung carcinoma), and HCT-116 (colon cancer). It is noteworthy that all of these cell lines can be considered SMER-sensitive, as enoxacin has previously demonstrated efficacy against them.<sup>20</sup> Interestingly, treatment with 33 (30  $\mu$ M) for 72 hours resulted

in a significant inhibitory effect on cell proliferation in all three cell lines (Fig. S2†), indicating that compound 33 possesses broad-spectrum anticancer potential.

### Compound 33 induced A2780 cell death and sensitized cisplatin-resistant A2780 cells to cisplatin

We next tested whether the growth inhibition exerted by 33 on SKOV-3 and A2780 cells was accompanied by induction of cell death. Thus, we treated both cell lines with 33 at 30  $\mu$ M for 72 hours and cells were counted using trypan blue solution in order to assess cell viability. Fig. 6A shows that after treatment, the total number of cells was greatly reduced for both cell lines, while only for A2780 was the percentage of dead cells increased to a great extent. This result was confirmed by the increased levels of cleaved PARP1 (c-PARP), a marker of apoptosis,<sup>45</sup> and by the downregulation of the anti-apoptotic protein Bcl-X<sub>L</sub><sup>46</sup> in treated A2780 cells (Fig. 6B). No changes were observed in SKOV-3 cells, where compound 33 may exert a cytostatic effect as previously shown for cisplatin treatment.<sup>47</sup>

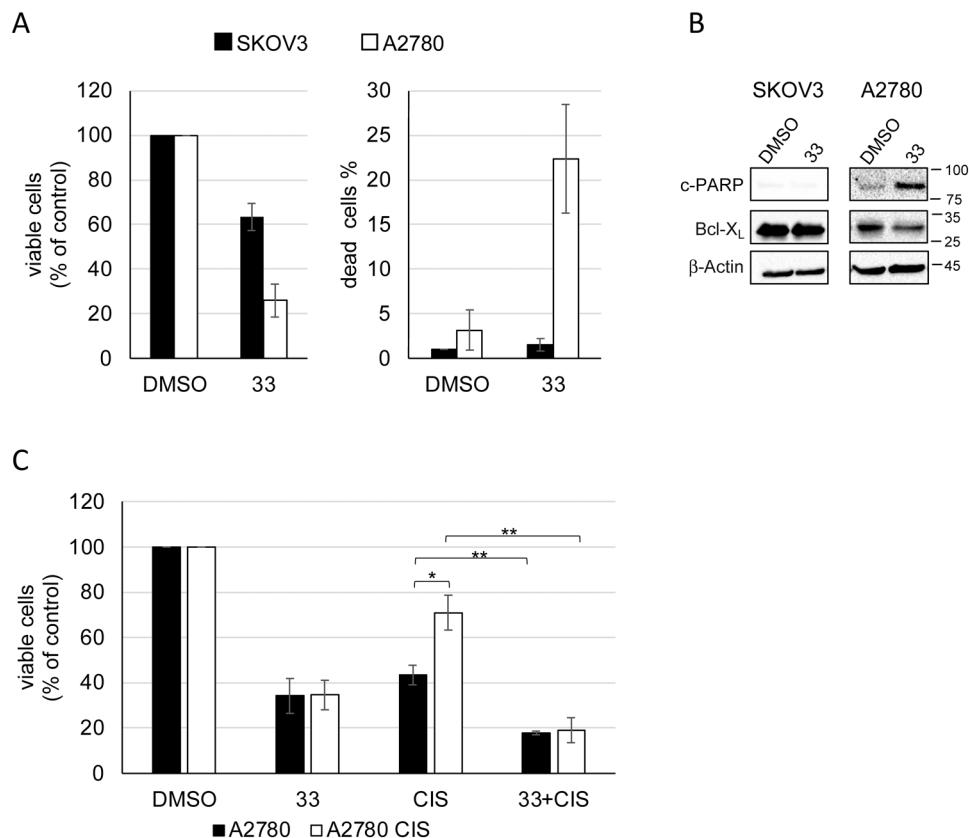
In consideration of the observed capacity of compound 33 to markedly elicit A2780 cell death, we proceeded to examine the potential synergistic effects of 33 in combination with cisplatin in view of a potential future combined treatment. Cisplatin is an effective first-line treatment for OC, but resistance is common. Although about 80% of OC patients are initially sensitive to cisplatin, many develop resistance, leading to high mortality within a few years. Various cellular processes contribute to platinum drug-resistance in OC including processes regulated by miRNAs. Indeed, it has been reported that modulation of the expression of specific miRNAs can reverse cisplatin resistance in human OC cells.<sup>48</sup>

Therefore, cisplatin-resistant A2780 cells (A2780 CIS)<sup>49</sup> were treated with 33, cisplatin and with a combination of the two compounds for 48 hours. In parallel, the same experiments were performed using the A2780 cell line. Fig. 6C illustrates that, as expected, A2780 CIS exhibited significantly greater resistance to cisplatin compared to A2780, while both cell lines showed equal sensitivity to treatment with compound 33. Notably, the combination of cisplatin and compound 33 sensitized A2780 CIS to cisplatin and produced a more pronounced inhibitory effect on A2780 proliferation, suggesting a synergistic mechanism of action.

### Conclusions

In this study, we aimed to identify new SMER compounds by exploiting our in-house library of quinolone derivatives. Preliminary evaluation of the antiproliferative activity of in-house derivatives against the OC SKOV-3 cell line led us to identify the 6-amino quinolone derivative 5 as a starting hit worthy of further exploration. Our medicinal chemistry efforts culminated in the identification of 33 as the best compound of the new series, exhibiting a  $GI_{50}$  of 13.52  $\mu$ M





**Fig. 6** Compound 33 inhibited cell growth and induced apoptosis of A2780 cells and sensitized A2780 CIS to cisplatin. A) Left graph: Reduction of number of SKOV-3 and A2780 viable cells upon treatment with compound 33 at 30  $\mu$ M for 72 hours. The number of viable cells is expressed as percentage with respect to DMSO condition set to a value of 100%; right graph: percentage of SKOV-3 and A2780 dead cells upon treatment described in “A”; B) representative Western blot analysis of protein extracts from SKOV-3 and A2780 cells treated as in “A”.  $\beta$ -Actin levels are used as a control for protein loading. C) Graph showing the number of viable cells of a culture of A2780 and A2780 CIS treated with 33 (30  $\mu$ M), cisplatin (CIS, 1  $\mu$ M) and a combination of the two compounds (33 + CIS). The number of viable cells is expressed as percentage with respect to DMSO condition set to a value of 100%. Error bars represent SE of at least three independent experiments.  $p$ -Values were calculated using paired two-tailed Student's  $t$ -test (\*  $p$  < 0.05; \*\*  $p$  < 0.01).

against SKOV-3 cells, about 9-fold lower than the known SMER enoxacin and less toxic against non-tumor cells ( $CC_{50}$  on Wi-38 = 142.9  $\mu$ M). Although featured by the presence of the most studied C-6 fluorine atom, like for enoxacin, compound 33 is characterized by an atypical base (tetrahydroisoquinoline moiety) at the C-7 position. Quinolone 33 demonstrated the ability to bind TRBP by both SPR and cellular thermal shift analyses, to modulate RNAi enhancing activity by promoting shRNA to siRNA maturation, and to enhance endogenous pre-miRNA processing. Furthermore, the anti-proliferative effect of 33 was also TRBP-dependent in the cell context; indeed, it lost its cytotoxic effect in TRBP KO SK-Hep-1 and HCCLM3 cells. Notably, in the A2780 cancer cell line, this anti-proliferative effect is also accompanied by the induction of the apoptotic process, as evidenced by the activation of PARP1 cleavage and the downregulation of Bcl-X<sub>L</sub> protein. In addition, compound 33 sensitized A2780 CIS to cisplatin and led to a more effective cisplatin inhibitory effect on A2780 proliferation, suggesting that, by enhancing the biogenesis of specific miRNAs, 33 may impact those cellular processes that

promote proliferation and sustain cisplatin resistance. Finally, compound 33 exhibited antiproliferative activity against other cancer types, including breast, lung, and colon cancers, indicating a broader applicability of its anticancer properties.

In conclusion, here, we demonstrate that enoxacin is not the sole member of the quinolone class to exert anticancer activity through the modulation of the miRNA/ RNAi machinery (SMER activity). Furthermore, the novel quinolone derivative 33 exhibited enhanced potency and a more robust binding affinity for TRBP compared to enoxacin. These data provide further support for the innovative concept of targeting miRNA maturation by small molecules as a potential OC therapy. We are aware that compound 33 is not yet a viable preclinical candidate and further studies will be conducted to identify more potent and cancer-specific agents capable of interacting with TRBP. However, this represents the first study of medicinal chemistry to identify quinolone agents that act as antitumor agents through an innovative mechanism of action.

# Materials and methods

## Chemistry

Unless otherwise stated, all starting materials were commercially available. Reagents and solvents were purchased from common commercial suppliers and used as received. Organic solutions were dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated under reduced pressure using a Büchi rotary evaporator. All reactions were routinely checked by TLC on silica gel 60<sub>F254</sub> (Merck) and visualized by UV and iodine. Purifications were performed by flash column chromatography separations on Merck silica gel 60 (mesh 230–400) or BUCHI Reveleris-X2 Flash Chromatography. Yields are based on purified products and are not optimized.  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded at 200 or 400 and 100 MHz, respectively, using Bruker Advance DRX-200 and DRX-400 instruments. Chemical shifts ( $\delta$ ) are reported in ppm relative to TMS and calibrated using residual undeuterated solvent as an internal reference. Coupling constants ( $J$ ) are expressed in Hz. Spectra were acquired at 298 K. Data processing was performed using Bruker TopSpin 4.3.0 software, and the spectral data are consistent with the assigned structures. The purity (>95%) of the compounds was evaluated by HPLC analysis using an Agilent 1290 Infinity System instrument equipped with a DAD detector from 190 to 640 nm. Purity was determined at 254 nm using a Phenomenex AERIS Widepore C4, 4.6 mm, 100 mm (6.6 lm) at a flow rate of 0.85 mL min<sup>-1</sup>; acquisition time: 10 min; gradient: acetonitrile in water containing 0.1% formic acid (0–100% in 10 min); oven temperature, 30 °C. Peak retention time is given in minutes. HRMS detection was based on electrospray ionization (ESI) in positive polarity, using an Agilent 1290 Infinity System equipped with an Agilent 6540UHD Accurate Mass Q-TOF MS detector.

**Ethyl (2E/Z)-2-(2-chloro-4-fluoro-3-methyl-5-nitrobenzoyl)-3-(ethylamino)acrylate (35).** To a solution of 34 (ref. 33) (1.00 g, 2.78 mmol) in a mixture of  $\text{Et}_2\text{O}$  (12 mL) and EtOH (32 mL), 2 M  $\text{EtNH}_2$  in THF (2.26 mL, 4.46 mmol) was added dropwise, and the reaction mixture was stirred at rt for 15 min. After evaporating two-thirds of the reaction volume under vacuum, a precipitate was observed and then filtered to give a yellow solid in 95% yield (0.95 g).  $^1\text{H}$  NMR (200 MHz, acetone- $d_6$ ):  $\delta$  0.91 (t,  $J$  = 7.1 Hz, 3H,  $\text{NHCH}_2\text{CH}_3$ ), 1.29 (t,  $J$  = 7.2 Hz, 3H,  $\text{OCH}_2\text{CH}_3$ ), 2.36 (d,  $J$  = 9.4 Hz, 3H,  $\text{CH}_3$ ), 3.57–3.69 (m, 2H,  $\text{NHCH}_2\text{CH}_3$ ), 3.81 (q,  $J$  = 7.0 Hz, 2H,  $\text{OCH}_2\text{CH}_3$ ), 7.71 (d,  $J$  = 6.0 Hz, 1H, CH), 8.23–8.32 (m, 1H, H-6), 11.00 (bs, 1H, NH).

**Ethyl 1-ethyl-7-fluoro-8-methyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate (36).** To a solution of 35 (0.75 g, 2.10 mmol) in DMF (15 mL),  $\text{K}_2\text{CO}_3$  (0.58 g, 4.20 mmol) was added portionwise and the reaction was stirred at 100 °C for 1 h. Then, the mixture was poured into ice/water and the obtained precipitate was filtered to give a yellow solid in 82% yield (0.55 g).  $^1\text{H}$  NMR (200 MHz, DMSO- $d_6$ ):  $\delta$  1.20–1.29 (m, 6H,  $\text{NCH}_2\text{CH}_3$  and  $\text{OCH}_2\text{CH}_3$ ), 2.53 (d,  $J$  = 9.5 Hz, 3H,  $\text{CH}_3$ ), 4.17 (q,  $J$  = 7.2 Hz, 2H,  $\text{OCH}_2\text{CH}_3$ ), 4.44 (q,  $J$  = 7.9 Hz, 2H,  $\text{NCH}_2\text{CH}_3$ ), 8.63–8.71 (m, 2H, H-2 and H-5).

**General procedure A (acidic hydrolysis).** To a solution of the ester derivative (1 equiv.) in EtOH (3.5 mL per mmol), 6 N HCl (3.5 mL per mmol) was added, and the reaction was stirred at reflux. Then, the mixture was allowed to cool at rt, and the obtained precipitate was filtered to give a solid that was either purified, as described below for each compound, or used as such for the next reaction.

**1-Ethyl-7-fluoro-8-methyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (37).** General procedure A: starting from 36 (reaction time: 1 h), compound 37 was obtained as a light brown solid in 93% yield (0.42 g).  $^1\text{H}$  NMR (200 MHz, DMSO- $d_6$ ):  $\delta$  1.32 (t,  $J$  = 7.8 Hz, 3H,  $\text{NCH}_2\text{CH}_3$ ), 2.58 (d,  $J$  = 10.4 Hz, 3H,  $\text{CH}_3$ ), 4.60 (q,  $J$  = 6.4 Hz, 2H,  $\text{NCH}_2\text{CH}_3$ ), 8.80 (d,  $J$  = 8.5 Hz, 1H, H-5), 8.98 (s, 1H, H-2), 14.14 (bs, 1H,  $\text{CO}_2\text{H}$ ).

**General procedure B (nucleophilic substitution).** Under  $\text{N}_2$  atmosphere, to a solution of the compound (1 equiv.) in dry DMF or DMSO (7 mL per 1 mmol), the desired nucleophile (3–5 equiv.) and  $\text{Et}_3\text{N}$  (3 equiv.) were added, and the reaction was stirred at 80–110 °C. Then, the mixture was poured into ice/water, pH adjusted to 3 with 2 N HCl, and the obtained precipitate was filtered to give a solid that was either purified, as described below for each compound, or used as such for the next reaction.

