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Ethyl 1,8-Naphthyridone-3-carboxylates Downregulate Human Papillomavirus-16 (HPV-16) E6 and E7 Oncogenes Expression

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ABSTRACT

Strong epidemiological and molecular data associate cervical cancer (CC) to high-risk human papillomavirus (HPV) infections. Carcinogenic mechanism depends mainly on the expression of E6 and E7 oncoproteins encoded by the viral genome. Using a cell-based high-throughput assay, an in-house library of compounds was screened identifying the 1,8-naphthyridone **1** that efficiently inhibited the transcription driven by the long control region of HPV genome. A series of analogues was then synthesized obtaining more potent derivatives able to downregulate E6 and E7 transcripts in HPV-16 positive CC CaSki cells. An unusual structural insight emerged for the C-3 position of the 1,8-naphthyridone core, where the ethyl carboxylate esters, but not the carboxylic acids, are responsible for the activity. In vitro uptake studies showed that the 3-ethyl carboxylates do not act as pro-drugs. The 1,8-naphthyridones emerged as valid starting points for the development of innovative agents potentially useful for the treatment of HPV-induced CC.

INTRODUCTION

Human papillomaviruses (HPV) are small double-stranded DNA viruses that infect the epithelial cells of the skin and the anogenital or oropharyngeal mucosa, causing benign and malignant neoplastic lesions. Infection with high-risk HPV types (e.g. HPV-16, HPV-18, HPV-31, and HPV-45) is a major cause of cervical cancer (CC), one of the most common types of gynecological malignancies among women worldwide[1,2] with more than 528,000 estimated new cases every year, and fourth for mortality with 266,000 deaths in 2012.[3,4] Epidemiologically, HPV-16 is the most prevalent in CC accounting for 65.2% of all genotypes.[5] Surgery is the current treatment for HPV-associated cancers, but the virus can persist in healthy tissues and favor tumor growth. Significant benefits in incidence of CC and patients survival have been achieved through screening programs that allow the detection of epithelial alterations and lesions in early stages, and, recently, through the introduction of prophylactic vaccines that prevent infections from four HPV types that cause about 70% of CCs (types 16 and 18) and 90% of genital warts (types 6 and 11) worldwide.[6]

However these achievements are insufficient and high mortality from CC still persists. Furthermore, the currently used therapy is non-specific with many associated toxicity problems[7,8] and no drugs directed against viral targets actually exist.

The continuous expression of E6 and E7 proteins encoded by high-risk HPVs plays a critical role in CC contributing to the maintenance of proliferation, to genomic instability, and to the transformation of primary human keratinocytes into malignant cells.[9-11] The physical and functional interaction of E6 and E7 oncoproteins with tumor suppressor proteins p53 and pRb leads to the subversion of cellular functions such as cell-cycle control, apoptosis, senescence, DNA repair, and genomic stability.[12-14] Since most HPV-induced tumors still contain wild-type p53 and pRb, the inhibition of E6 and E7 expression may rescue the tumor suppressor protein pathways, resulting in the inhibition of cell proliferation and induction of cellular senescence or apoptosis. Therefore, the two viral oncoproteins and their expression are considered the “Achilles’ heel” of CC and thus the ideal molecular targets for the development of innovative therapies. Selective silencing of E6 and E7 expression by macromolecule-based approaches such as rybozymes, antisense RNA and siRNA,[15-20] provided the proof of concept, but their use is limited by difficulties in delivering therapeutically active concentrations at specific anatomic sites. Ideally, the identification of small molecule inhibitors of E6 and E7 expression would facilitate the development of a drug against the HPV-induced CC. However, agents aimed at targeting directly or indirectly these CC-driven viral oncogenes are in very early stages of development.[21-23]

The expression of HPV-16 E6 and E7 is controlled by the long control region (LCR), a complex regulatory DNA sequence located immediately upstream of the E6 gene in the HPV genome.[14,24] In a previous paper, we described the development of a cell-based high-throughput assay to screen small molecules as inhibitors of HPV-16 LCR transcriptional activity.[25]

In the present study, this assay was used to screen a set of compounds selected within a proprietary library. Compound **1** (Table 1) emerged as a very promising inhibitor of E6 and E7 oncogene expression prompting the design, synthesis, and biological characterization of a series of analogues.

HIT IDENTIFICATION

To identify small molecule inhibitors of HPV-16 LCR transcriptional activity, we focused the attention on quinolone-based derivatives thanks to their capability to inhibit the transcription processes of different viruses. The ability of the 6-desfluoroquinolones (6-DFQs) to inhibit human immunodeficiency virus type 1 (HIV-1) gene expression by selectively interfering with the Tat-mediated transcription step is well documented,[26-29] as well as their anti-human cytomegalovirus (HCMV) activity ascribable to the specific inhibition of the immediate early 2-mediated transactivation of viral promoters.[30,31] In particular, 22 compounds (Table 1) were selected including mainly anti-HIV and anti-HCMV quinolones and few intermediates (compounds **1-18**), together with two antibacterial quinolones (compounds **19** and **20**), and two anti hepatitis C virus acridones (compounds **21** and **22**).

The ability of the compounds to inhibit the HPV-16 LCR-driven transcription was assayed using P21 cells that are a clone of keratinocytes stably transfected with a reporter construct containing the HPV-16 LCR positioned upstream from the firefly luciferase gene.[25] The cells were initially treated with two different concentrations of compounds (100 μM and 30 μM) and then assayed for luciferase activity reported as the percentage of LCR inhibition (Table S1). Many of the compounds were essentially inactive even at 100 μM while others showed a weak inhibitory activity at 100 μM but were inactive at 30 μM. However, compound **1** exhibited a high percentage of inhibition both at 100 μM (98.8%) and at the lower concentration of 30 μM (92%). TGF-β1 and IL-4, known inhibitors of HPV-16 LCR activity used as internal references,[32,33] reduced the LCR activity by 70.5 % and 69.8 %, respectively, when used at 50 ng/mL (data not shown).

When assayed in P21 cells at different concentrations (between 0.01 and 100 μM), compound **1** inhibited the LCR transcriptional activity in a concentration-dependent manner with an IC_{50} value of 1.12 μM (Figure 1). Compound **1** did not show any antiproliferative activity on the same cell line at 100 μM concentration, indicating that the inhibition of the LCR activity was not a consequence of a reduced cell viability.

STRUCTURAL MODIFICATIONS OF COMPOUND 1

According to the IC_{50} value, compound **1** induced potent inhibition of the LCR activity without showing appreciable toxicity, prompting the design and synthesis of additional derivatives in order to identify the structural features responsible for this ability. The activity of **1**, the only 1,8-naphthyridone derivative assayed within the initial set of compounds, was quite unexpected. Indeed, it bears an unusual benzyl moiety at the N-1 position but most importantly, it is a C-3 ester derivative. In fact, the vast majority of the 6-fluoroquinolones and 6-DFQs until now reported as antibacterial,[34-39] including antitubercular,[40-42] anti-HIV,[26] and anti-HCMV agents,[30,31,43] are characterized by a carboxylic acid group at the C-3 position. Of note, compound **3** (Table 1), the other ester derivative tested, did not show any inhibitory activity.

Starting from the hit **1**, a first set of naphthyridone analogues was designed maintaining both the ethyl carboxylate group and the 3-chloro-2-fluorobenzyl moiety at C-3 and N-1 position, respectively, while modifying the 2-pyridilpiperazine ring at the C-7 position (Figure 2). In particular, the pyridine moiety was replaced by some of the heterocyclic bases that usually impart antiviral activity to the quinolone compounds, such as 1,3-benzothiazole, quinoline, 1,3-thiazole, 1,3-benzoxazole, and 3-trifluoromethylphenyl rings, synthesizing derivatives **23-27**. With the aim to investigate the role played by the C-3 ester moiety, the corresponding acid derivatives (compounds **29-33**) were also synthesized together with compound **28**, the acid counterpart of the hit **1**.

The biological evaluation of this first set of naphthyridone analogues led to the identification of the 7-benzothiazolpiperazine derivative **23** as an extremely interesting new molecule, which was five times more active than compound **1** while maintaining the absence of cytotoxicity up to the maximum concentration tested of 100 μM. Thus, a second set of compounds was designed starting from the new hit **23**, keeping fixed the 1,3-benzothiazolpiperazine moiety at the C-7 position while varying the N-1 substituent (Figure 2). In particular, the role of the halogens on the benzyl group was studied by synthesizing compounds lacking the 3-chloro (compound **34**), the 2-fluoro

(compound **35**), and both the halogens (compound **36**). To explore the C-4 position of the N-1 benzyl ring, the chlorine derivative **37** as well as the 4-trifluoromethyl derivative **38** were prepared. Then, the 3-chloro-2-fluorophenyl moiety was replaced by various heteroaromatic rings, such as 2-pyridine (compound **39**), 2-furan (compound **40**), 5-indole (compound **41**), and 2-benzoimidazole (compound **42**). Finally, the aromatic ring of compounds **34**, **37**, and **39** was spaced from the 1,8-naphthyridone scaffold by inserting an additional methylene unit obtaining compounds **43-45**. Few additional 3-carboxylic acid analogues (compounds **46-49**) were synthesized within this second set of compounds.

CHEMISTRY

All the 1,8-naphthyridones synthesized in this study were prepared following the cycloaracylation procedure, as depicted in Scheme 1. Thus, acrylate **50**[44] was reacted with variously substituted arylalkylamines in Et_2O/EtOH mixture, to give intermediates **51-63**, which were then cyclized in the presence of K_2CO_3 in DMF to give synthones **64-76**.