**7-(3,4-Dihydroisoquinolin-2(1H)-yl)-1-ethyl-8-methyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (38).** General procedure B: starting from 37 and using 1,2,3,4-tetrahydroisoquinoline (5 equiv.) as a nucleophile (reaction time: 1 h; temperature: 80 °C; solvent: DMF), compound 38, after crystallization by EtOH/DMF, was obtained as a light brown solid in 32% yield (0.20 g).  $^1\text{H}$  NMR (200 MHz, DMSO- $d_6$ ):  $\delta$  1.27 (t,  $J$  = 7.1 Hz, 3H,  $\text{NCH}_2\text{CH}_3$ ), 2.49 (s, 3H,  $\text{CH}_3$ ), 2.78–2.87 (m, 2H, tetrahydroisoquinoline- $\text{CH}_2$ ), 3.15–3.25 (m, 2H, tetrahydroisoquinoline- $\text{CH}_2$ ), 4.30–4.38 (m, 2H, tetrahydroisoquinoline- $\text{CH}_2$ ), 4.51 (q,  $J$  = 6.6 Hz, 2H,  $\text{NCH}_2\text{CH}_3$ ), 7.08–7.24 (m, 4H, Ar-H), 8.52 (s, 1H, H-2), 8.93 (s, 1H, H-5), 14.42 (s, 1H,  $\text{CO}_2\text{H}$ ).

**General procedure C (nitro reduction).** To a solution of the nitro-derivative (1 equiv.) in DMF (25 mL per mmol), RANEY®/Ni (10% w/w) was added portionwise and the reaction was stirred at rt under  $\text{H}_2$  flux. Then, the mixture was filtered over Celite™, and the filtrate was evaporated to dryness to give a solid that was either purified, as described below for each compound, or used as such for the next reaction.

**6-Amino-7-(3,4-dihydroisoquinolin-2(1H)-yl)-1-ethyl-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (11).** General procedure C: starting from 38 (reaction time: 30 min), compound 11, after crystallization by EtOH/DMF, was obtained as a yellow solid in 78% yield (0.18 g).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  1.21 (t,  $J$  = 7.0 Hz, 3H,  $\text{NCH}_2\text{CH}_3$ ), 2.46 (s, 3H,  $\text{CH}_3$ ), 2.81–2.84 (m, 1H, tetrahydroisoquinoline- $\text{CH}_2$   $\times \frac{1}{2}$ ), 2.94–2.97 (m, 1H, tetrahydroisoquinoline- $\text{CH}_2$   $\times \frac{1}{2}$ ), 3.38–3.42 (m, 2H, tetrahydroisoquinoline- $\text{CH}_2$ ), 4.21–4.28 (m, 2H,  $\text{NCH}_2\text{CH}_3$ ), 4.58–4.62 (m, 2H, tetrahydroisoquinoline- $\text{CH}_2$ ), 5.32 (bs, 2H, NH<sub>2</sub>), 7.09–7.15 (m, 4H, Ar-H), 7.44 (s, 1H, H-5), 8.69 (s, 1H, H-2), 15.73 (bs, 1H,  $\text{CO}_2\text{H}$ ).  $^{13}\text{C}$  NMR (100 MHz,



DMSO-*d*<sub>6</sub>):  $\delta$  15.93, 18.99, 30.75, 47.90, 49.60, 52.78, 105.16, 106.84, 125.57, 125.87, 126.07, 126.47, 126.54, 129.64, 133.96, 135.12, 135.25, 143.83, 144.78, 148.66, 167.14, 177.08. HPLC: *r*<sub>t</sub> 11.338 min. HRMS (ESI) calculated for C<sub>22</sub>H<sub>23</sub>N<sub>3</sub>O<sub>3</sub> [M + H]<sup>+</sup> 378.1812, found 378.1813.

**1-Cyclopropyl-7-fluoro-8-methyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (40).**<sup>33</sup> General procedure A: starting from 39 (ref. 33) (reaction time: 1 h), compound 40 (ref. 33) was obtained as a light brown solid in 86% yield (1.00 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.21–1.24 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.29–1.41 (m, 2H, cyclopropyl-CH<sub>2</sub>), 2.95 (d, *J* = 3.7 Hz, 3H, CH<sub>3</sub>), 4.48–4.53 (m, 1H, CH), 8.87 (d, *J* = 8.4 Hz, 1H, H-5), 8.96 (s, 1H, H-2), 14.18 (bs, 1H, CO<sub>2</sub>H).

**1-Cyclopropyl-8-methyl-6-nitro-4-oxo-7-(4-phenylpiperidin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (41).** General procedure B: starting from 40 (ref. 33) and using 4-phenylpiperidine (4 equiv.) as a nucleophile (reaction time: 2 h; temperature: 100 °C; solvent: DMSO), compound 41, after purification by flash chromatography column eluting with CHCl<sub>3</sub>/MeOH 99:1, was obtained as a brown solid in 30% yield (0.30 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.83–0.86 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.15–1.20 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.80–1.85 (m, 4H, piperidine-CH<sub>2</sub> × 2), 2.71–2.74 (m, 4H, piperidine-CH and CH<sub>3</sub>), 3.11–3.15 (m, 2H, piperidine-NCH<sub>2</sub>), 3.20–3.26 (m, 2H, piperidine-NCH<sub>2</sub>), 4.30–4.35 (m, 1H, CH), 7.17–7.20 (m, 1H, H-4'), 7.27–7.35 (m, 4H, Ar-H), 8.42 (s, 1H, H-2), 8.85 (s, 1H, H-5), 14.41 (bs, 1H, CO<sub>2</sub>H).

**1-Cyclopropyl-7-(1,3-dihydro-2H-isoindol-2-yl)-8-methyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (42).** General procedure B: starting from 40 (ref. 33) and using isoindoline (4 equiv.) as a nucleophile (reaction time: 90 min; temperature: 100 °C; solvent: DMSO), compound 42, after crystallization by EtOH/DMF, was obtained as a brown solid in 74% yield (0.73 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.90–0.94 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.18–1.21 (m, 2H, cyclopropyl-CH<sub>2</sub>), 2.69 (s, 3H, CH<sub>3</sub>), 4.32–4.36 (m, 1H, CH), 4.57 (m, 4H, isoindoline-CH<sub>2</sub> × 2), 7.27–7.31 (m, 2H, Ar-H), 7.32–7.36 (m, 2H, Ar-H), 8.48 (s, 1H, H-2), 8.86 (s, 1H, H-5), 14.83 (bs, 1H, CO<sub>2</sub>H).

**6-Amino-1-cyclopropyl-8-methyl-4-oxo-7-(4-phenylpiperidin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (12).** General procedure C: starting from 41 (reaction time: 12 h), compound 12, after purification by flash chromatography column eluting with CHCl<sub>3</sub>/MeOH 98:2, was obtained as a brown solid in 20% yield (0.08 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.77–0.86 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.12–1.17 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.73–1.79 (m, 2H, piperidine-CH<sub>2</sub>), 1.87–1.96 (m, 2H, piperidine-CH<sub>2</sub>), 2.56 (s, 3H, CH<sub>3</sub>), 2.67 (t, *J* = 11.4 Hz, 1H, piperidine-CH), 3.10–3.17 (m, 2H, piperidine-NCH<sub>2</sub>), 3.22–3.37 (m, 2H, piperidine-NCH<sub>2</sub>), 4.25 (s, 1H, CH), 5.40 (s, 2H, NH<sub>2</sub>), 7.17 (t, *J* = 7.1 Hz, 1H, H-4'), 7.28 (t, *J* = 7.4 Hz, 2H, H-3' and H-5'), 7.31–7.39 (m, 3H, H-5, H-2' and H-6'), 8.59 (s, 1H, H-2), 15.68 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  10.76, 19.24, 34.46, 41.02, 42.48, 49.98, 104.08, 106.43, 124.39, 125.41, 126.44, 127.39, 128.67, 135.70, 144.41, 145.17, 146.93, 148.98, 166.91, 176.96. HPLC: *r*<sub>t</sub> 10.398 min.

HRMS (ESI) calculated for C<sub>25</sub>H<sub>27</sub>N<sub>3</sub>O<sub>3</sub> [M + H]<sup>+</sup> 418.2130, found 418.21288.

**6-Amino-1-cyclopropyl-7-(1,3-dihydro-2H-isoindol-2-yl)-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (13).** General procedure C: starting from 42 (reaction time: 12 h), compound 13, after purification by chromatography (Reveleris-X2) eluting with CHCl<sub>3</sub>/MeOH 98:2 to 95:5, was obtained as a brown solid in 14% yield (0.05 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.85–0.91 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.13–1.19 (m, 2H, cyclopropyl-CH<sub>2</sub>), 2.53 (s, 3H, CH<sub>3</sub>), 4.18–4.29 (m, 1H, CH), 4.57 (s, 4H, isoindoline-CH<sub>2</sub> × 2), 5.60 (s, 2H, NH<sub>2</sub>), 7.25–7.28 (m, 2H, Ar-H), 7.31–7.35 (m, 2H, Ar-H), 7.40 (s, 1H, H-5), 8.59 (s, 1H, H-2), 15.60 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  10.92, 18.12, 41.30, 55.70, 104.49, 106.14, 123.03, 125.70, 127.17, 128.82, 134.82, 139.60, 140.55, 146.57, 149.02, 166.87, 177.23. HPLC: *r*<sub>t</sub> 5.385 min. HRMS (ESI) calculated for C<sub>22</sub>H<sub>21</sub>N<sub>3</sub>O<sub>3</sub> [M + H]<sup>+</sup> 376.1661, found 376.16651.

**Ethyl 1-cyclopropyl-8-methyl-6-nitro-4-oxo-7-[(2-piperidin-1-ylethyl)amino]-1,4-dihydroquinoline-3-carboxylate (43).** General procedure B: starting from 39 (ref. 33) and using 1-(2-aminoethyl)piperidine (3 equiv.) as a nucleophile (reaction time: 2 h; temperature: 100 °C; solvent: DMSO), compound 43, after crystallization by EtOH/DMF, was obtained as an orange solid in 86% yield (0.34 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.87–0.91 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.10–1.16 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.35–1.39 (m, 5H, piperidine-CH<sub>2</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 1.50–1.55 (m, 4H, piperidine-CH<sub>2</sub> × 2), 2.33–2.36 (m, 4H, piperidine-NCH<sub>2</sub> × 2), 2.49 (t, *J* = 5.9 Hz, 2H, -CH<sub>2</sub>N), 2.55 (s, 3H, CH<sub>3</sub>), 3.30 (q, *J* = 5.5 Hz, 2H, NHCH<sub>2</sub>–), 3.86–3.89 (m, 1H, CH), 4.35 (q, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 7.74 (t, *J* = 4.3 Hz, 1H, NHCH<sub>2</sub>–), 8.42 (s, 1H, H-5), 8.57 (s, 1H, H-2).

**Ethyl 6-amino-1-cyclopropyl-8-methyl-4-oxo-7-[(2-piperidin-1-ylethyl)amino]-1,4-dihydroquinoline-3-carboxylate (44).** General procedure C: starting from 43 (reaction time: 2 h), compound 44, after purification by flash column chromatography eluting with CHCl<sub>3</sub>/MeOH 95:5, was obtained as an orange solid in 43% yield (0.08 g). <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>):  $\delta$  0.85–0.88 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.08–1.13 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.36–1.59 (m, 9H, piperidine-CH<sub>2</sub> × 3 and OCH<sub>2</sub>CH<sub>3</sub>), 2.38–2.41 (m, 6H, piperidine-NCH<sub>2</sub> × 2 and NHCH<sub>2</sub>–), 2.66 (s, 3H, CH<sub>3</sub>), 3.21 (t, *J* = 5.0 Hz, 2H, -CH<sub>2</sub>N), 3.91–3.97 (m, 3H, CH and NH<sub>2</sub>), 4.35 (q, *J* = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 7.60 (s, 1H, H-5), 8.58 (s, 1H, H-2).

**General procedure D (basic hydrolysis).** To a solution of the ester derivative (1 equiv.) in MeOH (5 mL per mmol), a 10% aqueous solution of NaOH was added, and the reaction was stirred at reflux. Then, the mixture was concentrated under vacuum, poured into ice/water, and pH was adjusted with 2 N HCl. The acid derivative was collected by filtration or extraction with EtOAc and then purified as described below for each compound.

**6-Amino-1-cyclopropyl-8-methyl-4-oxo-7-[(2-piperidin-1-ylethyl)amino]-1,4-dihydroquinoline-3-carboxylic acid (14).**

General procedure D: starting from **44** (reaction time: 1 h; work-up: extraction at pH = 7), compound **14**, after crystallization by Et<sub>2</sub>O/EtOH, was obtained as a yellow solid in 65% yield (0.04 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.75–0.87 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.11–1.25 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.31–1.39 (m, 2H, piperidine-CH<sub>2</sub>), 1.41–1.52 (m, 4H, piperidine-CH<sub>2</sub>  $\times$ 2), 2.26–2.40 (m, 6H, NCH<sub>2</sub>– and piperidine-NCH<sub>2</sub>  $\times$ 2), 2.61 (s, 3H, CH<sub>3</sub>), 3.16–3.22 (m, 2H, –CH<sub>2</sub>NH), 4.26–4.32 (m, 1H, CH), 4.60 (t, *J* = 6.1 Hz, 1H, NH), 5.55 (s, 2H, NH<sub>2</sub>), 7.33 (s, 1H, H-2), 8.57 (s, 1H, H-5), 15.98 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  10.84, 18.37, 24.25, 25.81, 41.15, 44.17, 54.38, 58.61, 104.72, 106.04, 117.52, 121.72, 136.57, 140.97, 144.53, 148.70, 167.30, 176.45. HPLC: *t*<sub>r</sub> 2.187 min. HRMS (ESI) calculated for C<sub>21</sub>H<sub>28</sub>N<sub>4</sub>O<sub>3</sub> [M + H]<sup>+</sup> 385.2239, found 385.22379.

**Ethyl 1-cyclopropyl-7-(3,4-dihydroisoquinolin-2(1*H*)-yl)-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate** (**47**). General procedure B: starting from **45** (ref. 31) and using 1,2,3,4-tetrahydroisoquinoline (5 equiv.) as a nucleophile (reaction time: 90 min; temperature: 100 °C; solvent: DMSO), compound **47** was obtained as a yellow solid in 78% yield (0.61 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.79–0.95 (m, 2H, cyclopropyl-CH<sub>2</sub>), 0.97–1.10 (m, 5H, cyclopropyl-CH<sub>2</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 2.60–2.80 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.21 (*t*, *J* = 5.4 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.30–3.50 (m, 1H, CH), 3.97 (q, *J* = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.21 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 6.80–7.05 (m, 4H, Ar-H), 7.26 (s, 1H, H-8), 8.22 (s, 1H, H-2), 8.29 (s, 1H, H-5).