The nucleophilic reaction of N-1 3-chloro-2-fluorobenzyl naphthyridone **64** with selected arylpiperazines gave ester derivatives **1** and **23-27** that were then hydrolysed in basic conditions to the corresponding acids **28-33**. On the other hand, the nucleophilic reaction of 7-chloro synthones **65**, **66**, **67**,[45] and **68-76**, differently functionalized at the N-1 position, with 2-(piperazin-1-yl)benzothiazole[46] furnished the ester derivatives **34-45**, of which, compounds **36**, **37**, **39**, and **45**, were then hydrolysed to the corresponding acids **46-49**.

RESULTS AND DISCUSSION

All the new synthesized compounds were initially assayed for their ability to inhibit HPV-16 LCR activity at 100 µM and 30 µM concentrations in P21 cells. Unfortunately, the poor solubility of compounds **32** and **47** in DMSO hampered their biological evaluation. In addition, compounds **27**,

33, **40**, and **43** caused a noticeable alteration of P21 cultured cells, thus preventing their evaluation in the luciferase assay.

Analyzing the data summarized in Table 2, it clearly emerged that many of the new derivatives were active in inhibiting the LCR activity at both the concentrations tested. By selecting the compounds with the higher percentage of inhibition, the IC_{50} value was determined for 14 derivatives. They reduced the LCR activity in a concentration-dependent fashion (data not shown) with IC_{50} values ranging from 0.13 to 10.90 µM (Table 2). In parallel, the compounds were also tested for the antiproliferative activity in the same cell line. In a few cases they exhibited some cytotoxicity but always at concentrations higher than those producing LCR inhibition. Comparing the biological data obtained for the 14 derivatives with those of compound **1**, some structural considerations can be highlighted. The modifications made at the C-7 position in most cases were very productive improving the activity as in compounds **23**, **24**, and **26**. In particular, the benzothiazolpiperazine derivative **23** was endowed with the best profile showing an IC_{50} = 0.26 µM that, coupled with the lack of cytotoxicity (CC_{50} > 100 µM ; CC_{50} is the concentration of compound that reduced cellular viability by 50%) led to a selectivity index (SI, ratio of CC_{50} to IC_{50}) > 385. A similar behaviour was exhibited by its 4-benzoxazol bioisoster **26**. A comparable potency was shown by compound **24** (IC_{50} = 0.21 µM), even if coupled with some cytotoxicity (CC_{50} = 59.3 µM). The thiazolpiperazine appeared a less suitable C-7 substituent, since compound **25** showed the same activity (IC_{50} = 1.21 µM) but a markedly higher antiproliferative effect (CC_{50} = 25.3 µM) than that of compound **1**. Unfortunately, the high toxicity of compound **27** hampered the evaluation of the contribute of the 3-(trifluoromethyl)phenylpiperazine.

Electing **23** as a new hit compound, the modifications then involved the 3-chloro-2-fluorobenzyl group at the N-1 position. The removal of the chlorine (compound **34**) or the fluorine (compound **35**) atom, proved to be unsuccessful. On the contrary, compound **36**, lacking both the halogens, exhibited the same good activity (IC_{50} = 0.23 µM) of compound **23** (IC_{50} = 0.26 µM) although coupled with a higher toxicity (CC_{50} = 61.7 µM). This data suggested that the presence of both

halogens is important to improve the selectivity. The presence of a substituent at the para position of the benzyl group decreased the activity as in the *p*-chloro derivative **37** (IC_{50} value of 2.37 μM) and even more in the *p*-trifluoromethyl derivative **38** (IC_{50} = 10.90 μM).

The insertion of various heteroaromatic rings at the N-1 position instead of the 3-chloro-2-fluorophenyl moiety gave contrasting results. While the presence of furan (compound **40**), 2-benzoimidazole (compound **42**), and 2-pyridine (compound **39**) were not fruitful, the 5-indole moiety markedly improved the activity. Indeed, with an IC_{50} = 0.13 μM, derivative **41** emerged as the most active and endowed with the highest SI value (> 769). Finally, the spacing of the *o*-fluorobenzyl, *p*-chlorobenzyl, and pyridine moieties from the N-1 position by an additional methylenic unit was not productive, being derivatives **43-45** inactive or toxic.

For some of the 3-ethyl carboxylate target compounds, the corresponding acids were also synthesized and tested, permitting to obtain clear SAR insights for the C-3 position. Analyzing the data reported in Table 2 for the ester (**1**, **23-26**, **36**, **39**, and **45**)/acids (**28-31**, **48**, and **49**) pairs, the ester derivatives always resulted in more potent activity, while some 3-carboxylic acids were even completely inactive. This is true also for compound **28**, the acid analogue of the hit **1**.

Since the end-point of the primary luciferase assay used for hits identification is measured enzymatically, a subsequent confirmatory assay was run to eliminate potential false positives caused by luciferase inhibition, a possibility reported by previous literature.[47] Therefore, in the next step of the study, we tested the ability of the molecules to repress HPV-16 oncogene transcription in the HPV-16-positive cervical carcinoma-derived CaSki cell line. In particular, we evaluated the effect of the molecules that showed the best biological profile (compounds **1**, **23-26**, **35-37**, and **41**), together with their corresponding 3-carboxylic acids (compounds **28-31** and **46**) on HPV-16 E6 and E7 mRNA levels. To this aim, the cells were treated with 10 μM of compounds, a concentration that reduced the luciferase activity more than 80%, for 24 hours, and then the levels of E6 and E7 mRNAs were examined by RT-PCR. Figure 3 shows that, with the exception of compound **37**, all the 1,8-naphthyridone 3-ethyl esters downregulated, in a statistically significant manner, the

expression of E6 and E7 transcripts, though to a different extent. In particular, quinolinylpiperazine derivative **24** and 1,3-thiazolylpiperazine derivative **25** strongly suppressed HPV transcription inhibiting E6 mRNA levels by 61.5% and 71.7%, and E7 mRNA levels by 57.4% and 72.7%, respectively. TGF-β1,[32,33] used as a positive control, showed 65.3% and 60.7% of inhibition of the E6 and E7 transcripts, respectively. Of note, none of the compounds tested showed any antiproliferative effect on CaSki cells at the concentration and time point chosen for the RT-PCR (data not shown). Confirming the data measured in the luciferase assay, all the tested 3-carboxylic acids failed to inhibit E6 and E7 mRNA levels. Overall, the secondary screening based on the RT-PCR assay, independently confirmed the hit compounds identified by the primary assay and put forward the luciferase-based assay as a reliable tool for the initial identification of candidate small molecules capable of downregulating HPV-16 oncogene expression in HPV-16-positive human cancer cells.

Since the quinolone derivatives are able to inhibit the transcription processes of different viruses, depending on the substitution patterns, we decided to investigate the selectivity of the derivatives herein identified. Thus, hit compound **23** was tested against the DNA virus herpes simplex type 2 (HSV-2) and some RNA viruses namely HIV-1, vesicular stomatitis virus, respiratory syncytial virus, and human rotavirus (VSV, RSV, and HRV, respectively). The antiviral assays showed that compound **23** is inactive against HIV-1 and HRV, barely active against RSV, while it exerts a moderate activity against HSV-2 and VSV (see Table S2).

As mentioned before, the greater activity of the 3-ethyl ester derivatives compared to the acid analogues is quite unusual in the quinolone field, since the majority of the biologically active quinolone-based compounds bear a carboxylic acid group at the C-3 position.[48] Its key role in the mechanism of action of the antibacterial quinolone drugs, where a Mg^{2+} bridge is formed between the quinolone 4-keto-3-carboxyl moiety and the DNA phosphodiester backbone, has been extensively reported.[49-50] Most of the modifications of the carboxylic acid moiety produced no

active compounds[51] with few exceptions, such as certain carboxylate esters which are converted in vivo back to a carboxylic acid.[52]

To investigate the role played by the C-3 substituent in the naphthyridones targeting HPV E6 and E7 oncogene expression, we performed an uptake study to understand whether the higher activity of the ethyl 3-carboxylates might be due to a better intracellular penetration and to clarify whether they are the active forms in the cellular context. To this end, CaSki cells were treated with three pairs of derivatives, the esters **23-25**, selected among the most active compounds in real-time PCR assays, and the corresponding acid derivatives, **29-31**, at fixed concentrations and different times of incubation up to 16 h. After incubation with compounds, cells were washed twice with Phosphate Buffered Saline (PBS) and lysed with a solution containing an excess of ammonium sulfate; then cell lysates were subjected to quantitative chromatographic analyses as described in experimental section.

As shown in Figure 4, after cell incubation with acid derivatives, a marked uptake (about 20%) was detected by the first hour. The intracellular percentage of the acid derivative increased over time for all the compounds and reached 35%, 38%, and 63%, for **29**, **30**, and **31**, respectively, after 16 h. These results clearly demonstrated that the acids are able to penetrate CaSki cells, and thus their inactivity is most likely due to the unsuitability of the 3-carboxylic group to properly interact with the specific target. The results obtained with ester derivatives **23-25** showed a different behavior. Indeed, after 1 h of incubation no carboxylate ester was detected in cell lysates. The intracellular amount of all the esters tested increased over time reaching 15%, 40%, and 28% for compounds **23**, **24**, and **25**, respectively, after 16 h. Interestingly, when the cells were incubated with ester derivatives, a remarkable intracellular percentage of the acid counterpart was detected at each time point in the chromatogram, reaching 30%, 52%, and 70%, for compounds **23**, **24**, and **25**, respectively, at 16 h. These data suggest that the cell uptake of ester derivatives seems favored but, as soon as they are internalized, the metabolism to the corresponding acid forms occurs.

Considering that the ester amount inside the cells increases over time while only the acid counterpart is initially detectable, the enzymatic hydrolysis appears as a saturable process.