**Ethyl 1-cyclopropyl-7-(7,8-dihydropyrido[4,3-*d*]pyrimidin-6(5*H*)-yl)-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate** (**48**). General procedure B: starting from **45** (ref. 31) and using 5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine (3 equiv.) as a nucleophile (reaction time: 24 h; temperature: 80 °C; solvent: DMSO), compound **48**, after purification by flash chromatography column eluting with CHCl<sub>3</sub>/MeOH 97:3, was obtained as a light brown solid in 61% yield (0.57 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.99–1.15 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.23–1.27 (m, 5H, cyclopropyl-CH<sub>2</sub>, and OCH<sub>2</sub>CH<sub>3</sub>), 3.00 (*t*, *J* = 5.1 Hz, 2H, 5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine-CH<sub>2</sub>), 3.60–3.65 (m, 3H, CH and 5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine-CH<sub>2</sub>), 4.16 (q, *J* = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.47 (s, 2H, 5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidine-CH<sub>2</sub>), 7.57 (s, 1H, H-8), 8.40 (s, 1H, H-2), 8.51 (s, 1H, H-5), 8.65 (s, 1H, Ar-H), 8.94 (s, 1H, Ar-H).

**Ethyl 1-cyclopropyl-7-(7,8-dihydropyrido[3,4-*b*]pyrazin-6(5*H*)-yl)-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate** (**49**). General procedure B: starting from **45** (ref. 31) and using 5,6,7,8-tetrahydropyrido[3,4-*b*]pyrazine (3 equiv.) as a nucleophile (reaction time: 18 h; temperature: 100 °C; solvent: DMF), compound **49**, after purification by flash chromatography column eluting with CHCl<sub>3</sub>/MeOH 90:10, was obtained as a light brown solid in 78% yield (0.90 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.00–1.30 (m, 7H, cyclopropyl-CH<sub>2</sub>  $\times$ 2 and OCH<sub>2</sub>CH<sub>3</sub>), 3.00–3.10 (m, 2H, tetrahydropyrido[3,4-*b*]pyrazine-CH<sub>2</sub>), 3.48–3.70 (m, 3H, tetrahydropyrido[3,4-*b*]pyrazine-CH<sub>2</sub> and CH), 4.20 (q, *J* = 7.0

Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.50 (s, 2H, tetrahydropyrido[3,4-*b*]pyrazine-CH<sub>2</sub>), 7.60 (s, 1H, H-8), 8.28–8.55 (m, 4H, H-2, H-5 and Ar-H).

**Ethyl 1-cyclopropyl-7-(3,4-dihydroquinoxalin-1(2*H*)-yl)-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate** (**50**). General procedure B: starting from **45** (ref. 31) and using 1,2,3,4-tetrahydroquinoxaline (3 equiv.) as a nucleophile (reaction time: 10 h; temperature: 100 °C; solvent: DMSO), compound **50**, after purification by flash chromatography column eluting with CHCl<sub>3</sub>/acetone 98:2 to 95:5, was obtained as a light brown solid in 34% yield (0.37 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.06–1.10 (m, 4H, cyclopropyl-CH<sub>2</sub>  $\times$ 2), 1.24 (t, *J* = 7.1 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 3.30–3.33 (m, 2H, tetrahydroquinoxaline-CH<sub>2</sub>), 3.53–3.61 (m, 3H, tetrahydroquinoxaline-CH<sub>2</sub> and cyclopropyl-CH), 4.17 (q, *J* = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 6.04 (s, 1H, NH), 6.31 (t, *J* = 7.8 Hz, 1H, Ar-H), 6.55–6.60 (m, 2H, Ar-H), 6.68 (t, *J* = 7.5 Hz, 1H, Ar-H), 7.82 (s, 1H, H-8), 8.42 (s, 1H, H-2), 8.52 (s, 1H, H-5).

**Ethyl 1-cyclopropyl-7-(6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl)-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate** (**51**). General procedure B: starting from **45** (ref. 31) and using 4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine (3 equiv.) as a nucleophile (reaction time: 3 h; temperature: 100 °C; solvent: DMSO), compound **51** was obtained as a yellow solid in 94% yield (1.14 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.05–1.09 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.21–1.29 (m, 5H, cyclopropyl-CH<sub>2</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 2.92–2.96 (m, 2H, tetrahydrothieno[3,2-*c*]pyridine-CH<sub>2</sub>), 3.53 (*t*, *J* = 5.2 Hz, 2H, tetrahydrothieno[3,2-*c*]pyridine-CH<sub>2</sub>), 3.58–3.62 (m, 1H, CH), 4.17 (q, *J* = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.31–4.35 (m, 2H, tetrahydrothieno[3,2-*c*]pyridine-CH<sub>2</sub>), 6.91 (d, *J* = 5.2 Hz, 1H, Ar-H), 7.35 (d, *J* = 5.1 Hz, 1H, Ar-H), 7.53 (s, 1H, H-8), 8.40 (s, 1H, H-2), 8.49 (s, 1H, H-5).

**Ethyl 1-cyclopropyl-7-(1-methyl-3,4-dihydroisoquinolin-2(1*H*)-yl)-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate** (**52**). General procedure B: starting from **45** (ref. 31) and using 1-methyl-1,2,3,4-tetrahydroisoquinoline (3 equiv.) as a nucleophile (reaction time: 4 h; temperature: 110 °C; solvent: DMSO), compound **52** was obtained as a brown solid in 72% yield (0.48 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.93–0.96 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.11–1.23 (m, 5H, cyclopropyl-CH<sub>2</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 1.47 (d, *J* = 15.0 Hz, 3H, CH<sub>3</sub>), 2.75–2.81 (m, 1H, tetrahydroisoquinoline-CH<sub>2</sub>  $\times$ 2), 2.93–2.97 (m, 1H, tetrahydroisoquinoline-CH<sub>2</sub>  $\times$ 2), 3.36–3.41 (m, 1H, tetrahydroisoquinoline-CH<sub>2</sub>  $\times$ 2), 3.58–3.62 (m, 2H, cyclopropyl-CH and tetrahydroisoquinoline-CH<sub>2</sub>  $\times$ 2), 4.17 (q, *J* = 7.0 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.78 (q, *J* = 6.3 Hz, 1H, tetrahydroisoquinoline-NCH), 7.15–7.23 (m, 4H, Ar-H), 7.58 (s, 1H, H-8), 8.39 (s, 1H, H-2), 8.43 (s, 1H, H-5).

**Ethyl 1-cyclopropyl-7-[(3*S*)-3-(hydroxymethyl)-3,4-dihydroisoquinolin-2(1*H*)-yl]-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate** (**53**). General procedure B: starting from **45** (ref. 31) and using (3*S*)-1,2,3,4-tetrahydroisoquinolin-3-ylmethanol (3 equiv.) as a nucleophile (reaction time: 34 h; temperature: 110 °C; solvent: DMSO), compound **57**, after purification by flash chromatography column eluting with CHCl<sub>3</sub>/MeOH 99:1 to



98:2, was obtained as a yellow solid in 69% yield (0.48 g).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  1.05–1.09 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.19–1.28 (m, 5H, cyclopropyl-CH<sub>2</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 2.71–2.73 (m, 1H, tetrahydroisoquinoline-CH), 3.16–3.21 (m, 1H,  $\frac{1}{2}$  CH<sub>2</sub>OH), 3.42–3.46 (m, 2H,  $\frac{1}{2}$  CH<sub>2</sub>OH and cyclopropyl-CH), 3.59–3.63 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.18 (q,  $J$  = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.31 (d,  $J$  = 16.2 Hz, 1H, tetrahydroisoquinoline-CH<sub>2</sub>  $\times 2$ ), 4.62 (d,  $J$  = 16.1 Hz, 1H, tetrahydroisoquinoline-CH<sub>2</sub>  $\times 2$ ), 4.75 (t,  $J$  = 5.2 Hz, 1H, OH), 7.18–7.23 (m, 4H, Ar-H), 7.58 (s, 1H, H-8), 8.42 (s, 1H, H-2), 8.49 (s, 1H, H-5).

**Ethyl 1-cyclopropyl-6-nitro-4-oxo-7-(4-phenylpiperidin-1-yl)-1,4-dihydroquinoline-3-carboxylate (54).** General procedure B: starting from 45 (ref. 31) and using 4-phenylpiperidine (3 equiv.) as a nucleophile (reaction time: 2 h; temperature: 100 °C; solvent: DMSO), compound 54 was obtained as a yellow solid in 90% yield (0.62 g).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  1.07–1.11 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.18–1.28 (m, 5H, cyclopropyl-CH<sub>2</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 1.73–1.88 (m, 4H, piperidine-CH<sub>2</sub>  $\times 2$ ), 2.71–2.73 (m, 1H, piperidine-CH), 3.08 (t,  $J$  = 10.9 Hz, 2H, piperidine-NCH<sub>2</sub>), 3.46 (d,  $J$  = 13.5 Hz, 2H, piperidine-NCH<sub>2</sub>), 3.60–3.63 (m, 1H, CH), 4.18 (q,  $J$  = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 7.15–7.20 (m, 1H, Ar-H), 7.25–7.31 (m, 4H, Ar-H), 7.50 (s, 1H, H-8), 8.41 (s, 1H, H-2), 8.48 (s, 1H, H-5).

**Ethyl 7-[benzyl(ethyl)amino]-1-cyclopropyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate (55).** General procedure B: starting from 45 (ref. 31) and using *N*-benzylethanolamine (3 equiv.) as a nucleophile (reaction time: 48 h; temperature: 100 °C; solvent: DMSO), compound 55 was obtained as a yellow solid in 90% yield (0.90 g).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  0.78–0.82 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.03–1.04 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.12 (t,  $J$  = 6.3 Hz, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 1.21 (t,  $J$  = 6.8 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 3.22 (q,  $J$  = 6.7 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 3.43–3.46 (m, 1H, CH), 4.15 (q,  $J$  = 6.8 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.56 (s, 2H, CH<sub>2</sub>), 7.19–7.34 (m, 6H, Ar-H), 8.33 (s, 1H, H-2), 8.37 (s, 1H, H-5).

**Ethyl 7-[benzyl(2-hydroxyethyl)amino]-1-cyclopropyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate (56).** General procedure B: starting from 45 (ref. 31) and using 2-(benzylamino)ethanol (3 equiv.) as a nucleophile (reaction time: 24 h; temperature: 100 °C; solvent: DMSO), compound 56, after crystallization by EtOH/DMF, was obtained as a yellow solid in 50% yield (0.65 g).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  0.77–0.81 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.04–1.08 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.22 (t,  $J$  = 7.1 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 3.24 (t,  $J$  = 5.7 Hz, 2H, NCH<sub>2</sub>CH<sub>2</sub>OH), 3.41–3.45 (m, 1H, CH), 3.57 (q,  $J$  = 5.4 Hz, 2H, NCH<sub>2</sub>CH<sub>2</sub>OH), 4.16 (q,  $J$  = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.68–4.72 (m, 2H, CH and OH), 7.18–7.22 (m, 1H, Ar-H), 7.27–7.31 (m, 2H, Ar-H), 7.36–7.37 (m, 2H, Ar-H), 7.41 (s, 1H, H-8), 8.34 (s, 1H, H-2), 8.38 (s, 1H, H-5).

**Ethyl 6-amino-1-cyclopropyl-7-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (57).** General procedure C: starting from 47 (reaction time: 90 min), compound 57, after crystallization by EtOH, was obtained as a white solid in 85% yield (0.48 g).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  0.91–0.97 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.03–

1.15 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.24 (t,  $J$  = 7.0 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 2.91–3.02 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.23–3.30 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.45–3.55 (m, 1H, CH), 4.14 (q,  $J$  = 7.0 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.22 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 5.16 (s, 2H, NH<sub>2</sub>), 7.09–7.22 (m, 4H, Ar-H), 7.40 (s, 1H, H-8), 7.50 (s, 1H, H-5), 8.25 (s, 1H, H-2).

**Ethyl 6-amino-1-cyclopropyl-7-(7,8-dihydropyrido[4,3-*d*]pyrimidin-6(5*H*)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (58).** General procedure C: starting from 48 (reaction time: 3 h), compound 58, after crystallization by Et<sub>2</sub>O/EtOH, was obtained as a white solid in 66% yield (0.24 g).  $^1\text{H}$  NMR (200 MHz, DMSO- $d_6$ ):  $\delta$  0.85–0.99 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.00–1.23 (m, 5H, cyclopropyl-CH<sub>2</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 2.90–3.10 (m, 2H, tetrahydropyrido[4,3-*d*]pyrimidine-CH<sub>2</sub>), 3.30–3.40 (m, 2H, tetrahydropyrido[4,3-*d*]pyrimidine-CH<sub>2</sub>), 3.50–3.60 (m, 1H, CH), 4.00–4.35 (m, 4H, OCH<sub>2</sub>CH<sub>3</sub> and tetrahydropyrido[4,3-*d*]pyrimidine-CH<sub>2</sub>), 5.27 (s, 2H, NH<sub>2</sub>), 7.44 (s, 1H, H-8), 7.48 (s, 1H, H-5), 8.26 (s, 1H, H-2), 8.60 (s, 1H, Ar-H), 8.93 (s, 1H, Ar-H).

**Ethyl 6-amino-1-cyclopropyl-7-(7,8-dihydropyrido[3,4-*b*]pyrazin-6(5*H*)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (59).**

General procedure C: starting from 49 (reaction time: 2 h), compound 59, after purification by flash chromatography column eluting with CHCl<sub>3</sub>/MeOH 94:6, was obtained as a whitish solid in 40% yield (0.12 g).  $^1\text{H}$  NMR (200 MHz, DMSO- $d_6$ ):  $\delta$  0.90–1.05 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.07–1.27 (m, 5H, cyclopropyl-CH<sub>2</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 3.00–3.15 (m, 2H, tetrahydropyrido[3,4-*b*]pyrazine-CH<sub>2</sub>), 3.39 (t,  $J$  = 5.3 Hz, 2H, tetrahydropyrido[3,4-*b*]pyrazine-CH<sub>2</sub>), 3.45–3.55 (m, 1H, CH), 4.12 (q,  $J$  = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.29 (s, 2H, tetrahydropyrido[3,4-*b*]pyrazine-CH<sub>2</sub>), 5.23 (s, 2H, NH<sub>2</sub>), 7.45 (s, 1H, H-8), 7.49 (s, 1H, H-5), 8.25 (s, 1H, H-2), 8.45 (d,  $J$  = 2.5 Hz, 1H, Ar-H), 8.49 (d,  $J$  = 2.5 Hz, 1H, Ar-H).