In summary, the uptake studies show that both acid and ester derivatives are able to cross the cell membranes and the ethyl 1,8-naphthyridone-3-carboxylates are not pro-drugs but they are the biologically active form. Actually, since the acids are inactive although they are capable of permeating CaSki cells, the ethyl esters seem the sole active forms. Most likely, they would be even more potent if they were not so extensively metabolized. The synthesis of different naphthyridone ester derivatives less prone to the enzymatic hydrolysis is required to validate this hypothesis.

CONCLUSIONS

Although the prophylactic vaccines recently introduced prevent the infections from HPV type 16 and 18, high mortality from CC still persists. Furthermore, the currently used therapeutic approach is non-specific and associated to toxicity problems and no drugs directed against specific viral targets actually exist. Thus, the medical need for new anti-HPV drugs is still urgent with a preference for small molecules targeting viral components. The 1,8-naphthyridone derivatives identified in this study could meet this urgent request. Indeed, when properly functionalized at the N-1, C-3 and C-7 positions, they are able to inhibit the expression of HPV-16 E6 and E7 oncogenes at subcytotoxic concentrations. The best compounds are characterized by a 3-chloro-2-fluorobenzyl moiety at the N-1 position coupled with a quinolinpiperazine (compound **24**) or a 1,3-thiazolpiperazine (compound **25**) at the C-7 position. An interesting SAR insight emerged for the C-3 position of the 1,8-naphthyridone scaffold, where, in contrast to most of the biologically active quinolones, an ethyl carboxylate ester, instead of the usual 3-carboxylic moiety, is crucial for the activity. However, a few recent publications reporting quinolone carboxylate esters endowed with different biological activity are emerging.[53-56]

Uptake studies performed on 1,8-naphthyridone acid/ester pairs, clearly showed that the ethyl esters are the active forms responsible for the anti-HPV activity and do not act as pro-drugs.

Within the limited landscape of compounds that target the oncogenic effects of HPV E6 and E7,[21-23,57] the quinolone-based derivatives herein identified are a rare example of small molecules able to downregulate the expression of CC-driven players E6 and E7. Although particularly promising, preliminary studies showed that they are not potent enough to significantly restore the cell growth control and induce the senescent phenotype in HPV-16 positive CC cell lines (data not shown). Thus, these hit compounds ask for further structural optimization, which is mandatory to achieve more potent analogues able to rescue p53- and Rb-dependent responses and thus potentially useful for the treatment of CC as well as of others HPV-induced anogenital and oropharyngeal cancers.

EXPERIMENTAL SECTION

Chemistry. All reactions were routinely checked by TLC on silica gel $60F_{254}$ (Merck) and visualized by using UV or iodine. Flash column chromatography separations were carried out on Merck silica gel 60 (mesh 230-400). Melting points were determined in capillary tubes (Büchi Electrothermal Mod. 9100) and are uncorrected. The purity of the compounds was determined by combustion analysis employing a Fisons elemental analyzer, model EA1108CHN, and data for C, H, and N are within 0.4% of the theoretical values (purity of ≥ 95%). ^{1}H NMR and ^{13}C NMR spectra were recorded at 200 MHz (Bruker Avance DPX-200) and 400 MHz (Bruker Avance DRX-400) using residual solvents such as chloroform (δ = 7.26) or dimethylsulfoxide (δ = 2.48) as an internal standard. Chemical shifts are given in ppm (δ) and the spectral data are consistent with the assigned structures. For the microwave synthesis, a Biotage Initiator Sixty (400 W) was used. Reagents and solvents were purchased from common commercial suppliers and were used as such. After extraction, organic solutions were dried over anhydrous Na_2SO_4, filtered, and concentrated with a Büchi rotary evaporator at reduced pressure. Yields are of purified product and were not optimized. All starting materials were commercially available unless otherwise indicated.

Preparation of ethyl 3-[(3-chloro-2-fluorobenzyl)amino]-2-[(2,6-dichloropyridin-3-yl)carbonyl]acrylate (51, Method A). A mixture of ethyl 2-[(2,6-dichloropyridin-3-yl)carbonyl]-3-

(dimethylamino)acrylate (**50**)[44] (1.0 g, 3.1 mmol) and (3-chloro-2-fluorophenyl)methanamine (0.6 g, 3.8 mmol) in Et_2O/EtOH (4:1, 15 mL), was stirred at room temperature for 2 h. The reaction mixture was then evaporated to dryness to give a reside, which after treatment with cyclohexane gave a solid, which was filtered to give **51** (1.27 g, 95%): mp 155-156 °C; ^{1}H-NMR (DMSO-d_6) δ 0.80 (t, J = 7.1 Hz, 3H, CH_2*CH_3*), 3.75 (q, J = 7.1 Hz, 2H, *CH_2*CH_3), 4.75 (bs, 2H, CH_2), 7.20-7.45 (m, 2H, aromatic CH), 7.50-7.60 (m, 2H, H-5'and aromatic CH), 7.75 (d, J = 7.9 Hz, 1H, H-4'), 8.35 (d, J = 13.8 Hz, 1H, H-3), 11.00-11.10 (m, 1H, NH).

Ethyl 2-[(2,6-dichloropyridin-3-yl)carbonyl]-3-[(2-fluorobenzyl)amino]acrylate (52). The title compound was prepared starting from **50**[44] through method A (20 min), using (2-fluorobenzyl)amine, in 75% yield: mp 161-162 °C; ^{1}H-NMR (DMSO-d_6) 0.68 and 0.85 (each t, J = 7.1 Hz, 0.65 and 2.35 H, CH_2*CH_3*), 3.80 (q, J = 7.1 Hz, 2H, *CH_2*CH_3), 4.60 (bd, J = 6.0 Hz, 2H, CH_2), 7.10-7.45 (m, 4H, aromatic CH), 7.51 (d, J = 8.0 Hz, 1H, H-4'), 7.75 (d, J = 8.0 Hz, 1H, H-5'), 8.30 (d, J = 14.0 Hz, 1H, H-3), 9.80-9.95 and 11.00-11.20 (each m, 0.2 and 0.8 H, NH).

Ethyl 3-[(3-chlorobenzyl)amino]-2-[(2,6-dichloropyridin-3-yl)carbonyl]acrylate (53). The title compound was prepared starting from **50**[44] through method A (40 min), using (3-chlorobenzyl)amine, in 77% yield: mp 156-157 °C; ^{1}H-NMR (DMSO-d_6) 0.75 and 0.90 (each t, J = 7.1 Hz, 0.7 and 2.3 H, CH_2*CH_3*), 3.80-3.90 (m, 2H, *CH_2*CH_3), 4.70-4.75 (m, 2H, CH_2), 7.32-7.50 (m, 4H, aromatic CH), 7.55 (d, J = 8.0 Hz, 1H, H-5'), 7.80 (d, J = 8.0 Hz, 1H, H-4'), 8.40 (d, J = 14.0 Hz, 1H, H-3), 9.95-10.00 and 11.10-11.15 (each m, 0.25 and 0.75 H, NH).

Ethyl 3-(benzylamino)-2-[(2,6-dichloropyridin-3-yl)carbonyl]acrylate (54). The title compound was prepared starting from **50**[44] through Method A (40 min), using 1-phenylmethanamine, in 70% yield: mp 123-124 °C; ^{1}H-NMR (DMSO-d_6) 0.65 and 0.80 (each t, J = 7.1 Hz, 0.77 and 2.23 H, CH_2*CH_3*), 3.75-3.80 (m, 2H, *CH_2*CH_3), 4.65-4.75 (m, 2H, CH_2), 7.25-7.40 (m, 5H, aromatic CH), 7.55 (d, J =8.0 Hz, 1H, H-5'), 7.75 (d, J = 8.0 Hz, 1H, H-4'), 8.30 (d, J = 14.6 Hz, 1H, H-3), 9.85-10.00 and 11.00-11.10 (each m, 0.22 and 0.78H, NH).

Ethyl 3-[(4-chlorobenzyl)amino]-2-[(2,6-dichloropyridin-3-yl)carbonyl]acrylate (55). The title compound was prepared starting from **50**[44] through Method A (1 h), using (4-chlorobenzyl)amine hydrochloride, in 66% yield: mp 135-136 °C; ^{1}H-NMR (DMSO-d_6) 0.65 and 0.85 (each t, J = 7.1, 1 and 2H, CH_2*CH_3*), 3.75-3.90 (m, 2H, *CH_2*CH_3), 4.65 (bd, J = 6.0 Hz, 2H, CH_2), 7.30-7.45 (m, 4H, aromatic CH), 7.50 (d, J = 8.0 Hz, 1H, H-5'), 7.70 (d, J = 8.0 Hz, 1H, H-4'), 8.30 (d, J = 14.0 Hz, 1H, H-3), 9.80-10.00 and 11.00-11.20 (each m, 0.3 and 0.7H, NH).

Ethyl 2-[(2,6-dichloropyridin-3-yl)carbonyl]-3-{[4-(trifluoromethyl)benzyl]amino}acrylate (56). The title compound was prepared starting from **50**[44] through Method A (6 h), using [4-(trifluoromethyl)benzyl]amine, in 90% yield: mp 110-112 °C; ^{1}H-NMR (DMSO-d_6) 0.75 and 0.90 (each t, J = 7.1 Hz, 0.8 and 2.2H, CH_2*CH_3*), 3.85-3.90 (m, 2H, *CH_2*CH_3), 4.80 (bd, J = 5.8 Hz, 2H, CH_2), 7.55-7.65 (m, 3H, H-5' and aromatic CH), 7.70-7.80 (m, 3H, H-4' and aromatic CH), 8.40 (d, J = 14.5 Hz, 1H, H-3), 10.00-10.05 and 11.10-11.20 (each m, 0.3 and 0.7H, NH).