**Ethyl 6-amino-1-cyclopropyl-7-(3,4-dihydroquinoxalin-1(2*H*)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (60).**

General procedure C: starting from 50 (reaction time: 4 h), compound 60, after purification by flash chromatography column eluting with CHCl<sub>3</sub>/MeOH 99:1, was obtained as a whitish solid in 32% yield (0.06 g).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  0.94–1.06 (m, 4H, cyclopropyl-CH<sub>2</sub>  $\times 2$ ), 1.22 (t,  $J$  = 7.0 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 3.38–3.41 (m, 2H, dihydroquinoxaline-CH<sub>2</sub>), 3.47–3.50 (m, 3H, dihydroquinoxaline-CH<sub>2</sub> and cyclopropyl-CH), 4.15 (q,  $J$  = 6.9 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 5.17 (s, 2H, NH<sub>2</sub>), 5.74 (s, 1H, NH), 6.09 (d,  $J$  = 7.8 Hz, 1H, Ar-H), 6.29 (t,  $J$  = 5.5 Hz, 1H, Ar-H), 6.50–6.55 (m, 2H, Ar-H), 7.49 (s, 1H, H-5), 7.55 (s, 1H, H-8), 8.28 (s, 1H, H-2).

**Ethyl 6-amino-1-cyclopropyl-7-(1-methyl-3,4-dihydroisoquinolin-2(1*H*)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (61).**

General procedure C: starting from 52 (reaction time: 1 h), compound 61, after crystallization by EtOH, was obtained as a brown solid in 75% yield (0.35 g).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  0.73–0.82 (m, 2H, cyclopropyl-CH<sub>2</sub>), 0.93–1.05 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.22 (t,  $J$  = 7.2 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 1.28 (d,  $J$  = 6.5 Hz, 3H, CH<sub>3</sub>), 2.73–



2.79 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.25–3.27 (m, 1H, tetrahydroisoquinoline-CH<sub>2</sub> ×<sup>1</sup>), 3.35–3.38 (m, 1H, tetrahydroisoquinoline-CH<sub>2</sub> ×<sup>1</sup>), 3.46–3.49 (m, 1H, CH), 4.14 (q, *J* = 7.0 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.65 (q, *J* = 6.4 Hz, 1H, tetrahydroisoquinoline-CH), 5.19 (s, 2H, NH<sub>2</sub>), 7.09–7.23 (m, 4H, Ar-H), 7.38 (s, 1H, H-5), 7.43 (s, 1H, H-8), 8.23 (s, 1H, H-2).

**Ethyl 6-amino-1-cyclopropyl-7-[(3*S*)-3-(hydroxymethyl)-3,4-dihydroisoquinolin-2(1*H*)-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylate (62).** General procedure C: starting from 53 (reaction time: 2 h), compound **62** was obtained as a brown solid in 90% yield (0.42 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.71–0.78 (m, 2H, cyclopropyl-CH<sub>2</sub>), 0.86–1.01 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.21 (t, *J* = 6.6 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 2.61–2.66 (m, 1H, tetrahydroisoquinoline-CH<sub>2</sub> ×<sup>1</sup>), 2.97–3.00 (m, 1H, tetrahydroisoquinoline-CH<sub>2</sub> ×<sup>1</sup>), 3.82–3.89 (m, 2H, tetrahydroisoquinoline-CH and cyclopropyl-CH), 3.55–3.58 (m, 1H, CH<sub>2</sub>OH ×<sup>1</sup>), 3.71–3.75 (m, 1H, CH<sub>2</sub>OH ×<sup>1</sup>), 4.08–4.14 (m, 3H, OCH<sub>2</sub>CH<sub>3</sub> and tetrahydroisoquinoline-CH<sub>2</sub> ×<sup>1</sup>), 4.52–4.56 (m, 1H, tetrahydroisoquinoline-CH<sub>2</sub> ×<sup>1</sup>), 4.83 (bs, 1H, OH), 5.26 (s, 2H, NH<sub>2</sub>), 7.07–7.21 (m, 4H, Ar-H), 7.37–7.41 (m, 2H, H-5 and H-8), 8.21 (s, 1H, H-2).

**Ethyl 6-amino-1-cyclopropyl-4-oxo-7-(4-phenylpiperidin-1-yl)-1,4-dihydroquinoline-3-carboxylate (63).** General procedure C: starting from 54 (reaction time: 2 h), compound **63**, after crystallization by EtOH, was obtained as a white solid in 72% yield (0.37 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.02–1.04 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.17–1.25 (m, 5H, cyclopropyl-CH<sub>2</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 1.84–1.99 (m, 4H, piperidine-CH<sub>2</sub> ×<sup>2</sup>), 2.65–2.73 (m, 3H, piperidine-NCH<sub>2</sub> and -CH), 3.39–3.42 (m, 2H, piperidine-NCH<sub>2</sub>), 3.55–3.59 (m, 1H, CH), 4.15 (q, *J* = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 5.14 (s, 2H, NH<sub>2</sub>), 7.15–7.20 (m, 1H, Ar-H), 7.29–7.30 (m, 4H, Ar-H), 7.43 (s, 1H, H-5), 7.45 (s, 1H, H-8), 8.28 (s, 1H, H-2).

**Ethyl 6-amino-7-[benzyl(ethyl)amino]-1-cyclopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (64).** General procedure C: starting from 55 (reaction time: 90 min), compound **64** was obtained as an orange solid in 57% yield (0.20 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.80–0.86 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.02 (t, *J* = 6.9 Hz, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 1.12–1.17 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.21 (t, *J* = 7.1 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 3.05 (q, *J* = 7.1 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 3.44–3.46 (m, 1H, CH), 4.13 (q, *J* = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.25 (s, 2H, CH<sub>2</sub>), 5.23 (s, 2H, NH<sub>2</sub>), 7.12–7.17 (m, 1H, Ar-H), 7.20–7.24 (m, 2H, Ar-H), 7.29–7.31 (m, 2H, Ar-H), 7.35 (s, 1H, H-5), 7.40 (s, 1H, H-8), 8.23 (s, 1H, H-2).

**Ethyl 6-amino-7-[benzyl(2-hydroxyethyl)amino]-1-cyclopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (65).** General procedure C: starting from 56 (reaction time: 1 h), compound **65** after crystallization by EtOH, was obtained as a yellow solid in 53% yield (0.32 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.72–0.77 (m, 2H, cyclopropyl-CH<sub>2</sub>), 0.99–1.04 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.20 (t, *J* = 7.1 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 3.07 (t, *J* = 5.3 Hz, 2H, NCH<sub>2</sub>CH<sub>2</sub>OH), 3.39–3.43 (m, 1H, CH), 3.62 (q, *J* = 5.1 Hz, 2H, NCH<sub>2</sub>CH<sub>2</sub>OH), 4.12 (q, *J* = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.36 (s, 2H, CH<sub>2</sub>), 4.92 (t, *J* = 4.9 Hz, 1H, OH), 5.55 (s,

2H, NH<sub>2</sub>), 7.09–7.13 (m, 1H, Ar-H), 7.17–7.22 (m, 2H, Ar-H), 7.28–7.30 (m, 2H, Ar-H), 7.34 (s, 1H, H-5), 7.35 (s, 1H, H-8), 8.19 (s, 1H, H-2).

**6-Amino-1-cyclopropyl-7-(3,4-dihydroisoquinolin-2(1*H*)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (15).** General procedure A: starting from 57 (reaction time: 9 h), compound **15**, after crystallization by EtOH/DMF, was obtained as a pale yellow solid in 82% yield (0.30 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.98–1.04 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.15–1.25 (m, 2H, cyclopropyl-CH<sub>2</sub>), 2.90–3.00 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.36 (t, *J* = 5.4 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.65–3.75 (m, 1H, CH), 4.29 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 5.44 (s, 2H, NH<sub>2</sub>), 7.10–7.19 (m, 4H, Ar-H), 7.50 (s, 1H, H-8), 7.55 (s, 1H, H-5), 8.48 (s, 1H, H-2), 15.98 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.76, 28.56, 36.22, 47.85, 52.59, 106.25, 106.95, 107.87, 122.03, 126.25, 126.81 (2C), 129.17, 134.29, 134.53, 134.67, 142.60, 145.02, 145.71, 167.22, 176.73. HPLC: *t*<sub>r</sub> 5.4300 min. HRMS-ESI *m/z* [M + H]<sup>+</sup> calcd. for C<sub>22</sub>H<sub>21</sub>N<sub>3</sub>O<sub>3</sub>: 376.1661, found: 376.1657.

**6-Amino-1-cyclopropyl-7-(7,8-dihdropyrido[4,3-*d*]pyrimidin-6(5*H*)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (18).** General procedure D: starting from 58 (reaction time: 1 h; work-up: filtration at pH = 5), compound **18**, after purification by flash column chromatography eluting with CHCl<sub>3</sub>/MeOH 93:7, was obtained as a pale yellow solid in 80% yield (0.14 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.00–1.30 (m, 4H, cyclopropyl-CH<sub>2</sub> ×<sup>2</sup>), 2.98–3.18 (m, 2H, tetrahydropyrido[4,3-*d*]pyrimidine-CH<sub>2</sub>), 3.40–3.50 (m, 2H, tetrahydropyrido[4,3-*d*]pyrimidine-CH<sub>2</sub>), 3.70–3.78 (m, 1H, CH), 4.29 (s, 2H, tetrahydropyrido[4,3-*d*]pyrimidine-CH<sub>2</sub>), 5.50 (s, 2H, NH<sub>2</sub>), 7.50 (s, 1H, H-8), 7.62 (s, 1H, H-5), 8.50 (s, 1H, H-2), 8.60 (s, 1H, Ar-H), 9.00 (s, 1H, Ar-H), 15.00 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.84, 31.71, 35.99, 47.31, 49.10, 106.54, 107.36, 108.21, 122.61, 128.70, 134.26, 142.63, 145.18, 145.20, 155.08, 156.95, 163.63, 167.05, 176.92. HPLC: *t*<sub>r</sub>: 3.5000 min. HRMS-ESI *m/z* [M + H]<sup>+</sup> calcd. for C<sub>20</sub>H<sub>19</sub>N<sub>5</sub>O<sub>3</sub>, 378.1566, found: 378.1560.

**6-Amino-1-cyclopropyl-7-(7,8-dihdropyrido[3,4-*b*]pyrazin-6(5*H*)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (19).** General procedure D: starting from 59 (reaction time: 90 min; work-up: filtration at pH = 5), compound **19**, after crystallization by EtOH/DMF, was obtained as a pale yellow solid in 93% yield (0.09 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.00–1.30 (m, 4H, cyclopropyl-CH<sub>2</sub> ×<sup>2</sup>), 3.19 (t, *J* = 5.3 Hz, 2H, tetrahydropyrido[3,4-*b*]pyrazine-CH<sub>2</sub>), 3.50 (t, *J* = 5.3 Hz, 2H, tetrahydropyrido[3,4-*b*]pyrazine-CH<sub>2</sub>), 3.70–3.80 (m, 1H, CH), 4.41 (s, 2H, tetrahydropyrido[3,4-*b*]pyrazine-CH<sub>2</sub>), 5.56 (s, 2H, NH<sub>2</sub>), 7.57 (s, 1H, H-8), 7.68 (s, 1H, H-5), 8.45–8.52 (m, 3H, Ar-H), 15.86 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.83, 31.33, 36.06, 47.39, 53.89, 106.25, 107.13, 108.05, 122.31, 134.17, 142.28, 142.65, 143.28, 144.83, 145.18, 150.34, 151.20, 167.20, 176.76. HPLC: *t*<sub>r</sub>: 3.7700 min. HRMS-ESI *m/z* [M + H]<sup>+</sup> calcd for C<sub>20</sub>H<sub>19</sub>N<sub>5</sub>O<sub>3</sub>, 378.1566, found: 378.1562.

**6-Amino-1-cyclopropyl-7-(3,4-dihydroquinoxalin-1(2*H*)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (20).** General procedure D: starting from **60** (reaction time: 2 h; work-up:



extraction at pH = 3), compound **20**, after crystallization by EtOH/DMF, was obtained as a yellow solid in 25% yield (0.06 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.97–1.12 (m, 4H, cyclopropyl-CH<sub>2</sub> ×2), 3.37–3.41 (m, 2H, dihydroquinoxaline CH<sub>2</sub>), 3.51–3.58 (s, 2H, dihydroquinoxaline CH<sub>2</sub>), 3.63–3.69 (m, 1H, CH), 5.52 (s, 2H, NH<sub>2</sub>), 5.84 (s, 1H, NH), 6.16 (d,  $J$  = 7.7 Hz, 1H, Ar-H), 6.32 (t,  $J$  = 6.8 Hz, 1H, Ar-H), 6.53 (d,  $J$  = 6.5 Hz, 1H, Ar-H), 6.59 (t,  $J$  = 7.4 Hz, 1H, Ar-H), 7.55 (s, 1H, H-5), 7.76 (s, 1H, H-8), 8.47 (s, 1H, H-2), 15.74 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.76, 35.96, 47.51 (2C), 106.09, 107.26, 114.40, 114.63, 116.37, 116.80, 121.28, 123.44, 130.27, 133.62, 137.12, 140.97, 143.90, 145.32, 167.06, 176.86. HPLC: *r*<sub>t</sub> 5.782 min. HRMS (ESI) calculated for C<sub>21</sub>H<sub>20</sub>N<sub>4</sub>O<sub>3</sub> [M + H]<sup>+</sup> 377.1613, found 377.16151.

**6-Amino-1-cyclopropyl-7-(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid** (**21**). Under N<sub>2</sub> atmosphere, to a solution of **51** (0.22 g, 0.49 mmol) in EtOH (20 mL), SnCl<sub>2</sub> × 2 H<sub>2</sub>O (0.55 g, 2.45 mmol) was added portionwise and the reaction was stirred at reflux for 2 h. Then, EtOH was evaporated under vacuum and the resulting mixture was poured into ice/water to give a precipitate that was collected by filtration. After purification by flash chromatography column eluting with CHCl<sub>3</sub>/MeOH 99:1, compound **21** was obtained as a yellow solid in 21% yield. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.03–1.09 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.12–1.18 (m, 2H, cyclopropyl-CH<sub>2</sub>), 2.91–2.97 (m, 2H, dihydrothienopyridine CH<sub>2</sub>), 3.39 (t,  $J$  = 5.4 Hz, 2H, dihydrothienopyridine CH<sub>2</sub>), 3.61–3.75 (m, 1H, CH), 4.23 (s, 2H, dihydrothienopyridine CH<sub>2</sub>), 5.45 (s, 2H, NH<sub>2</sub>), 6.92 (d,  $J$  = 5.1 Hz, 1H, Ar-H), 7.34 (d,  $J$  = 5.1 Hz, 1H, Ar-H), 7.52 (s, 1H, H-5), 7.60 (s, 1H, H-8), 8.46 (s, 1H, H-2), 15.86 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.71, 25.06, 35.91, 47.72, 50.09, 106.17, 106.98, 107.97, 121.98, 123.74, 125.76, 133.48, 133.62, 134.21, 142.58, 145.00, 145.36, 167.16, 176.66. HPLC: *r*<sub>t</sub> 4.900 min. HRMS (ESI) calculated for C<sub>20</sub>H<sub>19</sub>N<sub>3</sub>O<sub>3</sub>S [M + H]<sup>+</sup> 382.1225, found 382.12217.

**6-Amino-1-cyclopropyl-7-(1-methyl-3,4-dihydroisoquinolin-2(1H)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid** (**22**). General procedure D: starting from **61** (reaction time: 2 h; work-up: extraction at pH = 3), compound **22**, after crystallization by EtOH/DMF, was obtained as a yellow solid in 63% yield (0.18 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.81–0.87 (m, 2H, cyclopropyl-CH<sub>2</sub>), 0.99–1.15 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.31 (d,  $J$  = 6.7 Hz, 3H, CH<sub>3</sub>), 2.78 (t,  $J$  = 7.0 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.31–3.40 (m, 1H, CH), 3.53–3.61 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.73 (q,  $J$  = 6.6 Hz, 1H, tetrahydroisoquinoline-NCH), 5.44 (s, 2H, NH<sub>2</sub>), 7.11 (t,  $J$  = 8.9 Hz, 1H, Ar-H), 7.14–7.21 (m, 2H, Ar-H), 7.21–7.26 (m, 1H, Ar-H), 7.50 (s, 1H, H-5), 7.53 (s, 1H, H-8), 8.43 (s, 1H, H-2), 15.83 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.54, 21.38, 27.55, 35.75, 42.28, 54.14, 106.12, 106.69, 110.43, 122.13, 126.30, 126.53, 127.26, 129.19, 133.91, 134.10, 139.85, 143.03, 144.87, 145.02, 167.14, 176.71. HPLC: *r*<sub>t</sub> 9.269 min. HRMS (ESI) calculated for C<sub>23</sub>H<sub>23</sub>N<sub>3</sub>O<sub>3</sub> [M + H]<sup>+</sup> 390.1817, found 390.18118.

**6-Amino-1-cyclopropyl-7-[(3*S*)-3-(hydroxymethyl)-3,4-dihydroisoquinolin-2(1*H*)-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid** (**23**). General procedure D: starting from **62** (reaction time: 2 h; work-up: extraction at pH = 3), compound **23**, after crystallization by EtOH/DMF, was obtained as a yellow solid in 21% yield (0.06 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.77–0.86 (m, 2H, cyclopropyl-CH<sub>2</sub>), 0.97–1.15 (m, 2H, cyclopropyl-CH<sub>2</sub>), 2.65 (d,  $J$  = 16.9 Hz, 1H, tetrahydroisoquinoline-CH<sub>2</sub> ×<sub>2</sub><sup>1</sup>), 3.04 (dd,  $J$  = 17.0, 6.4 Hz, 1H, tetrahydroisoquinoline-CH<sub>2</sub> ×<sub>2</sub><sup>1</sup>), 3.36–3.47 (m, 1H, tetrahydroisoquinoline-CH), 3.49–3.66 (m, 2H, tetrahydroisoquinoline-NCH<sub>2</sub>), 3.81–3.87 (m, 1H, CH), 4.17 (d,  $J$  = 16.8 Hz, 1H, CH<sub>2</sub>OH ×<sub>2</sub><sup>1</sup>), 4.60 (d,  $J$  = 16.8 Hz, 1H, CH<sub>2</sub>-OH ×<sub>2</sub><sup>1</sup>), 4.89 (t,  $J$  = 5.4 Hz, 1H, OH), 5.56 (s, 2H, NH<sub>2</sub>), 7.07–7.24 (m, 4H, Ar-H), 7.48 (s, 1H, H-5), 7.52 (s, 1H, H-8), 8.41 (s, 1H, H-2), 15.88 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.48, 7.59, 27.59, 35.71, 47.62, 54.79, 60.57, 106.08, 106.65, 109.23, 121.76, 126.24, 126.33, 126.86, 129.61, 133.56, 134.01, 134.07, 142.72, 144.92, 145.36, 167.18, 176.58. HPLC: *r*<sub>t</sub> 7.109 min. HRMS (ESI) calculated for C<sub>23</sub>H<sub>23</sub>N<sub>3</sub>O<sub>4</sub> [M + H]<sup>+</sup> 406.1767, found 406.17645.

**6-Amino-1-cyclopropyl-4-oxo-7-(4-phenylpiperidin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid** (**24**). General procedure D: starting from **63** (reaction time: 1 h; work-up: extraction at pH = 3), compound **24**, after crystallization by EtOH/DMF, was obtained as a yellow solid in 33% yield (0.16 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.08–1.15 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.22–1.29 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.83–1.89 (m, 2H, piperidine-CH<sub>2</sub>), 1.92–2.03 (m, 2H, piperidine-CH<sub>2</sub>), 2.64–2.82 (m, 3H, piperidine-NCH<sub>2</sub> and -CH), 3.43–3.51 (m, 2H, piperidine-NCH<sub>2</sub>), 3.74–3.83 (m, 1H, CH), 5.44 (s, 2H, NH<sub>2</sub>), 7.15–7.20 (m, 1H, H-4'), 7.30 (d,  $J$  = 4.3 Hz, 4H, H-2', H-3', H-5' and H-6'), 7.50 (s, 1H, H-5), 7.58 (s, 1H, H-8), 8.48 (s, 1H, H-2), 15.89 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.79, 33.42, 35.99, 42.01, 51.07, 106.15, 106.52, 107.45, 121.85, 126.54, 127.20, 128.78, 134.28, 142.59, 144.90, 146.51, 146.76, 167.21, 176.65. HPLC: *r*<sub>t</sub> 5.672 min. HRMS (ESI) calculated for C<sub>24</sub>H<sub>25</sub>N<sub>3</sub>O<sub>3</sub> [M + H]<sup>+</sup> 404.1974, found 404.1971.

**6-Amino-7-[benzyl(ethyl)amino]-1-cyclopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid** (**25**). General procedure D: starting from **64** (reaction time: 2 h; work-up: extraction at pH = 3), compound **25**, after crystallization by EtOH/DMF, was obtained as a pale yellow solid in 70% yield (0.12 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.87–0.93 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.05 (t,  $J$  = 7.0 Hz, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 1.16–1.25 (m, 2H, cyclopropyl-CH<sub>2</sub>), 3.12 (q,  $J$  = 6.9 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 3.58–3.73 (m, 1H, CH), 4.33 (s, 2H, NCH<sub>2</sub>), 5.51 (s, 2H, NH<sub>2</sub>), 7.14 (t,  $J$  = 7.2 Hz, 1H, H-4'), 7.22 (t,  $J$  = 7.4 Hz, 2H, H-3' and H-5'), 7.30 (d,  $J$  = 7.1 Hz, 2H, H-2' and H-6'), 7.45–7.49 (m, 2H, H-5 and H-8), 8.42 (s, 1H, H-2), 15.83 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.82, 11.93, 35.94, 45.74, 54.12, 105.82, 106.68, 110.57, 121.98, 127.43, 128.63, 128.69, 133.74, 138.27, 143.47, 143.68, 145.18, 167.33, 176.68. HPLC: *r*<sub>t</sub> 5.114 min. HRMS (ESI) calculated for C<sub>22</sub>H<sub>23</sub>N<sub>3</sub>O<sub>3</sub> [M + H]<sup>+</sup> 378.1817, found 378.18165.



**6-Amino-7-[benzyl(2-hydroxyethyl)amino]-1-cyclopropyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (26).** General procedure D: starting from **65** (reaction time: 2 h; work-up: extraction at pH = 3), compound **26**, after crystallization by EtOH/DMF, was obtained as a yellow solid in 58% yield (0.14 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.81–0.88 (s, 2H, cyclopropyl-CH<sub>2</sub>), 1.16 (q, *J* = 6.5 Hz, 2H, cyclopropyl-CH<sub>2</sub>), 3.15 (t, *J* = 5.1 Hz, 2H, CH<sub>2</sub>CH<sub>2</sub>OH), 3.53–3.62 (m, 1H, CH), 3.63–3.69 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>OH), 4.45 (s, 2H, CH<sub>2</sub>), 5.07 (t, *J* = 4.6 Hz, 1H, OH), 5.91 (s, 2H, NH<sub>2</sub>), 7.11 (t, *J* = 7.2 Hz, 1H, H-4'), 7.20 (t, *J* = 7.5 Hz, 2H, H-3' and H-5'), 7.31 (d, *J* = 7.4 Hz, 2H, H-2' and H-6'), 7.39 (s, 1H, H-5), 7.48 (s, 1H, H-8), 8.38 (s, 1H, H-2), 15.88 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.75, 35.85, 53.75, 54.65, 58.54, 105.91, 106.17, 109.63, 121.61, 127.22, 128.48 (2C), 133.41, 138.51, 143.31, 143.73, 144.68, 167.20, 176.45. HPLC: *r*<sub>t</sub> 6.778 min. HRMS (ESI) calculated for C<sub>22</sub>H<sub>23</sub>N<sub>3</sub>O<sub>4</sub> [M + H]<sup>+</sup> 394.1767, found 394.1766.

**7-Chloro-1-cyclopropyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (66).** General procedure A: starting from **45** (ref. 31) (reaction time: 3 h), compound **66** was obtained as a yellow solid in 95% yield (1.30 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.11–1.19 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.25–1.29 (m, 2H, cyclopropyl-CH<sub>2</sub>), 3.81–3.69 (m, 1H, CH), 8.51 (s, 1H, H-8), 8.76 (s, 1H, H-5), 8.87 (s, 1H, H-2), 13.94 (bs, 1H, CO<sub>2</sub>H).

**7-Chloro-1-cyclopropyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxamide (67).** SOCl<sub>2</sub> (5 mL) was added to acidic derivative **66** (0.50 g, 1.62 mmol) and the reaction mixture was stirred at reflux for 2 h. Then, the excess of SOCl<sub>2</sub> was removed under vacuum and the resulting yellow oil was dissolved in dry DMF (10 mL) and added dropwise at 0 °C to a solution of 7 N NH<sub>3</sub> in MeOH. The reaction mixture was allowed to warm at rt and stirred for 3 h and then it was poured into ice/water to give a precipitate that was collected by filtration. After trituration with a mixture of Et<sub>2</sub>O/EtOH, compound **67** was obtained as a yellow solid in 92% yield. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.10–1.13 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.24–1.29 (m, 2H, cyclopropyl-CH<sub>2</sub>), 3.73–3.76 (m, 1H, CH), 7.67–7.68 (m, 1H, NH<sub>2</sub> ×<sub>2</sub>), 8.37 (s, 1H, H-8), 8.68 (s, 1H, H-5), 8.82 (s, 1H, H-2), 78.81–8.83 (m, 1H, NH<sub>2</sub> ×<sub>2</sub>).

**1-Cyclopropyl-7-(3,4-dihydroisoquinolin-2(1H)-yl)-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxamide (68).** General procedure B: starting from **67** and using 1,2,3,4-tetrahydroisoquinoline (3 equiv.) as a nucleophile (reaction time: 3 h; temperature: 90 °C; solvent: DMSO), compound **68** was obtained as a yellow solid in 96% yield (0.19 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.03–1.09 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.26–1.28 (m, 2H, cyclopropyl-CH<sub>2</sub>), 2.87–2.96 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.43 (t, *J* = 7.3 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.70–3.74 (m, 1H, CH), 1.91–1.97 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 7.17–7.21 (m, 3H, Ar-H), 7.22–7.25 (m, 1H, Ar-H), 7.53–7.54 (m, 1H, NH<sub>2</sub> ×<sub>2</sub>), 7.59 (s, 1H, H-8), 8.58–8.61 (m, 2H, H-2 and H-5), 8.96–8.97 (m, 1H, NH<sub>2</sub> ×<sub>2</sub>).

**6-Amino-1-cyclopropyl-7-(3,4-dihydroisoquinolin-2(1H)-yl)-4-oxo-1,4-dihydroquinoline-3-carboxamide (17).** General

procedure C: starting from **68** (reaction time: 5 h), compound **17**, after purification by chromatography column (Reveleris-X2) eluting with CHCl<sub>3</sub>/MeOH 97:3 to 95:5, was obtained as a yellow solid in 25% yield (0.07 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.91–1.01 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.12–1.16 (m, 2H, cyclopropyl-CH<sub>2</sub>), 2.95 (t, *J* = 5.5 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.33 (t, *J* = 5.7 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.52–3.62 (m, 1H, CH), 4.24 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 5.20 (s, 2H, NH<sub>2</sub>), 7.13–7.17 (m, 4H, Ar-H), 7.30 (d, *J* = 4.8 Hz, 1H, CONH<sub>2</sub> ×<sub>2</sub>), 7.51–7.54 (m, 2H, H-5 and H-8), 8.46 (s, 1H, H-2), 9.43 (d, *J* = 4.9 Hz, 1H, CONH<sub>2</sub> ×<sub>2</sub>). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.67, 28.63, 34.97, 47.97, 52.66, 107.33, 108.23, 109.72, 124.07, 126.14, 126.70, 126.77, 129.12, 133.68, 134.52, 134.83, 141.36, 144.39, 144.81, 166.56, 174.96. HPLC: *r*<sub>t</sub> 4.557 min. HRMS (ESI) calculated for C<sub>22</sub>H<sub>22</sub>N<sub>3</sub>O<sub>2</sub> [M + Na]<sup>+</sup> 397.16407, found 397.16376.

**Ethyl 7-(3,4-dihydroisoquinolin-2(1H)-yl)-1-ethyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate (69).** General procedure B: starting from **46** (ref. 37) and using 1,2,3,4-tetrahydroisoquinoline (3 equiv.) as nucleophile (reaction time: 2 h; temperature: 90 °C; solvent: DMSO), compound **69** was obtained as a yellow solid in 88% yield (0.50 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.18–1.45 (m, 6H, OCH<sub>2</sub>CH<sub>3</sub> and NCH<sub>2</sub>CH<sub>3</sub>), 2.89–3.02 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.43 (t, *J* = 5.6 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.26 (q, *J* = 6.9 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.27–4.53 (m, 4H, NCH<sub>2</sub>CH<sub>3</sub> and tetrahydroisoquinoline-CH<sub>2</sub>), 7.00–7.25 (m, 5H, Ar-H), 8.50 (s, 1H, H-2), 8.70 (s, 1H, H-5).