Ethyl 2-[(2,6-dichloropyridin-3-yl)carbonyl]-3-[(pyridin-2-ylmethyl)amino]acrylate (57). The title compound was prepared starting from **50**[44] through Method A (4 h), using (pyridin-2-ylmethyl)amine, in 91% yield: mp 157-158 °C; ^{1}H-NMR (DMSO-d_6) δ 0.75 and 0.90 (each t, J = 7.0 Hz, 1 and 2H, CH_2*CH_3*), 3.80 (q, J = 7.0 Hz, 2H, *CH_2*CH_3), 4.80 (bs, 2H, CH_2), 7.25-7.35 (m, 2H, pyridine CH), 7.55 (d, J = 8.0 Hz, 1H, H-5'), 7.60-7.75 (m, 2H, H-4'and pyridine CH), 8.40 (d, J = 14.4 Hz, 1H, H-3), 8.45-8.55 (m, 1H, pyridine CH), 9.90-10.00 and 11.20-11.30 (each m, 0.2 and 0.8H, NH).

Ethyl 2-[(2,6-dichloropyridin-3-yl)carbonyl]-3-[(2-furylmethyl)amino]acrylate (58). The title compound was prepared starting from **50**[44] through Method A (30 min), using (2-furylmethyl)amine, in 87% yield: mp 132-134 °C; ^{1}H-NMR (DMSO-d_6) 0.85 (t, J = 7.0 Hz, 3H, CH_2*CH_3*), 3.80 (q, J = 7.0 Hz, 2H, *CH_2*CH_3), 4.70 (bs, 2H, CH_2), 6.30-6.45 (m, 2H, furan CH), 7.55 (d, J = 12.0 Hz, 1H, H-5'), 7.60-7.65 (m, 1H, furan CH), 7.70-7.80 (m, 1H, H-4'), 8.25 (d, J = 14.7 Hz, 1H, H-3), 10.90-11.00 (m, 1H, NH).

Ethyl 2-[(2,6-dichloropyridin-3-yl)carbonyl]-3-[(1*H*-indol-5-ylmethyl)amino]acrylate (59). The title compound was prepared starting from **50**[44] through Method A (1.2 h), using (1*H*-indol-5-ylmethyl)amine, in 96% yield: mp 141-144 °C; ^{1}H-NMR (DMSO-d_6) 0.85 and 0.90 (each t, J = 7.0 Hz, 0.7 and 2.3H, CH_2CH_3), 3.80 (q, J = 7.0 Hz, 2H, CH_2CH_3), 4.70 (bs, 2H, CH_2), 6.35-6.45 and 7.0-7.10 (m, each 1H, indol CH), 7.20-7.40 (m, 2H, indol CH), 7.40-7.55 (m, 2H, H-5' and indol CH), 7.75 (d, J = 8.0 Hz 1H, H-4'), 8.30 (d, J = 14.4 Hz, 1H, H-3), 11.10 (bs, 2H, NH and indol NH).

Ethyl 3-[(1*H*-benzimidazol-2-ylmethyl)amino]-2-[(2,6-dichloropyridin-3-yl)carbonyl]acrylate (60). The title compound was prepared starting from **50**[44] through Method A (12 h), using (1*H*-benzimidazol-2-ylmethyl)amine, with the exception that the compound was further purified by flash chromatography eluting with MeOH/$CHCl_3$ (3%), in 67% yield: mp 110-113 °C; ^{1}H-NMR (DMSO-d_6) 0.85 and 1.00 (each t, J = 7.0 Hz, 3H, CH_2CH_3), 3.80 (q, J = 7.0 Hz, 2H, CH_2CH_3), 5.0 (bs, 2H, CH_2), 7.10-7.25 (m, 2H, benzimidazole CH), 7.50-7.60 (m, 3H, H-5' and benzimidazole CH), 7.75-7.80 (m, 1H, H-4'), 8.45 (d, J = 14.4 Hz, 1H, H-3), 11.05-11.10 (m, 1H, NH), 12.20 (bs, 1H, benzimidazole NH).

Ethyl 2-[(2,6-dichloropyridin-3-yl)carbonyl]-3-{[2-(2-fluorophenyl)ethyl]amino}acrylate (61). The title compound was prepared starting from **50**[44] through Method A (50 min), using [2-(2-fluorophenyl)ethyl]amine, in 75% yield: mp 61-62 °C; ^{1}H-NMR (DMSO-d_6) 0.75 and 0.90 (each t, J = 7.1 Hz, 0.6 and 2.4 H, CH_2CH_3), 2.80-3.10 (m, 2H, CH_2), 3.70-3.75 (m, 2H, NCH_2), 3.80-3.90 (m, 2H, CH_2CH_3), 7.10-7.20 and 7.25-7.35 (m, each 2H, aromatic CH), 7.55 (d, J = 8.0 Hz, 1H, H-5'), 7.70 (d, J = 8.0 Hz, 1H, H-4'), 8.00 (d, J = 14.0 Hz, 1H, H-3), 9.60-9.70 and 10.80-10.90 (each m, 0.2 and 0.8H, NH).

Ethyl 3-{[2-(4-chlorophenyl)ethyl]amino}-2-[(2,6-dichloropyridin-3-yl)carbonyl]acrylate (62). The title compound was prepared starting from **50**[44] through Method A (1 h), using [2-(4-chlorophenyl)ethyl]amine, in 62% yield: mp 128-130 °C; ^{1}H-NMR (DMSO-d_6) 0.65 and 0.85 (each t, J = 7.0 Hz, 0.5 and 2.5 H, CH_2CH_3), 2.85 (t, J =6.5 Hz, 2H, CH_2), 3.60-3.70 (m, 2H, NCH_2), 3.80

(q, J = 8.0 Hz, 2H, CH_2CH$_3$), 7.20-7.25 and 7.30-7.35 (m, each 2H, aromatic CH), 7.70 (d, J = 8.0 Hz, 1H, H-5'), 7.50 (d, J = 8.0 Hz, 1H, H-4'), 8.00 (d, J = 14.0 Hz, 1H, H-3), 10.75-10.85 (m, 1H, NH).

Ethyl 2-[(2,6-dichloropyridin-3-yl)carbonyl]-3-[(2-pyridin-2-ylethyl)amino]acrylate (63). The title compound was prepared starting from **50**[44] through Method A (4 h) using (2-pyridin-2-ylethyl)amine, in 74% yield: mp 118-122 °C; ^{1}H-NMR (CDCl$_3$) δ 0.80-1.00 (m, 3H, CH$_2$$CH_3$), 3.10-3.20 (m, 2H, CH$_2$), 3.90-4.00 (m, 4H, CH_2CH$_3$ and NCH$_2$), 7.10-7.25 (m, 3H, pyridine CH), 7.45 (d, J = 8.2 Hz, H-5'), 7.60 (t, J = 7.3 Hz, pyridine CH), 8.15 (d, J = 14.0 Hz, 1H, H-3), 8.60-8.70 (m, 1H, H-4'), 9.95-10.00 and 11.20-11.25 (each m, 1H, NH).

Preparation of ethyl 7-chloro-1-(3-chloro-2-fluorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (64, Method B). A mixture of **51** (1.2 g, 2.9 mmol) and K$_2$CO$_3$ (1.2 g, 8.8 mmol) in DMF (15 mL) was maintained at 60 °C for 4 h. After cooling the reaction mixture was poured into ice/water, obtaining a precipitate which was filtered, washed with water and treated with Et$_2$O, to give **64** (1.1 g, 96%); mp 191-192 °C; ^{1}H-NMR (CDCl$_3$) δ 1.45 (t, J = 6.8 Hz, 3H, CH$_2$$CH_3$), 4.45 (q, J = 6.8 Hz, 2H, CH_2CH$_3$), 5.60 (bs, 2H, CH$_2$), 7.10-7.20 (m, 1H, aromatic CH), 7.35-7.45 (m, 3H, H-6 and aromatic CH), 8.70-8.80 (m, 2H, H-2 and H-5).

Ethyl 7-chloro-1-(2-fluorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (65). The title compound was prepared from **52** through method B (1 h) in 87% yield: mp 155-156 °C; ^{1}H-NMR (DMSO-d_6) δ 1.20 (t, J =7.0 Hz, 3H, CH$_2$$CH_3$), 4.20 (q, J =7.0 Hz, 2H, CH_2CH$_3$), 5.60 (s, 2H, CH$_2$), 7.00-7.35 (m, 4H, aromatic CH), 7.50 (d, J = 8.3 Hz, 1H, H-6), 8.48 (d, J = 8.3 Hz, 1H, H-5), 8.90 (s, 1H, H-2).

Ethyl 7-chloro-1-(3-chlorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (66). The title compound was prepared from **53** through method B (1 h) in 90% yield: mp 165-167 °C; ^{1}H-NMR (DMSO-d_6) δ 1.28 (t, J = 7.0 Hz, 3H, CH$_2$$CH_3$), 4.25 (q, J = 7.0 Hz, 2H, CH_2CH$_3$), 5.60 (s, 1H, CH$_2$), 7.25-7.40 (m, 3H, aromatic CH), 7.50 (bs, 1H, aromatic CH), 7.60 (d, J = 8.2 Hz, 1H, H-6), 8.55 (d, J = 8.2 Hz, 1H, H-5), 9.00 (s, 1H, H-2).

Ethyl 1-benzyl-7-chloro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (67).[45] The title compound was prepared from **54** through method B (2 h) in 80 % yield: mp 136-138; ^{1}H-NMR (DMSO-d_6) δ 1.23 (t, *J* = 7 Hz, 3H, CH_2*CH_3*), 4.20 (q, *J* =7 Hz, 2H, *CH_2*CH_3), 7.18-7.35 (m, 5H, aromatic CH), 7.55 (d, *J* =8.3 Hz, 1H, H-6), 8.45 (d, *J* =8.3 Hz, 1H, H-5), 8.90 (s, 1H, H-2).

Ethyl 7-chloro-1-(4-chlorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (68). The title compound was prepared from **55** through Method B (1.5 h) in 55% yield: mp 157-158 °C; ^{1}H-NMR ($CDCl_3$) δ 1.40 (t, *J* = 7.1 Hz, 3H, CH_2*CH_3*), 4.45 (q, *J* = 7.1 Hz, 2H, *CH_2*CH_3), 5.50 (s, 2H, CH_2), 7.30-7.40 (m, 5H, aromatic CH and H-6), 8.65-8.70 (m, 2H, H-5 and H-2).

Ethyl 7-chloro-4-oxo-1-[4-(trifluoromethyl)benzyl]-1,4-dihydro-1,8-naphthyridine-3-carboxylate (69). The title compound was prepared from **56** through Method B (4.5 h), with the exception that the solid obtained was purified by chromatography column eluting with MeOH/$CHCl_3$ (2%), to give **69** in 45 % yield: mp 191-192 °C; ^{1}H-NMR (DMSO-d_6) δ 1.28 (t, *J* = 7.1 Hz, 3H, CH_2*CH_3*), 4.24 (q, *J* = 7.1 Hz, 2H, *CH_2*CH_3), 5.70 (s, 2H, CH_2), 7.53 (d, *J* = 8.1 Hz, 2H, aromatic CH), 7.60 (d, *J* = 8.2 Hz, 1H, H-6), 7.70 (d, *J* = 8.1 Hz, 2H, aromatic CH), 8.55 (d, *J* = 8.2 Hz, 1H, H-5), 9.00 (s, 1H, H-2).

Ethyl 7-chloro-4-oxo-1-(pyridin-2-ylmethyl)-1,4-dihydro-1,8-naphthyridine-3-carboxylate (70). The title compound was prepared from **57** through Method B (4 h), in 81% yield: mp 128-129 °C; ^{1}H-NMR ($CDCl_3$) δ 1.50 (t, *J* = 6.8 Hz, 3H, CH_2*CH_3*), 4.45 (q, *J* = 6.8 Hz, 2H, *CH_2*CH_3), 5.75 (bs, 2H, CH_2), 7.35-7.50 (m, 3H, H-6 and pyridine CH), 7.70-7.80 and 8.55-8.65 (m, each 1H, pyridine CH), 8.75 (d, *J* = 8.1 Hz, 1H, H-5), 8.90 (s, 1H, H-2).

Ethyl 7-chloro-1-(2-furylmethyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (71). The title compound was prepared from **58** through Method B (1,5 h), with the exception that the solid obtained was purified by crystallization by cyclohexane/EtOAc, in 57% yield: mp 137-139 °C; ^{1}H-NMR ($CDCl_3$) δ 1.35 (t, *J* = 7.0 Hz, 3H, CH_2*CH_3*), 4.35 (q, *J* = 7.0 Hz, 2H, *CH_2*CH_3), 5.55 (bs, 2H, CH_2), 6.30-6.35 and 6.45-6.50 (m, each 1H, furan CH), 7.35-7.40 (m, 2H, H-6 and furan CH), 8.55-8.65 (m, 2H, H-5 and H-2).

Ethyl 7-chloro-1-(1*H*-indol-5-ylmethyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (72). The title compound was prepared from **59** through Method B (2 h), with the exception that the solid obtained was purified by flash chromatography eluting with MeOH/$CHCl_3$ (1%), in 40% yield: mp 222-224 °C; ^{1}H-NMR ($CDCl_3$) δ 1.35 (t, J = 7.0 Hz, 3H, $CH_2\mathit{CH_3}$), 4.35 (q, J = 7.0 Hz, 2H, $\mathit{CH_2}CH_3$), 5.60 (bs, 2H, CH_2), 6.45-6.55 (m, 1H, indol CH), 7.05-7.40 (m, 4H, indol CH), 7.55-7.65 (m, 1H, H-6), 8.3 (bs, 1H, indol NH), 8.55-8.70 (m, 2H, H-5 and H-2).

Ethyl 1-(1*H*-benzimidazol-2-ylmethyl)-7-chloro-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (73). The title compound was prepared from **60** through Method B (2 h), in 80% yield: mp 294-297 °C; ^{1}H-NMR ($CDCl_3$) δ 1.25 (t, J = 7.0 Hz, 3H, $CH_2\mathit{CH_3}$), 4.25 (q, J = 7.0 Hz, 2H, $\mathit{CH_2}CH_3$), 5.80 (bs, 2H, CH_2), 7.10-7.20 and 7.35-7.50 (m, each 2H, aromatic CH), 7.60 (d, J = 8.2 Hz, 1H, H-6), 8.60 (d, J = 8.2 Hz, 1H, H-5), 9.05 (s, 1H, H-2)

Ethyl 7-chloro-1-[2-(2-fluorophenyl)ethyl]-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (74). The title compound was prepared from **61** through Method B (1.5 h), in 90% yield: mp 123-124 °C; ^{1}H-NMR (DMSO-d_6) δ 1.15 (t, J = 7.1 Hz, 3H, $CH_2\mathit{CH_3}$), 3.05 (t, J = 6.5 Hz, 2H, CH_2), 4.10 (q, J = 7.1 Hz, 2H, $\mathit{CH_2}CH_3$), 4.55 (t, J = 6.5 Hz, 2H, NCH_2), 6.95-7.05 and 7.10-7.20 (m, each 2H, aromatic CH), 7.50 (d, J = 8.3 Hz, 1H, H-6), 8.45 (d, J = 8.3 Hz, 1H, H-5), 8.50 (s, 1H, H-2).

Ethyl 7-chloro-1-[2-(4-chlorophenyl)ethyl]-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (75). The title compound was prepared from **62** through Method B (2 h), in 85% yield: mp 108-109 °C; ^{1}H-NMR ($CDCl_3$) δ 1.30 (t, J =7.1 Hz, 3H, $CH_2\mathit{CH_3}$), 3.10 (t, J = 7.2 Hz, 2H, CH_2), 4.30 (q, J = 7.1 Hz, 2H, $\mathit{CH_2}CH_3$), 4.50 (t, J = 7.2 Hz, 2H, NCH_2), 7.00 and 7.20 (d, J = 8.3 Hz, each 2H, aromatic CH), 7.30 (d, J = 8.2 Hz, 1H, H-6), 8.20 (s, 1H, H-2), 8.65 (d, J =8.2 Hz, 1H, H-5).

Ethyl 7-chloro-4-oxo-1-(2-pyridin-2-ylethyl)-1,4-dihydro-1,8-naphthyridine-3-carboxylate (76) The title compound was prepared from **63** through Method B (3 h), in 70% yield: mp 158-159 °C; ^{1}H-NMR (DMSO-d_6) δ 1.25 (t, J = 7.0 Hz, 3H, $CH_2\mathit{CH_3}$), 3.25 (t, J = 6.7 Hz, 2H, $NCH_2\mathit{CH_2}$), 4.20

(q, J = 7.0 Hz, 2H, CH_2CH_3), 4.75 (t, J = 6.7 Hz, 2H, NCH_2), 7.15-7.25 (m, 2H, pyridine CH), 7.55 (d, J = 7.9 Hz, 1H, H-6), 7.60-7.65 and 8.35-8.40 (m, each 1H, pyridine CH), 8.50 (d, J = 8.2 Hz, 1H, H-5), 8.55 (s, 1H, H-2).

Preparation of ethyl 1-(3-chloro-2-fluorobenzyl)-4-oxo-7-(4-pyridin-2-ylpiperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxylate (1, Method C). A mixture of **64** (0.15g, 0.4 mmol) and 1-(pyridin-2-yl)piperazine (0.17 g, 0.8 mmol) in DMF (10 mL) was maintained at 80 °C for 6 h. After cooling the reaction mixture was poured into ice/water obtaining a precipitate which was filtered and crystallized by EtOH/DMF to give compound **1** (0.15g, 75%); mp 200-201 °C; ^{1}H-NMR (DMSO-d_6) δ 1.25 (t, J = 7.0 Hz, 3H, CH_2CH_3), 3.40-3.50 and 3.65-3.75 (m, each 4H, piperazine CH_2), 4.45 (q, J = 7.0 Hz, 2H, CH_2CH_3), 5.65 (s, 2H, CH_2), 6.65 (dd, J = 5.1 and 6.8 Hz, 1H, pyridine CH), 6.85 (d, J = 8.8 Hz, 1H, pyridine CH), 7.00 (d, J = 9.0 Hz, 1H, H-6), 7.15-7.20 (m, 2H, aromatic CH), 7.50-7.60 (m, 2H, aromatic CH and pyridine CH), 8.15 (d, J = 4.9 Hz, 1H, pyridine CH), 8.20 (d, J = 9.0 Hz, 1H, H-5), 8.85 (s, 1H, H-2). Anal. ($C_{27}H_{25}ClFN_5O_3$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-1-(3-chloro-2-fluorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (23). The title compound was prepared through the Method C (80 °C, 6 h) using 1-(1,3-benzothiazol-2-yl)piperazine,[46] in 78% yield: mp 250-251 °C; ^{1}H NMR (DMSO-d_6) δ 1.30 (t, J = 6.9 Hz, 3H, CH_2CH_3), 3.55-3.65 and 3.80-3.90 (m, each 4H, piperazine CH_2), 4.25 (q, J = 6.9 Hz, 2H, CH_2CH_3), 5.70 (s, 2H, CH_2), 7.05 (d, J = 8.7 Hz, 1H, H-6), 7.10 (t, J = 7.0 Hz, 1H, benzothiazole CH), 7.15-7.25 (m, 2H,aromatic CH), 7.30 (t, J = 7.0 Hz, 1H, benzothiazole CH), 7.45-7.60 (m, 2H, aromatic CH and benzothiazole CH), 7.80 (d, J = 7.7 Hz, 1H, benzothiazole CH), 8.20 (d, J = 8.7 Hz,1H, H-5), 8.80 (s, 1H, H-2). Anal. ($C_{29}H_{25}ClFN_5O_3S$) C, H, N.