**Ethyl 6-amino-7-(3,4-dihydroisoquinolin-2(1H)-yl)-1-ethyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (70).** General procedure C: starting from **69** (reaction time: 2 h), compound **70**, after trituration by EtOH, was obtained as a white solid in 70% yield (0.47 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.00–1.30 (m, 6H, OCH<sub>2</sub>CH<sub>3</sub> and NCH<sub>2</sub>CH<sub>3</sub>), 2.92–3.01 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.25–3.32 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.20–4.30 (m, 6H, NCH<sub>2</sub>CH<sub>3</sub>, OCH<sub>2</sub>CH<sub>3</sub> and tetrahydroisoquinoline-CH<sub>2</sub>), 5.18 (s, 2H, NH<sub>2</sub>), 7.05–7.25 (m, 4H, Ar-H), 7.45 (s, 1H, H-8), 7.48 (s, 1H, H-5), 8.27 (s, 1H, H-2).

**6-Amino-7-(3,4-dihydroisoquinolin-2(1H)-yl)-1-ethyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (16).** General procedure D: starting from **70** (reaction time: 4 h; work-up: extraction at pH = 5), compound **16**, after crystallization by EtOH/DMF, was obtained as a pale yellow solid in 76% yield (0.30 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.36 (t, *J* = 7.0 Hz, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 3.02 (t, *J* = 5.4 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.36 (t, *J* = 5.4 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.31 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.52 (q, *J* = 7.0 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 5.47 (s, 2H, NH<sub>2</sub>), 7.10–7.20 (m, 4H, Ar-H), 7.30 (s, 1H, H-8), 7.59 (s, 1H, H-5), 8.77 (s, 1H, H-2), 16.04 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  14.98, 28.89, 48.13, 49.25, 52.53, 106.52, 107.16, 107.41, 122.69, 126.18, 126.75, 126.79, 129.16, 132.19, 134.45, 134.75, 142.52, 145.51, 146.03, 167.41, 176.48. HPLC: *r*<sub>t</sub> 5.2700 min. HRMS (ESI) calculated for C<sub>21</sub>H<sub>21</sub>N<sub>3</sub>O<sub>3</sub> [M + H]<sup>+</sup> 364.1661, found 364.1659.

**Ethyl 7-chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (71).** To a solution of the commercially available ethyl 3-(2,6-dichloro-5-fluoropyridin-3-yl)-3-oxopropanoate (0.5 g, 2.0 mmol) in toluene (3 mL), DMF-DMA (0.3 mL, 2.2 mmol) and acetic acid (30  $\mu$ L, 0.5 mmol) were added and the reaction mixture was stirred at rt for 2 h. After the disappearance of the starting material as checked by TLC (cyclohexane/EtOAc 60:40), 2 M EtNH<sub>2</sub> in THF (1.1 mL, 2.2 mmol) was added and the reaction mixture was stirred for additional 30 min at rt. Then, 3 mL of aq. 10% citric acid were added, and the two phases were separated; the organic phase was washed with water and aq. 1.5 M BuN<sub>4</sub>OH (1.5 mL, 2.2 mmol) was added. The reaction mixture was stirred for 5 min until the formation of a precipitate, then it was concentrated under vacuum to half volume and neutralized by 2 N HCl and the precipitate was filtered under vacuum to afford compound 71 as a white solid in 63% yield. <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.19–1.38 (m, 6H, OCH<sub>2</sub>CH<sub>3</sub>, and NCH<sub>2</sub>CH<sub>3</sub>), 4.22 (q, *J* = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.40 (q, *J* = 7.1 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 8.43 (d, *J* = 7.9 Hz, 1H, H-5), 8.85 (s, 1H, H-2).

**Ethyl 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (72).** Following the same procedure used to prepare compound 71 and replacing EtNH<sub>2</sub> with cyclopropyl amine, ethyl ester intermediate 72, after purification by flash chromatography column eluting with CH<sub>2</sub>Cl<sub>2</sub>/MeOH 95:5, was obtained in 53% yield (0.72 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.93–1.06 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.09–1.16 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.25 (t, *J* = 7.1 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 3.61–3.64 (m, 1H, CH), 4.20 (q, *J* = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 8.39 (d, *J* = 7.8 Hz, 1H, H-5), 8.53 (s, 1H, H-2).

**Ethyl 7-(3,4-dihydroisoquinolin-2(1H)-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (73).** General procedure B: starting from 71 and using 1,2,3,4-tetrahydroisoquinoline (3 equiv.) as a nucleophile (reaction time: 2 h; temperature: 90 °C; solvent: DMF), compound 73 was obtained as a white solid in 83% yield (0.23 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.21–1.36 (m, 6H, OCH<sub>2</sub>CH<sub>3</sub>, and NCH<sub>2</sub>CH<sub>3</sub>), 2.96 (t, *J* = 5.8 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.96 (t, *J* = 5.8 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.17 (q, *J* = 7.0 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.40 (q, *J* = 7.1 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 4.87 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 7.10–7.30 (m, 4H, Ar-H), 7.91 (d, *J* = 13.6 Hz, 1H, H-5), 8.61 (s, 1H, H-2).

**Ethyl 1-cyclopropyl-7-(3,4-dihydroisoquinolin-2(1H)-yl)-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (74).** General procedure B: starting from 72 and using 1,2,3,4-tetrahydroisoquinoline (3 equiv.) as a nucleophile (reaction time: 3 h; temperature: 90 °C; solvent: DMF), compound 74 was obtained as a white solid in 94% yield (0.62 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.98–1.06 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.14–1.16 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.22 (t, *J* = 7.0 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 2.95–2.99 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.51–3.58 (m, 1H, CH), 3.99 (t, *J* = 7.2 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.15 (q, *J* = 7.0 Hz, 2H, OCH<sub>2</sub>-

CH<sub>3</sub>), 4.24 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 7.10–7.19 (m, 3H, Ar-H), 7.21–7.23 (m, 1H, Ar-H), 7.86 (d, *J* = 13.6 Hz, 1H, H-5), 8.34 (s, 1H, H-2).

**Ethyl 7-[benzyl(ethyl)amino]-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (75).** General procedure B: starting from 71 and using *N*-benzylethanamine (3 equiv.) as a nucleophile (reaction time: 3 h; temperature: 90 °C; solvent: DMF), compound 75 was obtained as a white solid in 74% yield (0.40 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.13 (t, *J* = 7.0 Hz, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 1.24–1.29 (m, 6H, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 3.69 (t, *J* = 6.3 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 4.20 (t, *J* = 7.0 Hz, NCH<sub>2</sub>CH<sub>3</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 4.90 (s, 2H, CH<sub>2</sub>), 7.24–7.36 (m, 5H, Ar-H), 7.90 (d, *J* = 14.0 Hz, 1H, H-5), 8.58 (s, 1H, H-2).

**Ethyl 1-ethyl-6-fluoro-4-oxo-7-(4-phenylpiperidin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxylate (76).** General procedure B: starting from 71 and using 4-phenylpiperidine (3 equiv.) as a nucleophile (reaction time: 3 h; temperature: 90 °C; solvent: DMF), compound 76 was obtained as a whitish solid in 78% yield (0.45 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.28 (t, *J* = 6.3 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 1.33–1.39 (m, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 1.75–1.77 (m, 2H, piperidine-CH<sub>2</sub>), 1.89–1.91 (m, 2H, piperidine-CH<sub>2</sub>), 2.88–2.919 (m, 1H, piperidine-CH), 3.20 (t, *J* = 2.7 Hz, 2H, piperidine-NCH<sub>2</sub>), 4.20–4.23 (m, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.35–4.39 (m, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 4.51–4.55 (m, 2H, piperidine-NCH<sub>2</sub>), 7.20–7.23 (m, 1H, Ar-H), 7.28–7.35 (m, 4H, Ar-H), 7.93 (d, *J* = 13.9 Hz, 1H, H-5), 8.66 (s, 1H, H-2).

**7-(3,4-Dihydroisoquinolin-2(1H)-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (27).** General procedure A: starting from 73 (reaction time: 4 h), compound 27, after crystallization by DMF, was obtained as a pale yellow solid in 40% yield (0.07 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.40 (t, *J* = 6.9 Hz, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 3.00 (t, *J* = 5.9 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.05 (t, *J* = 5.9 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.53 (q, *J* = 6.9 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 4.97 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 7.10–7.30 (m, 4H, Ar-H), 8.07 (d, *J* = 13.4 Hz, 1H, H-5), 8.97 (s, 1H, H-2), 15.37 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  15.17, 28.50, 45.49 (d, *J*<sub>C-F</sub> = 8.0 Hz), 47.69, 48.78 (d, *J*<sub>C-F</sub> = 7.0 Hz), 108.46, 112.74 (d, *J*<sub>C-F</sub> = 3.0 Hz), 119.56 (d, *J*<sub>C-F</sub> = 22.0 Hz), 126.71, 126.90, 127.21, 128.80, 133.96, 135.08, 145.39, 147.22 (d, *J*<sub>C-F</sub> = 257.0 Hz), 148.09, 150.47 (d, *J*<sub>C-F</sub> = 10.0 Hz), 166.36, 176.76. HPLC *t*<sub>r</sub>: 6.0900 min. HRMS-ESI *m/z* [M + H]<sup>+</sup> calcd for C<sub>20</sub>H<sub>18</sub>FN<sub>3</sub>O<sub>3</sub>, 368.1410, found: 368.1405.

**1-Cyclopropyl-7-(3,4-dihydroisoquinolin-2(1H)-yl)-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (28).** General procedure A: starting from 74 (reaction time: 6 h), compound 28, after crystallization by EtOH/DMF, was obtained as a white solid in 67% yield (0.25 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.01–1.10 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.18–1.22 (m, 2H, cyclopropyl-CH<sub>2</sub>), 2.99 (t, *J* = 5.8 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.62–3.76 (m, 1H, CH), 4.05 (t, *J* = 5.9 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.98 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 7.15–7.21 (m, 3H, Ar-H), 7.21–7.27 (m, 1H, Ar-H), 8.00 (d, *J* = 13.5 Hz, 1H, H-5), 8.55 (s, 1H, H-2), 15.18 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$



7.24, 28.41, 35.39, 45.35 (d,  $J_{C-F}$  = 9.1 Hz), 48.72 (d,  $J_{C-F}$  = 4.0 Hz), 107.95, 112.31, 119.44 (d,  $J_{C-F}$  = 21.2 Hz), 126.62, 126.85, 127.13, 128.80, 133.98, 133.00, 147.18, 147.19 (d,  $J_{C-F}$  = 260.6 Hz), 147.48, 150.13 (d,  $J_{C-F}$  = 9.1 Hz), 166.15, 176.80. HPLC:  $r_t$  10.251 min. HRMS (ESI) calculated for  $C_{21}H_{18}FN_3O_3$  [M + H]<sup>+</sup> 380.1410, found 380.14075.

**7-[Benzyl(ethyl)amino]-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (29).** General procedure D: starting from 75 (reaction time: 1 h; work-up: filtration at pH = 3), compound 29, after crystallization by EtOH, was obtained as a yellow solid in 77% yield (0.21 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.16–1.18 (m, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 1.28 (t,  $J$  = 7.7 Hz, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 3.74–3.77 (m, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 1.33–1.38 (m, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 4.96 (s, 2H, CH<sub>2</sub>), 7.25–7.37 (m, 5H, Ar-H), 8.05 (d,  $J$  = 13.7 Hz, 1H, H-5), 8.91 (s, 1H, H-2). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  14.07, 15.17, 46.22 (d,  $J_{C-F}$  = 8.0 Hz), 47.46, 53.32, 108.49, 112.34, 119.61 (d,  $J_{C-F}$  = 22.0 Hz), 126.95, 127.53, 129.13, 138.40, 145.51 (d,  $J_{C-F}$  = 22.0 Hz), 146.83 (d,  $J_{C-F}$  = 239.0 Hz), 148.02, 160.15 (d,  $J_{C-F}$  = 8.0 Hz), 166.47, 176.77. HPLC:  $r_t$  6.778 min. HRMS (ESI) calculated for  $C_{20}H_{20}FN_3O_3$  [M + H]<sup>+</sup> 370.1575, found 370.15737.

**1-Ethyl-6-fluoro-4-oxo-7-(4-phenylpiperidin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (30).** General procedure A: starting from 76 (reaction time: 16 h), compound 30, after crystallization by EtOH/DMF, was obtained as a white solid in 76% yield (0.29 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.41 (t,  $J$  = 7.0 Hz, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 1.75–1.82 (m, 2H, piperidine-CH<sub>2</sub>), 1.92–1.96 (m, 2H, piperidine-CH<sub>2</sub>), 2.94 (t,  $J$  = 12.0 Hz, 1H, piperidine-CH), 3.28 (t,  $J$  = 12.7 Hz, 2H, piperidine-NCH<sub>2</sub>), 4.50 (q,  $J$  = 6.7 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 4.64 (d,  $J$  = 13.1 Hz, 2H, piperidine-NCH<sub>2</sub>), 7.20 (t,  $J$  = 6.6 Hz, 1H, Ar-H), 7.25–7.32 (m, 4H, Ar-H), 8.05 (d,  $J$  = 13.7 Hz, 1H, H-5), 8.97 (s, 1H, H-2). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  15.28, 33.42, 42.26, 47.77, 48.13 (d,  $J_{C-F}$  = 8.1 Hz), 108.55, 112.96, 119.89 (d,  $J_{C-F}$  = 22.2 Hz), 126.81, 127.33, 128.99, 145.61, 146.28, 147.10 (d,  $J_{C-F}$  = 226.2 Hz), 150.48, 160.56, 166.42 (d,  $J_{C-F}$  = 14.0 Hz), 176.88. HPLC:  $r_t$  7.332 min. HRMS (ESI) calculated for  $C_{22}H_{22}FN_3O_3$  [M + H]<sup>+</sup> 396.1723, found 396.17333.

**Ethyl (2E/Z)-3-(dimethylamino)-2-(2,4,5-trifluoro-3-methylbenzoyl)acrylate (77).** Under N<sub>2</sub> atmosphere, to a mixture of 2,4,5-trifluoro-3-methylbenzoic acid (6.80 g, 35.8 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (120 mL), oxalyl chloride (3.7 mL, 42.9 mmol) and cat. dry DMF were added, and the reaction mixture was stirred at rt for 4 h. The solvent was removed under vacuum and the oil residue was dissolved in dry toluene (100 mL); the resulting solution was added dropwise, under N<sub>2</sub> atmosphere, to a mixture of ethyl *N,N*-dimethylaminoacrylate (6.7 mL, 46.5 mmol) and Et<sub>3</sub>N (7.46 mL, 53.7 mmol) in dry toluene (50 mL) and the reaction mixture was heated at 90 °C for 4 h. After cooling, the reaction mixture was filtered, and the filtrate was evaporated to give compound 77 as a pale brown solid in 70% yield. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>):  $\delta$  0.98 (t,  $J$  = 7.1 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 2.21 (t,  $J$  = 1.8 Hz, 3H, CH<sub>3</sub>), 2.60–3.40 (m, 6H, N(CH<sub>3</sub>)<sub>2</sub>), 4.00

(q,  $J$  = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 7.20–7.35 (m, 1H, Ar-H), 7.78 (s, 1H, CH).