Ethyl 1-(3-chloro-2-fluorobenzyl)-4-oxo-7-(4-quinolin-2-ylpiperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxylate (24). The title compound was prepared through Method C (80 °C, 5 h) using 2-piperazin-1-ylquinoline,[58] in 61% yield after crystallization by EtOH: mp 210-212 °C; ^{1}H-NMR ($CDCl_3$) δ 1.45 (t, J = 7.1 Hz, 3H, CH_2CH_3), 3.80 (bs, 8H, piperazine CH_2), 4.40 (q, J =

7.1, 2H, CH_2CH_3) 5.50 (s, 2H, CH_2), 6.75 (d, *J* = 9.0 Hz, 1H, H-6), 6.90-7.10 (m, 3H, aromatic CH and quinoline CH), 7.25-7.35 (m, 2H, quinoline CH), 7.60 (t, *J* = 7.4, 1H, quinoline CH), 7.65 (d, *J* = 7.9 Hz, 1H, quinoline CH), 7.75 (bs, 1H, aromatic CH), 7.95 (d, *J* = 9.0 Hz, 1H, quinoline CH), 8.50 (d, *J* = 9.0 Hz, H-5), 8.60 (s, 1H, H-2). Anal. ($C_{31}H_{27}ClFN_5O_3$) C, H, N.

1-(3-Chloro-2-fluorobenzyl)-4-oxo-7-[4-(1,3-thiazol-2-yl)piperazin-1-yl]-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (25). The title compound was prepared through Method C (80 °C, 5 h) using 1-(1,3-thiazol-2-yl)piperazine,[59] in 50% yield after crystallization by EtOH: mp 223-224 °C; ^{1}H-NMR ($CDCl_3$) δ 1.35 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 3.45-3.55 and 3.70-3.80 (m, each 4H, piperazine CH_2), 4.45 (q, *J* = 7.1 Hz, 2H, CH_2CH_3), 5.50 (s, 2H, CH_2), 6.60 (d, *J* = 4.0 Hz, 1H, thiazole CH) 6.70 (d, *J* = 9.0 Hz, 1H, H-6), 6.85-7.05 (m, 2H, aromatic CH) 7.20 (d, *J* = 4.0 Hz, 1H, thiazole CH), 7.30-7.40 (m, 1H, aromatic CH), 8.45 (d, *J* = 8 Hz, 1H, H-5), 8.50 (s, 1H, H-2). Anal. ($C_{25}H_{23}ClFN_5O_3S$) C, H, N.

7-[4-(1,3-Benzoxazol-2-yl)piperazin-1-yl]-1-(3-chloro-2-fluorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (26). The title compound was prepared through Method C (80 °C, 5 h) using 2-piperazin-1-yl-1,3-benzoxazole,[59] in 18% yield after crystallization by DMF: mp 282-284 °C; ^{1}H-NMR (DMSO-d_6) δ 1.35 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 3.50-3.60 and 3.80-3.90 (m, each 4H, piperazine CH_2), 4.30 (q, *J* = 7.1 Hz, 2H, CH_2CH_3), 5.50 (s, 1H, CH_2), 7.00-7.70 (m, 8H, H-6, benzoxazole CH, and aromatic CH), 8.20 (d, *J* =9.0 Hz, 1H, H-5), 8.90 (s, 1H, H-2). Anal. ($C_{29}H_{25}ClFN_5O_4$) C, H, N.

Ethyl 1-(3-chloro-2-fluorobenzyl)-4-oxo-7-{4-[3-(trifluoromethyl)phenyl]piperazin-1-yl}-1,4-dihydro-1,8-naphthyridine-3-carboxylate (27). The title compound was prepared through Method C (80 °C, 17 h) using 1-[3-(trifluoromethyl)phenyl]piperazine,[60] in 90% yield: mp 100-102 °C; ^{1}H-NMR (DMSO-d_6) δ 1.30 (t, *J* = 7.0 Hz, 3H, CH_2CH_3), 3.20-3.40 and 3.60-3.80 (m, each 4H, piperazine CH_2), 4.15 (q, *J* = 7.0 Hz, 2H, CH_2CH_3), 5.10 (s, 2H, CH_2), 6.90-7.50 (m, 8H, H-6 and aromatic CH), 8.10 (d, *J* = 8.7 Hz, 1H, H-5), 8.78 (s, 1H, H-2). Anal. ($C_{29}H_{25}ClF_4N_4O_3$) C, H, N.

Preparation of 1-(3-chloro-2-fluorobenzyl)-4-oxo-7-(4-pyridin-2-ylpiperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid hydrochloride (28, Method D). A suspension of **1** (0.10 g, 0.2 mmol) in 4% NaOH (4 mL) was refluxed for 11 h. After cooling, the reaction mixture was filtered and the solid was resuspended in 8N HCl and refluxed for 30 min. After cooling the reaction mixture was filtered, to give a solid which was crystallized by DMF/EtOH, to give **28** (0.96 g, 94%): mp 178-180 °C; ^{1}H-NMR (DMSO-d_6) δ 3.80-4.00 (m, 8H, piperazine CH_2), 5.85 (s, 2H, CH_2), 6.95 (t, J = 6.4 Hz, 1H, pyridine CH), 7.15-7.35 (m, 4H, aromatic CH, H-6, and pyridine CH) 7.50-7.60 (m, 1H, aromatic CH), 7.95-8.10 (m, 2H, pyridine CH and aromatic CH), 8.35 (d, J = 9.1 Hz, 1H, H-5), 9.20 (s, 1H, H-2). Anal. ($C_{25}H_{22}Cl_2FN_5O_3$) C, H, N.

7-[4-(1,3-Benzothiazol-2-yl)piperazin-1-yl]-1-(3-chloro-2-fluorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid hydrochloride (29). The title compound was prepared from **23** through Method D (24 h), in 82% yield: mp 281-282 °C; ^{1}H-NMR (DMSO-d_6) δ 3.60-3.70 and 3.85-3.95 (m, each 4H, piperazine CH_2), 5.80 (s, 2H, CH_2), 7.05-7.40 (m, 5H, H-6, aromatic CH, and benzothiazole CH), 7.45-7.55 (m, 2H, aromatic CH, and benzothiazole CH), 7.80 (d, J = 7.6 Hz, 1H, benzothiazole CH), 8.45 (d, J = 9.1 Hz, 1H, H-5), 9.20 (s, 1H, H-2). Anal. ($C_{27}H_{22}Cl_2FN_5O_3S$) C, H, N.

1-(3-Chloro-2-fluorobenzyl)-4-oxo-7-(4-quinolin-2-ylpiperazin-1-yl)-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (30). The title compound was prepared from **24** through Method D (48 h), in 77% yield: mp 220-221 °C; ^{1}H-NMR (DMSO-d_6) δ 3.35-3.55 and 3.85-4.05 (m, each 4H, piperazine CH_2), 5.80 (s, 2H, CH_2), 7.20-7.30 (m, 3H, H-6 and aromatic CH), 7.40-7.60 (m, 3H, aromatic CH and quinoline CH), 7.70-7.95 (m, 2H, quinoline CH), 8.10-8.40 (m, 3H, H-5 and quinoline CH), 9.15 (s, 1H, H-2), 15.50 (bs, 1H, COOH). Anal. ($C_{29}H_{23}ClFN_5O_3$) C, H, N.

1-(3-Chloro-2-fluorobenzyl)-4-oxo-7-[4-(1,3-thiazol-2-yl)piperazin-1-yl]-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (31). The title compound was prepared from **25** through Method D (18 h), in 38% yield: mp 268-269 °C; ^{1}H-NMR (DMSO-d_6) δ 3.40-3.50 and 3.80-3.90 (m, each 4H, piperazine CH_2), 5.77 (s, 2H, CH_2), 6.90 (d, J = 4.0, 1H, thiazole CH), 7.14-7.25 (m, 4H, H-6,

thiazole CH, and aromatic CH), 7.45-7.55 (m, 1H, aromatic CH), 8.30 (d, *J* = 9.1, 1H, H-5), 9.15 (s, 1H, H-2), 15.45 (s, 1H, COOH). Anal. ($C_{23}H_{19}ClFN_5O_3S$) C, H, N.

7-[4-(1,3-Benzoxazol-2-yl)piperazin-1-yl]-1-(3-chloro-2-fluorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (32). In a microwave oven tube, **26** (0.12 g, 0.213 mmol) was suspended in 4% NaOH (6 mL) and 1,4-dioxane (3 mL). The mixture was irradiated at 120 °C for 35 min employing the following experimental parameters: pressure 5 bar, cooling on, FHT on, pre-stirring 30 sec, very high absorption. After cooling, the sodium salt was filtered, suspended in water and then treated with 2N HCl to pH = 2. After stirring 30 min the mixture was filtered to give **32** (0.05 g, 48%): mp >300 °C; ^{1}H-NMR (DMSO-d_6) δ 3.50-3.60 and 3.80-3.90 (m, each 4H, piperazine CH_2), 5.75 (s, 2H, CH_2), 7.00-7.50 (m, 8H, H-6, benzoxazole CH, and aromatic CH), 8.27 (d, *J* = 9.0, 1H, H-5), 9.10 (s, 1H, H-2), 15.70 (bs, 1H, COOH). Anal. ($C_{27}H_{21}ClFN_5O_4$) C, H, N.

1-(3-Chloro-2-fluorobenzyl)-4-oxo-7-{4-[3-(trifluoromethyl)phenyl]piperazin-1-yl}-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (33). The title compound was prepared starting from **27** by using the same procedure as used for the synthesis of **32**, in 55% yeld: mp 210-212 °C; ^{1}H-NMR (DMSO-d_6) δ 3.20-3.30 and 3.75-3.85 (m, each 4H, piperazine CH_2), 5.75 (s, 2H, CH_2), 7.10 (d, *J* = 7.5, 1H, aromatic CH), 7.15-7.25 (m, 5H, H-6 and aromatic CH), 7.40 (t, *J* = 8.0, 1H, aromatic CH), 7.45-7.55 (m, 1H, aromatic CH), 8.30 (d, *J* = 9.1 Hz, 1H, H-5), 9.15 (s, 1H, H-2), 15.45 (s, 1H, COOH). Anal. ($C_{27}H_{21}ClF_4N_4O_3$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-1-(2-fluorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (34). The title compound was prepared from **65** through Method C (80 °C, 17 h) using 1-(1,3-benzothiazol-2-yl)piperazine,[46] in 86% yield: mp 270-272 °C; ^{1}H-NMR (DMSO-d_6) δ 1.20 (t, *J* = 7.1 Hz, 3H, $CH_2\mathit{CH_3}$), 3.40-3.60 and 3.60-3.20 (m, each 4H piperazine CH_2), 4.15 (q, *J* = 7.1 Hz, 2H, $\mathit{CH_2}CH_3$), 5.55 (s, 2H, CH_2), 6.90-7.30 (m, 7H, H-6, benzothiazole CH, and aromatic CH), 7.40 (bd, *J* = 7.6 Hz, 1H, benzothiazole CH), 7.70 (bd, *J* = 7.6 Hz, 1H, benzothiazole CH), 8.15 (d, *J* = 9.0 Hz, 1H, H-5), 8.75 (s, 1H, H-2). Anal. ($C_{29}H_{26}FN_5O_3S$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-1-(3-chlorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (35). The title compound was prepared from **66** through Method C (80 °C, 18 h) using 1-(1,3-benzothiazol-2-yl)piperazine[46] and adding K_2CO_3 (2 equiv), in 60% yield: mp 251-252 °C; ^{1}H-NMR (DMSO-d_6) δ 1.20 (t, J = 7.0 Hz, 3H, CH_2*CH_3*), 3.50-3.60 and 3.75-3.95 (m, each 4H, piperazine CH_2), 4.30 (q, J = 7.0, 2H, *CH_2*CH_3), 5.50 (s, 2H, CH_2), 6.90-7.05 (m, 2H, H-6 and aromatic CH), 7.15-7.35 (m, 4H, aromatic CH and benzothiazole CH), 7.40-7.45 (m, 2H, benzothiazole CH), 7.72 (d, J = 7.4 Hz, 1H, benzothiazole CH), 8.15 (d, J = 9.0 Hz, 1H, H-5), 8.80 (s, 1H, H-2). Anal. ($C_{29}H_{26}ClN_5O_3S$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-1-benzyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (36). The title compound was prepared from **67**[45] through Method C (80 °C, 4 h) using 1-(1,3-benzothiazol-2-yl)piperazine[46] and adding K_2CO_3 (2 equiv.), in 16% yield: mp 246-247 °C; ^{1}H-NMR ($CDCl_3$) δ 1.35 (t, J = 7.0 Hz, 3H, CH_2*CH_3*), 3.55-3.65 and 3.75-3.85 (m, each 4H, piperazine CH_2), 4.32 (q, J = 7.0 Hz, 2H, *CH_2*CH_3), 5.40 (s, 2H, CH_2), 6.68 (d, J = 9.0 Hz, 1H, H-6), 7.00-7.40 (m, 7H, aromatic CH and benzothiazole CH), 7.50-7.60 (m, 2H, benzothiazole CH), 8.45 (d, J = 9.0 Hz, 1H, H-5), 8.50 (s, 1H, H-2). Anal. ($C_{29}H_{27}N_5O_3S$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-1-(4-chlorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (37). The title compound was prepared from **68** through Method C (80 °C, 30 h) using 1-(1,3-benzothiazol-2-yl)piperazine,[46] in 23% yield: mp 204-206 °C; ^{1}H-NMR (DMSO-d_6) δ 1.30 (t, J = 7.0 Hz, 3H, CH_2*CH_3*), 3.55-3.65 and 3.80-3.90 (m, each 4H, piperazine CH_2), 4.25 (q, J = 7.0 Hz, 2H, *CH_2*CH_3), 5.55 (s, 2H, CH_2), 7.00 (d, J = 9.0 Hz, 1H, H-6), 7.10-7.15 (m, 1H, benzothiazole CH), 7.25-7.55 (m, 6H, aromatic CH and benzothiazole CH), 7.77 (d, J = 7.8 Hz, 1H, benzothiazole CH), 8.20 (d, J = 8.8 Hz, 1H, H-5), 8.82 (s, 1H, H-2). Anal. ($C_{29}H_{26}ClN_5O_3S$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-4-oxo-1-[4-(trifluoromethyl)benzyl]-1,4-dihydro-1,8-naphthyridine-3-carboxylate (38). The title compound was prepared from **69** through Method C (80 °C, 18 h) using 1-(1,3-benzothiazol-2-yl)piperazine,[46] in 66% yield: mp 205-

207 °C; ^{1}H-NMR (DMSO-d_6) δ 1.25 (t, J = 7.1 Hz, 3H, CH$_2$*CH$_3$*), 3.50-3.60 and 3.75-3.85 (m, each 4H, piperazine CH$_2$), 4.20 (q, J = 7.1 Hz, 2H, *CH$_2$*CH$_3$), 5.60 (s, 2H, CH$_2$) 6.95 (d, J = 9.0 Hz, 1H, H-6), 7.05 (d, J =7.5 Hz, 1H, benzothiazole CH), 7.20-7.30 (m, 1H, aromatic CH), 7.40-7.50 and 7.65-7.75 (m, each 3H, benzothiazole CH and aromatic CH), 8.15 (d, J = 9.0 Hz, 1H, H-5), 8.80 (s, 1H, H-2). Anal. ($C_{30}H_{29}F_3N_6O_3S$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-4-oxo-1-(pyridin-2-ylmethyl)-1,4-dihydro-1,8-naphthyridine-3-carboxylate (39). The title compound was prepared from **70** through Method C (80 °C, 20 h) using 1-(1,3-benzothiazol-2-yl)piperazine,[46] adding K_2CO_3 (2 equiv.), in 85% yield: mp 225-227 °C; ^{1}H-NMR (DMSO-d_6) δ 1.30 (t, J = 7.0 Hz, 3H, CH$_2$*CH$_3$*), 3.55-3.65 and 3.70-3.80 (m, each 4H, piperazine CH$_2$), 4.25 (q, J = 7.0 Hz, 2H, *CH$_2$*CH$_3$), 5.60 (s, 2H, CH$_2$), 6.95 (d, J = 9.0 Hz, 1H, H-6), 7.10 (t, J = 7.5 Hz, 1H, benzothiazole CH), 7.25-7.35, 7.40-7.50, and 7.75-7.85 (m, each 2H, pyridine CH and benzothiazole CH), 8.20 (d, J = 9.0 Hz, 1H, H-5), 8.45 (d, J = 4.6 Hz, 1H, pyridine CH), 8.80 (s, 1H, H-2). Anal. ($C_{28}H_{26}N_6O_3S$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-1-(2-furylmethyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (40). The title compound was prepared from **70** through Method C (80 °C, 8 h) using 1-(1,3-benzothiazol-2-yl)piperazine,[46] in 60% yield: mp 188-189 °C; ^{1}H-NMR (DMSO-d_6) δ 1.20 (t, J = 7.0 Hz, 3H, CH$_2$*CH$_3$*), 3.60-3.70 and 3.80-3.90 (m, each 4H, piperazine CH$_2$), 4.15 (q, J = 7.0 Hz, 2H, *CH$_2$*CH$_3$), 5.50 (bs, 2H, CH$_2$), 6.35-6.50 (m, 2H, furan CH), 6.95-7.10 (m, 2H, H-6 and benzothiazole CH), 7.25 (t, J = 7.8 Hz, 1H, benzothiazole CH), 7.45 (d, J = 7.8 Hz, 1H, benzothiazole CH), 7.55 (bs, 1H, furan CH), 7.75 (d, J = 7.5 Hz, 1H, benzothiazole CH), 8.15 (d, J = 9.0 Hz, 1H, H-5), 8.70 (s, 1H, H-2). Anal. ($C_{27}H_{25}N_5O_4S$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-1-(1*H*-indol-7-ylmethyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (41). The title compound was prepared from **72** through Method C (80 °C, 15 h) using 1-(1,3-benzothiazol-2-yl)piperazine,[46] in 30% yield: mp 290-293 °C; ^{1}H-NMR (DMSO-d_6) δ 1.20 (t, J = 7.0 Hz, 3H, CH$_2$*CH$_3$*), 3.55-3.65 and 3.80-3.90 (m, each 4H, piperazine CH$_2$), 4.15 (q, J = 7.0 Hz, 2H, *CH$_2$*CH$_3$), 5.60 (bs, 2H, CH$_2$), 6.35-6.40 (m, 1H, indole

CH), 6.95-7.10 (m, 3H, H-6 and benzothiazole CH, and indol CH), 7.20-7.30 (m, 3H, indole CH and benzothiazole CH), 7.40-7.50 (m, 2H, indole CH and benzothiazole CH), 7.75 (d, J = 7.5 Hz, 1H, benzothiazole CH), 8.15 (d, J = 9.0 Hz, 1H, H-5), 8.70 (s, 1H, H-2). Anal. ($C_{31}H_{28}N_6O_3S$) C, H, N.