**Ethyl (2E/Z)-3-(ethylamino)-2-(2,4,5-trifluoro-3-methylbenzoyl)acrylate (78).** Following the same procedure used to prepare compound 35 and starting from 77, intermediate 78 was obtained in 90% yield (0.51 g). <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>):  $\delta$  1.05 (t,  $J$  = 7.1 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 1.26 (t,  $J$  = 7.2 Hz, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 2.25 (t,  $J$  = 1.8 Hz, 3H, CH<sub>3</sub>), 3.50 (q,  $J$  = 7.2 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 4.00 (q,  $J$  = 7.1 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 7.20–7.35 (m, 1H, Ar-H), 7.78 (d,  $J$  = 14.1 Hz, 1H, CH), 10.80 (bs, 1H, NH).

**Ethyl (2E/Z)-3-(cyclopropylamino)-2-(2,4,5-trifluoro-3-methylbenzoyl)acrylate (79).** Following the same procedure used to prepare compound 35, replacing ethylamine with cyclopropyl amine and starting from 77, intermediate 79 was obtained in 99% yield (1.85 g). <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>):  $\delta$  0.81–0.99 (m, 4H, cyclopropyl-CH<sub>2</sub> × 2), 1.06 (t,  $J$  = 7.1 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 2.18–2.23 (m, 3H, CH<sub>3</sub>), 2.93–3.03 (m, 1H, CH), 4.06 (t,  $J$  = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 6.95–7.10 (m, 1H, Ar-H), 8.14–8.17 (m, 1H, CH), 10.81–10.87 (m, 1H, NH).

**Ethyl 1-ethyl-6,7-difluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (80).** Under N<sub>2</sub> atmosphere, to a mixture of 60% NaH (1.71 g, 43.0 mmol) in dry DMF (20 mL) cooled at 0 °C, a solution of acrylate intermediate 78 (4.5 g, 14.0 mmol) in dry DMF (50 mL) was added dropwise. After stirring at 0 °C for 3 h, the reaction mixture was poured into ice/water, acidified with 2 N HCl (pH = 4–5) and the precipitate was filtered under vacuum to afford compound 80 as a white solid in 90% yield. <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.22–1.30 (m, 6H, OCH<sub>2</sub>CH<sub>3</sub> and NCH<sub>2</sub>CH<sub>3</sub>), 2.59 (d,  $J$  = 3.0 Hz, 3H, CH<sub>3</sub>), 4.21 (q,  $J$  = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.49 (q,  $J$  = 7.3 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 7.92–8.02 (m, 1H, H-5), 8.62 (s, 1H, H-2).

**Ethyl 1-cyclopropyl-6,7-difluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (81).** Following the same procedure used to prepare compound 80 and starting from 79, intermediate 81 was obtained as a white solid in 97% yield (1.65 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.93–0.97 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.07–1.15 (m, 5H, cyclopropyl-CH<sub>2</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 2.77 (d,  $J$  = 3.4 Hz, 3H, CH<sub>3</sub>), 3.31–3.36 (m, 1H, CH), 4.21 (q,  $J$  = 7.0 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 7.87–7.95 (m, 1H, H-5), 8.59 (s, 1H, H-2).

**Ethyl 7-azido-1-ethyl-6-fluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (82).** To solution of intermediate 80 (3.00 g, 10.1 mmol) in DMF, NaN<sub>3</sub> (2.60 g, 40.4 mmol) was added, and the reaction mixture was heated at 90 °C for 24 h. After cooling, the mixture was poured into ice/water, acidified with 2 N HCl (pH = 5) and the precipitate was filtered under vacuum to afford compound 82 as a brown solid in 73% yield. <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.00–1.35 (m, 6H, OCH<sub>2</sub>CH<sub>3</sub> and NCH<sub>2</sub>CH<sub>3</sub>), 2.55 (s, 3H, CH<sub>3</sub>), 4.15 (q,  $J$  = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.40 (q,  $J$  = 7.3 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 7.86 (d,  $J$  = 11.4 Hz, 1H, H-5), 8.63 (s, 1H, H-2).

**Ethyl 7-azido-1-cyclopropyl-6-fluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (83).** Following the same procedure used to prepare compound 82 and starting from

**81**, intermediate **83** was obtained as a light brown solid in 74% yield (2.85 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.85–0.89 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.08–1.14 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.26 (t, *J* = 7.1 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 2.69 (s, 3H, CH<sub>3</sub>), 4.12–4.23 (m, 3H, CH and OCH<sub>2</sub>CH<sub>3</sub>), 7.79 (d, *J* = 11.8 Hz, 1H, H-5), 8.58 (s, 1H, H-2).

**Ethyl 7-amino-1-ethyl-6-fluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (84).** A solution of azido intermediate **82** (2.40 g, 7.5 mmol) in a mixture MeOH/THF (1:1) (300 mL) was hydrogenated over a catalytic amount of Pd/C (10% w/w) at rt for 3 h. Then, the reaction mixture was filtered over celite and the filtrate was evaporated to give, after purification by flash column chromatography eluting CHCl<sub>3</sub>/MeOH 95:5, compound **84** as a white solid in 53% yield. <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.05–1.30 (m, 6H, OCH<sub>2</sub>CH<sub>3</sub> and NCH<sub>2</sub>CH<sub>3</sub>), 2.25 (s, 3H, CH<sub>3</sub>), 4.10–4.40 (m, 4H, OCH<sub>2</sub>CH<sub>3</sub>, and NCH<sub>2</sub>CH<sub>3</sub>), 6.00 (s, 2H, NH<sub>2</sub>), 7.61 (d, *J* = 11.6 Hz, 1H, H-5), 8.50 (s, 1H, H-2).

**Ethyl 7-amino-1-cyclopropyl-6-fluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (85).** Following the same procedure used to prepare compound **84** and starting from **83**, derivative **85** was obtained as a yellow solid in 70% yield (1.15 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.72–0.80 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.08–1.14 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.23 (t, *J* = 6.9 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 2.49 (s, 3H, CH<sub>3</sub>), 4.15–4.21 (m, 3H, CH and OCH<sub>2</sub>CH<sub>3</sub>), 5.96 (bs, 2H, NH<sub>2</sub>), 7.56 (d, *J* = 11.3 Hz, 1H, H-5), 8.50 (s, 1H, H-2).

**Ethyl 7-bromo-1-ethyl-6-fluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (86).** To a mixture of amino intermediate **84** (1.30 g, 4.4 mmol) and CuBr<sub>2</sub> (4.90 g, 22.0 mmol) in aq. 5% HBr (50 mL) cooled at 0 °C, aq. 2 M NaNO<sub>2</sub> (4.40 mL, 8.8 mmol) was added dropwise, and the reaction mixture was stirred at rt for 30 min. Then, the mixture was poured into ice/water and the precipitate was filtered to give compound **86** as a pale brown solid in 82% yield. <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.15–1.30 (m, 6H, OCH<sub>2</sub>CH<sub>3</sub> and NCH<sub>2</sub>CH<sub>3</sub>), 2.75 (s, 3H, CH<sub>3</sub>), 4.23 (q, *J* = 7.1 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.40 (q, *J* = 7.3 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 7.85 (d, *J* = 11.6 Hz, 1H, H-5), 8.70 (s, 1H, H-2).

**Ethyl 7-bromo-1-cyclopropyl-6-fluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (87).** Following the same procedure used to prepare compound **86** and starting from **85**, derivative **87** was obtained as a yellow solid in 67% yield (0.43 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.76–0.77 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.06–1.15 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.23 (t, *J* = 7.1 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 2.89 (s, 3H, CH<sub>3</sub>), 4.13–4.27 (m, 3H, CH and OCH<sub>2</sub>CH<sub>3</sub>), 7.80 (d, *J* = 8.4 Hz, 1H, H-5), 8.62 (s, 1H, H-2).

**Ethyl 7-(3,4-dihydroisoquinolin-2(1H)-yl)-1-ethyl-6-fluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (88).** Under inert atmosphere, a mixture of intermediate **86** (0.33 g, 0.93 mmol), Pd<sub>2</sub>(dba)<sub>3</sub> (0.19 g, 0.23 mmol), *rac*-BINAP (0.39 g, 0.64 mmol), 1,2,3,4-tetrahydroisoquinoline (0.13 mL, 0.10 mmol), and Cs<sub>2</sub>CO<sub>3</sub> (0.55 g, 1.70 mmol) in dry toluene (30 mL) was degassed for 30 min at rt and then refluxed for 13 h. After cooling, the reaction mixture was concentrated and poured

into ice/water, the aqueous mixture was extracted with EtOAc ( $\times 3$ ), the collected organic layers were then washed with brine, dried, and evaporated under vacuum to give a yellow solid. After crystallization by EtOH/DMF, compound **88** was obtained as a grey solid in 61% yield. <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.10–1.30 (m, 6H, OCH<sub>2</sub>CH<sub>3</sub> and NCH<sub>2</sub>CH<sub>3</sub>), 2.54 (s, 3H, CH<sub>3</sub>), 2.85–3.00 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.45–3.55 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.21 (q, *J* = 6.5 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.25–4.55 (m, 4H, NCH<sub>2</sub>CH<sub>3</sub> and tetrahydroisoquinoline-CH<sub>2</sub>), 6.98–7.29 (m, 4H, Ar-H), 7.75 (d, *J* = 12.6 Hz, 1H, H-5), 8.60 (s, 1H, H-2).

**Ethyl 1-cyclopropyl-7-(3,4-dihydroisoquinolin-2(1H)-yl)-6-fluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (89).** Following the same procedure used to prepare compound **88** and starting from **87**, derivative **89**, after purification by flash column chromatography eluting CHCl<sub>3</sub>/MeOH 99:1, was obtained as an orange solid in 63% yield (0.60 g). <sup>1</sup>H NMR (200 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.75–0.80 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.00–1.30 (m, 5H, cyclopropyl-CH<sub>2</sub> and OCH<sub>2</sub>CH<sub>3</sub>), 2.70 (s, 3H, CH<sub>3</sub>), 2.95 (t, *J* = 5.8 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.50 (t, *J* = 5.8 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.05–4.26 (m, 3H, CH and OCH<sub>2</sub>CH<sub>3</sub>), 4.30 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 7.05–7.25 (m, 4H, Ar-H), 7.70 (d, *J* = 11.2 Hz, 1H, H-5), 8.60 (s, 1H, H-2).

**7-(3,4-Dihydroisoquinolin-2(1H)-yl)-1-ethyl-6-fluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (31).** General procedure A: starting from **88** (reaction time: 3 h), compound **31**, after crystallization by EtOH/DMF, was obtained as a pale yellow solid in 70% yield (0.07 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.27 (t, *J* = 7.0 Hz, 3H, NCH<sub>2</sub>CH<sub>3</sub>), 2.56 (s, 3H, CH<sub>3</sub>), 2.80–3.00 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.50–3.68 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.39 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.58 (q, *J* = 7.0 Hz, 2H, NCH<sub>2</sub>CH<sub>3</sub>), 7.00–7.20 (m, 4H, Ar-H), 7.98 (d, *J* = 12.2 Hz, 1H, H-5), 8.97 (s, 1H, H-2), 15.05 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  16.03, 19.05, 29.63, 49.48 (d, *J*<sub>C-F</sub> = 5.0 Hz), 52.79, 52.92 (d, *J*<sub>C-F</sub> = 5.0 Hz), 107.83, 109.65 (d, *J*<sub>C-F</sub> = 23.0 Hz), 123.58 (d, *J*<sub>C-F</sub> = 8.0 Hz), 126.17, 126.39, 126.61 (d, *J*<sub>C-F</sub> = 4.0 Hz), 126.67, 129.53, 134.42, 134.91, 139.71, 145.19 (d, *J*<sub>C-F</sub> = 12.0 Hz), 151.84, 156.68 (d, *J*<sub>C-F</sub> = 249.0 Hz), 166.29, 177.11 (d, *J*<sub>C-F</sub> = 2.0 Hz). HPLC *t*<sub>r</sub>: 6.2500 min. HRMS-ESI *m/z* [M + H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>21</sub>FN<sub>2</sub>O<sub>3</sub>, 381.1614, found: 381.1606.

**1-Cyclopropyl-7-(3,4-dihydroisoquinolin-2(1H)-yl)-6-fluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (32).** General procedure A: starting from **89** (reaction time: 1 h), compound **32**, after crystallization by EtOH/DMF, was obtained as a yellow solid in 74% yield (0.12 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  0.85–0.92 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.16–1.23 (m, 2H, cyclopropyl-CH<sub>2</sub>), 2.71 (s, 3H, CH<sub>3</sub>), 2.93 (t, *J* = 5.2 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.52 (t, *J* = 5.2 Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 4.25–4.35 (m, 1H, CH), 4.49 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 7.10–7.20 (m, 4H, Ar-H), 7.80 (d, *J* = 12.2 Hz, 1H, H-5), 8.88 (s, 1H, H-2), 15.00 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  10.95, 19.05, 29.67, 41.53, 49.57 (d, *J*<sub>C-F</sub> = 5.0 Hz), 52.90 (d, *J*<sub>C-F</sub> = 5.0 Hz), 107.43, 109.32 (d, *J*<sub>C-F</sub> = 23.0 Hz), 122.95 (d, *J*<sub>C-F</sub> = 8.0 Hz), 126.17,



126.47, 126.67, 127.23 (d,  $J_{C-F} = 4.0$  Hz), 129.52, 134.44, 134.91, 141.01, 145.20 (d,  $J_{C-F} = 12.0$  Hz), 152.54, 156.61 (d,  $J_{C-F} = 248.5$  Hz), 166.11, 177.20 (d,  $J_{C-F} = 2.0$  Hz). HPLC  $r_t$ : 6.5300 min. HRMS-ESI  $m/z$  [M + H]<sup>+</sup> calcd for C<sub>23</sub>H<sub>21</sub>FN<sub>2</sub>O<sub>3</sub>, 393.1614, found: 393.1609.

**Ethyl 1-cyclopropyl-7-(3,4-dihydroisoquinolin-2(1H)-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (91).** General procedure B: starting from **90** (ref. 43) and using 1,2,3,4-tetrahydroisoquinoline (3 equiv.) as a nucleophile (reaction time: 4 h; temperature: 100 °C; solvent: DMSO), compound **91** was obtained as a pale yellow solid in 67% yield (0.23 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.00–1.05 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.19–1.21 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.26 (t,  $J = 7.0$  Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 2.91–2.97 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.59–3.63 (m, 3H, tetrahydroisoquinoline-CH<sub>2</sub> and cyclopropyl-CH), 4.19 (q,  $J = 7.0$  Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.51 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 7.18–7.26 (m, 4H, Ar-H), 7.48 (d,  $J = 6.7$  Hz, 1H, H-8), 7.77 (d,  $J = 13.5$  Hz, 1H, H-5), 8.40 (s, 1H, H-2).

**1-Cyclopropyl-7-(3,4-dihydroisoquinolin-2(1H)-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (33).** General procedure A: starting from **91** (reaction time: 4 h), compound **33**, after crystallization by EtOH/DMF, was obtained as a yellow solid in 49% yield (0.10 g). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.00–1.08 (m, 2H, cyclopropyl-CH<sub>2</sub>), 1.18–1.25 (m, 2H, cyclopropyl-CH<sub>2</sub>), 2.94–2.96 (m, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.68 (t,  $J = 5.2$  Hz, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 3.71–3.75 (m, 1H, CH), 4.56 (s, 2H, tetrahydroisoquinoline-CH<sub>2</sub>), 7.17–7.25 (m, 4H, Ar-H), 7.55 (d,  $J = 7.3$  Hz, 1H, H-8), 7.85 (d,  $J = 13.5$  Hz, 1H, H-5), 8.58 (s, 1H, H-2), 15.21 (s, 1H, CO<sub>2</sub>H). <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  7.90, 29.40, 36.18, 48.25 (d,  $J_{C-F} = 7.0$  Hz), 51.32, 106.02, 107.01, 111.38 (d,  $J_{C-F} = 23.0$  Hz), 118.29 (d,  $J_{C-F} = 7.0$  Hz), 126.52, 126.85, 127.00, 129.00, 133.85, 134.54, 139.65, 144.87 (d,  $J_{C-F} = 10.0$  Hz), 148.27, 153.03 (d,  $J_{C-F} = 247.0$  Hz), 166.37, 176.67. HPLC  $r_t$ : 6.9470 min. HRMS-ESI  $m/z$  [M + H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>19</sub>FN<sub>2</sub>O<sub>3</sub>, 379.14527, found: 379.1464.

### Cell handling and treatment

SKOV-3, A2780 and A2780 CIS were cultured and maintained, as previously described,<sup>50</sup> at 37 °C in RPMI 1640 medium (Euroclone) supplemented with 10% fetal bovine serum (FBS; Euroclone), L-glutamine and penicillin/streptomycin (SIGMA-ALDRICH). OVCA433, HEK-293T, HeLa, HCCLM3, SK-Hep-1, MCF-7, A549 and HCT-116 cells were maintained in DMEM medium (Euroclone) containing 10% FBS (Euroclone), L-glutamine and penicillin/streptomycin (SIGMA-ALDRICH). Wi-38 cells were cultured in DMEM medium (Euroclone) supplemented with 10% FBS (Euroclone), L-glutamine and penicillin/streptomycin (SIGMA-ALDRICH), and 1% non-essential amino acids (NEAA; Euroclone). The cultures were maintained in a constant temperature incubator at 37 °C with 5% CO<sub>2</sub>.

For the evaluation of GI<sub>50</sub> and CC<sub>50</sub>, cells were counted and plated in equal numbers, amounting to 100 000 cells in

35 mm culture dish. Once cell adhesion had been promoted, the compounds were added to the culture medium. After 72 hours of treatment, the cells were counted, and the data analyzed by linear regression. Each experiment was carried out three times in triplicate and three separate cell counts were made for each sample.

HEK-293T\_shSCR and HEK-293T\_shGAPDH were treated with 50 μM of enoxacin and **33** for 48 hours before RNA extraction and expression analysis.

HeLa cells were transfected with 10 ng of psi-R21 or psi-CTRL using Lipofectamine<sup>TM</sup> 2000 (Thermo Fisher Scientific) and after 48 hours were treated with either enoxacin or **33** at 20 μM and 50 μM. After 6 hours Renilla luciferase (RLuc) and firefly luciferase (FLuc) activities were measured using the Dual-Luciferase<sup>®</sup> Reporter Assay System (Promega), following the manufacturer's instructions, and GloMax<sup>®</sup> Discover Microplate Reader (Promega).

HCCLM3 and SK-Hep-1 cells were seeded into 96-well plates at a density of 10 000 cells per well and allowed to adhere overnight. Subsequently, the cells were treated with the following substances: 0.1% DMSO as the negative control, various concentrations of the test compounds, and 125 μM enoxacin as the positive control. After 48 h of incubation, the original culture medium was removed, and the cells were washed twice with PBS. Then, 100 μL of DMEM culture medium containing 10% CCK-8 (Bimake, USA) was added to each well. The plates were incubated for 30 minutes at 37 °C. To determine the fluorescence intensity, measurements were taken at a wavelength of 450 nm using a Varioskan from Thermo Fisher Scientific.

A2780 and SKOV-3 cells were treated with 50 μM of **33** for 72 h before RNA and protein extraction and expression analyses. A2780 and A2780 CIS were treated with **33** (30 μM) and/or cisplatin (1 μM, Sigma-Aldrich) for 48 hours while MCF-7, A549 and HCT-116 were treated with **33** (30 μM) for 72 hours. Total cell number and viability have been assessed using Trypan Blue Solution (Thermo Fisher Scientific) and Countess<sup>TM</sup> 3 Automated Cell Counter (Thermo Fisher Scientific).

### Equation to evaluate GI<sub>50</sub> and CC<sub>50</sub>

The formula suggested by GraphPad titled 'Absolute IC<sub>50</sub>, X is the concentration' was used to assess GI<sub>50</sub> in SKOV-3, OVCA433 and A2780 cell lines and CC<sub>50</sub> in Wi-38 cell lines.

In detail, the formula is as follows:

$$\text{Fifty} = (\text{Top} + \text{Baseline})/2$$

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + ((\text{Top} - \text{Bottom})/(\text{Fifty} - \text{Bottom}) - 1) \times (\text{Absolute IC}_{50}/X)^{\text{HillSlope}})$$

Considering: X = Compound concentration;

Y = number of cells



Each compound was initially tested in a wide range of concentrations, up to 200  $\mu\text{M}$  (in SKOV-3, OVCA433 and A2780) and up to 250  $\mu\text{M}$  (in Wi-38). Afterwards, the concentration range was narrowed to the relative value obtained in the first evaluation in order to obtain an accurate value.

The confidence intervals (CI) of the parameters were calculated with a 95% confidence level. The goodness-of-fit was quantified by means of  $R$  squared and sum-of-squares.

### Surface plasmon resonance (SPR) assay

Following the manufacturer's instructions, the BIAcore Sensor Chip (CM5) underwent initial activation using a mixture of NHS and EDC. Subsequently, recombinant human TRBP proteins at a concentration of 20  $\mu\text{g mL}^{-1}$  (in 10 mM sodium acetate, pH 4.0) were injected over the CM5 chip. After reaching a coupling level of 12 000 RU, a 1 M ethanolamine hydrochloride was injected to block the chip, preventing nonspecific binding. All the compounds were diluted in HBS-EP buffer (10 mM HEPES, pH 7.4, 3 mM EDTA, 150 mM NaCl, and 0.005% surfactant P-20) with 1% DMSO. Samples were injected at a rate of 10  $\mu\text{L min}^{-1}$  for 120 seconds, followed by a dissociation phase of 90 seconds. After each cycle, a 50% DMSO solution was used to regenerate the chip surface and restore the sensorgram baseline. Binding analysis was performed using BIA evaluation version 3.2 software, determining the dissociation rate constant ( $K_D$ ).

### Plasmid construction

The binding site for miR-21 was cloned into psiCHECK™-2 Vector (Promega), downstream the Renilla cDNA, by inverted PCR using the following oligonucleotides: 21MRE\_FW CTGA TAAGCTA AACCTAGAGCGGCCGCTGGC; 21MRE\_REV ACTG ATGTTGA GGCTCGAGCGATCGCCTAGAA. The resulting construct was named psi-R21.

### Lentivirus packaging and viral transduction

Lentiviral particles were produced by calcium phosphate transient transfection of HEK-293T cells with specific lentiviral plasmids (shGAPDH: TRCN0000445616 and shSCR: TRCN0000015937 from SIGMA-ALDRICH) together with the packaging plasmids (pLP1 and pLP2) and the envelope plasmid pLP/VSVG. For each lentiviral plasmid one 150 mm plate of HEK293T cells was used for transfection. After 14–16 hours the medium was replaced with complete medium supplemented with 1 mM sodium butyrate (SIGMA-ALDRICH). The collection of medium containing lentiviral particles occurs 48 h and 72 h after transfection; complete medium supplemented with sodium butyrate was added after the first collection. Collected media were pulled and centrifuged at 1000 rpm for 5 min at room temperature. The supernatant was filtered through 0.45  $\mu\text{m}$  pore nitrocellulose filters and then ultracentrifuged at 20 000 rpm for 2 hours at 4 °C. The pellet containing lentiviral particles was then

resuspended in 25  $\mu\text{L}$  HBSS buffer (GIBCO ThermoFisher) and stored at -80 °C.

1  $\times$  10<sup>6</sup> of HEK-293T cells were resuspended in 1 ml of serum-free and antibiotic-free medium supplemented with polybrene at 4  $\mu\text{g mL}^{-1}$  and infected with 5  $\mu\text{L}$  of the lentiviral particles in HBSS buffer. After 6 h one volume of medium with FBS 2% and penicillin/streptomycin 2 $\times$  was added and after additional 24 h the medium was replaced with complete medium. 48 h after viral transduction, HEK-293T\_shSCR and HEK-293T\_shGAPDH transduced cells were selected using 3  $\mu\text{g mL}^{-1}$  of puromycin (SIGMA-ALDRICH).

### RNA extraction and analysis

Total RNA was extracted using the Direct-zol RNA MiniPrep kit (Zymo Research) with on-column DNase treatment, according to the manufacturer's instructions. Reverse transcription was carried out with SuperScript VILO cDNA Synthesis Kit (Life Technologies) and the cDNA samples were analyzed by qRT-PCR using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). For miR-21 expression analysis total RNA was retrotranscribed using miRCURY® LNA® RT Kit (Qiagen) and the cDNA was analyzed by using miRCURY® LNA® miRNA PCR Assay (Qiagen). The oligonucleotides used are the following: GAPDH\_FW ACCCACTCCTCACCTTTGA; GAPDH\_REV TCCACCACCCCTGTTGCTGA; ATP5O\_FW ACTC GGGTTTGACCTACAGC; ATP5O\_REV GGTACTGAAGCATCGC ACCT; hsa-miR-21-5p miRCURY LNA miRNA PCR Assay (YP00204230); U6 snRNA (v2) miRCURY LNA miRNA PCR Assay (YP02119464).

### Cell thermal shift assay

HEK-293T cells were harvested in PBS supplemented with protease inhibitor cocktail (complete, EDTA-free, Roche). Cell suspensions were freeze-thawed three times in liquid nitrogen and then centrifuged (20 000g) for 10 min at 4 °C. The lysates were diluted at concentration of 2  $\mu\text{g mL}^{-1}$  and then treated with DMSO and 50  $\mu\text{M}$  of 33 or with increasing concentration of 33 for 30 min at 37 °C. After this incubation the lysates were divided into equal parts (50  $\mu\text{L}$ ) and heated individually at designated temperatures for 3 min. The heated samples were cooled at room temperature for 3 min and centrifuged (20 000g) for 10 min at 4 °C. The supernatants were transferred to new tubes and analyzed by use for western blotting.

### Western blotting

Lysates were loaded on 4–12% bis-tris-acrylamide gel (Life Technologies) and transferred to a nitrocellulose membrane. The membrane was blocked in 5% milk and hybridized with  $\alpha$ -TRBP (15753-1-AP Proteintech),  $\alpha$ -GAPDH (sc-25778, Santa Cruz Biotechnology),  $\alpha$ -c-PARP (Bioss Antibodies, BSM-52408R),  $\alpha$ -BCLXL (mAb #2764, Cell Signaling Technology) and  $\alpha$ - $\beta$ -actin (A3854, Sigma) antibodies. All the images were captured using the ChemiDocTM Touch Imaging System



(Bio-Rad), and the densitometric analyses were performed using the associated Image Lab software (Bio-Rad).

### Detection of hTOP1 and hTOP2 $\alpha$ cleavage complexes (TOP1ces and TOP2ccs) *in vivo*

After compound treatments,  $1 \times 10^6$  human cells in 35 mm dish per sample were washed with 1× phosphate-buffered saline (PBS) and lysed with 600  $\mu$ L of DNAzol (Invitrogen), followed by precipitation with 300  $\mu$ L of 200 proof ethanol. The nucleic acids were collected, washed with 75% ethanol, resuspended in 200  $\mu$ L of TE buffer, and then heated at 65 °C for 15 min, followed by shearing with sonication (40% output for 10 s pulse and 10 s rest for four times). The samples were centrifuged at 15 000 rpm for 5 min at 4 °C, and the supernatant were collected. 1  $\mu$ L of each sample was removed for spectrophotometric measurement of absorbance at 260 nm to quantitate DNA content (NanoDrop). 2  $\mu$ g of each sample was subjected to slot-blot for immunoblotting using anti-hTOP1 antibody (BD Pharmingen, 556597) and anti-hTOP2 $\alpha$  antibody (Santa Cruz biotechnology, 365916).<sup>51</sup>

### Abbreviation list

GI <sub>50</sub>	50% growth inhibition
HGSC	High-grade serous ovarian carcinoma
KO	Knock out
OC	Ovarian cancer
SMER	Small-molecule enhancer of miRNA
SPR	Surface plasmon resonance
Tops	Topoisomerases
TRBP	TAR RNA-binding protein
WT	Wild type

### Data availability

The data supporting this article have been included as part of the ESI.<sup>†</sup>

### Author contributions

Conceptualization: T. F., N. D. I., M. M., G. S., G. M.; validation: T. F., N. D. I., M. A. D. F., S. P., J. Y., Y. S.; formal analysis and investigation: T. F., N. D. I., D. P.; S. P., M. P., J. Y., Y. S., S. M., M. L. B., S. S., O. T., V. C., M. M.; resources: M. M., G. S., G. M.; writing – original draft: T. F., N. D. I.; writing – review & editing: T. F., N. D. I., M. A. D. F., V. C., F. W., Y. P., M. M., G. S., G. M.; supervision: V. C., F. W., Y. P., M. M., G. S., G. M.; project administration: M. M., G. S., G. M.

### Conflicts of 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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