Ethyl 1-(1*H*-benzimidazol-2-ylmethyl)-7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (42). The title compound was prepared from **73** through Method C (80 °C, 8 h) using 1-(1,3-benzothiazol-2-yl)piperazine,[46] in 50% yield: mp 295-297 °C; ^{1}H-NMR (DMSO-d_6) δ 1.20 (t, J = 7.0 Hz, 3H, CH_2CH_3), 3.30-3.40 and 3.65-3.75 (m, each 4H, piperazine CH_2), 4.20 (q, J = 7.0 Hz, 2H, CH_2CH_3), 5.65 (bs, 2H, CH_2), 6.90 (d, J = 9.0 Hz, benzimidazole CH), 6.95-7.10 (m, 3H, H-6, benzothiazole CH, and benzimidazole CH), 7.25 (t, J = 7.8 Hz, 1H, benzothiazole CH), 7.40-7.50 (m, 2H, benzimidazole CH and benzothiazole CH), 7.75 (d, J = 7.5 Hz, 1H, benzothiazole CH), 8.15 (d, J = 9.0 Hz, 1H, H-5), 8.80 (s, 1H, H-2). Anal. ($C_{30}H_{27}N_7O_3S$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-1-[2-(2-fluorophenyl)ethyl]-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (43). The title compound was prepared from **74** through Method C (80 °C, 16 h) using 1-(1,3-benzothiazol-2-yl)piperazine,[46] in 39% yield: mp 168-170 °C; ^{1}H-NMR (DMSO-d_6) δ 1.20 (t, J = 7.1 Hz, 3H, CH_2CH_3), 3.10 (bt, J = 6.5 Hz, 2H, CH_2), 3.60-3.70 and 3.85-3.95 (m, each 4H, piperazine CH_2), 4.15 (q, J = 7.1, 2H, CH_2CH_3), 4.50 (bt, J = 6.5 Hz, 2H, NCH_2), 6.90-7.30 (m, 7H, H-6, benzothiazole CH, and aromatic CH), 7.45 and 7.75 (d, J = 7.9 Hz, each 1H, benzothiazole CH), 7.15 (d, J = 9.0 Hz, 1H, H-5), 8.35 (s, 1H, H-2). Anal. ($C_{30}H_{28}FN_5O_3S$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-1-[2-(4-chlorophenyl)ethyl]-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate (44). The title compound was prepared from **75** through Method C (80 °C, 15 h) using 1-(1,3-benzothiazol-2-yl)piperazine,[46] in 47% yield: mp 261-263; ^{1}H-NMR (DMSO-d_6) δ 1.30 (t, J = 7.1 Hz, 3H, CH_2CH_3), 3.05 (t, J = 6.9, 2H, CH_2), 3.70-3.80 and 3.85-3.95 (m, each 4H, piperazine CH_2), 4.30 (q, J = 7.1 Hz, 2H, CH_2CH_3), 4.45 (t, J = 6.9 Hz,

2H, NCH_2), 6.72 (d, *J* = 9.0 Hz, 1H, H-6), 6.95-7.05 (m, 2H, aromatic CH) 7.10 (dt, *J* = 1.3 and 7.5 Hz, 1H, benzothiazole CH), 7.20-7.35 (m, 3H, aromatic CH and benzothiazole CH), 7.50-7.65 (m, 2H, benzothiazole CH), 8.10 (s, 1H, H-2), 8.50 (d, *J* = 9.0 Hz, 1H, H-5). Anal. ($C_{30}H_{28}ClN_5O_3S$) C, H, N.

Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-4-oxo-1-(2-pyridin-2-ylethyl)-1,4-dihydro-1,8-naphthyridine-3-carboxylate (45). The title compound was prepared from **76** through Method C (80 °C, 4 h) using 1-(1,3-benzothiazol-2-yl)piperazine[46] and adding K_2CO_3 (2 equiv.), in 96% yield: mp 138-139; ^{1}H-NMR (DMSO-d_6) δ 1.25 (t, *J* = 7.0 Hz, 3H, $CH_2\mathit{CH_3}$), 3.25 (t, *J* = 6.7 Hz, 2H, CH_2), 3.65-3.75 and 3.85-3.95 (m, each 4H, piperazine CH_2), 4.15 (q, *J* = 7.0 Hz, 2H, $\mathit{CH_2}CH_3$), 4.70 (t, *J* = 6.7 Hz, 2H, NCH_2), 7.00-7.15 (m, 2H, pyridine CH and benzothiazole CH), 7.20-7.30 (m, 3H, H-6, pyridine CH and benzothiazole CH), 7.45 (d, *J* = 7.9 Hz, 1H, benzothiazole CH), 7.70 (t, *J* = 7.7 Hz, 1H, pyridine CH), 7.75 (d, *J* = 7.9 Hz, 1H, benzothiazole CH), 8.20 (d, *J* = 8.8 Hz, H-5), 8.35 (s, 1H, H-2), 8.45-8.50 (m, 1H, pyridine CH). Anal. ($C_{29}H_{28}N_6O_3S$) C, H, N.

7-[4-(1,3-Benzothiazol-2-yl)piperazin-1-yl]-1-benzyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (46). The title compound was prepared from **36** through Method D (48 h), in 42% yield: mp 287-288 °C; ^{1}H-NMR (DMSO-d_6) δ 3.60-3.70 and 3.90-4.00 (m, each 4H, piperazine CH_2), 5.70 (s, 2H CH_2), 7.10 (t, *J* = 7.5 Hz, 1H, benzothiazole CH), 7.20 (d, *J* = 9.0 Hz, 1H, H-6), 7.25-7.40 (m, 6H, aromatic CH and benzothiazole CH), 7.50 and 7.80 (d, *J* = 7.8 Hz, each 1H, benzothiazole CH), 8.30 (d, *J* = 9.0 Hz, 1H, H-5), 9.15 (s, 1H, H-2), 15.50 (s, 1H, COOH). Anal. ($C_{27}H_{23}N_5O_3S$) C, H, N.

7-[4-(1,3-Benzothiazol-2-yl)piperazin-1-yl]-1-(4-chlorobenzyl)-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (47). The title compound was prepared from **37** through Method D (72 h), in 75% yield: mp > 300 °C; ^{1}H-NMR (DMSO-d_6) δ 3.60-3.70 and 3.80-3.90 (m, each 4H, piperazine CH_2), 5.65 (s, 2H, CH_2), 7.05 (t, *J* = 7.5 Hz, 1H, benzothiazole CH), 7.15 (d, *J* = 8.9 Hz, 1H, H-6), 7.25-7.30 (m, 1H, benzothiazole CH), 7.35-7.40 (m, 4H, aromatic CH), 7.50 and 7.75 (d,

J = 7.5, each 1H, benzothiazole CH), 8.30 (d, J = 8.9 Hz, 1H, H-5), 9.15 (s, 1H, H-2), 15.50 (s, 1H, COOH). Anal. ($C_{27}H_{22}ClN_5O_3S$) C, H, N.

7-[4-(1,3-Benzothiazol-2-yl)piperazin-1-yl]-4-oxo-1-(pyridin-2-ylmethyl)-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid hydrochloride (48). The title compound was prepared from **39** through Method D (4 h), in 96% yield: mp 257-258 °C; ^{1}H-NMR (DMSO-d_6) δ 3.55-3.65 and 3.80-3.90 (m, each 4H, piperazine CH_2), 5.95 (s, 2H, CH_2), 7.10-7.20 (m, 2H, H-6 and benzothiazole CH), 7.30 (t, J = 7.1 Hz, 1H, benzothiazole CH), 7.45-7.55 (m, 2H, benzothiazole CH and pyridine CH), 7.70 (d, J = 7.2 Hz, 1H, pyridine CH), 7.80 (d, J = 7.5 Hz, 1H, benzothiazole CH), 8.05-8.15 (m, 1H, pyridine CH), 8.30 (d, J = 8.5 Hz, 1H, H-5), 8.65 (s, 1H, pyridine CH), 9.20 (s, 1H, H-2). Anal. ($C_{26}H_{23}ClN_6O_3S$) C, H, N.

7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-4-oxo-1-(2-pyridin-2-ylethyl)-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (49). The title compound was prepared from **45** through Method D (1 h), in 91% yield: mp > 300 °C; ^{1}H-NMR (DMSO-d_6) δ 3.60 (t, J = 6.7 Hz, 2H, CH_2), 3.65-3.75 and 3.85-3.95 (m, each 4H, piperazine CH_2), 4.95 (t, J = 6.7 Hz, 2H, NCH_2), 7.05-7.15 (m, 2H, pyridine CH and benzothiazole CH), 7.30 (t, J = 7.7 Hz, 1H, benzothiazole CH), 7.50 (d, J = 7.9 Hz, 1H, benzothiazole CH), 7.77-7.85 (m, 3H, H-6, pyridine CH and benzothiazole CH), 8.25 (d, J = 8.8 Hz, H-5), 8.35 (t, J = 7.5 Hz, 1H, pyridine CH), 8.75 (d, J = 5.3 Hz, 1H, pyridine CH), 8.90 (s, 1H, H-2). Anal. ($C_{22}H_{27}N_7O_3S$) C, H, N.

Biology. Cells. P21 is a HaCat-derived stably transfected cell clone with integrated reporter plasmid pALuc HPV-16-LCR, that we previously generated.[25] This cell line grows as monolayers in Dulbecco's modified Eagle's medium (DMEM) (SIGMA) supplemented with 10% fetal calf serum (SIGMA), 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany) and 0.2 mg/mL of G418 (Gibco/BRL). CaSki is a HPV-16-positive human cervical carcinoma cell line purchased from American Type Culture Collection (*ATCC*, Rockville, MD, USA) and grown in DMEM High Glucose (4.5 g/l) (SIGMA) supplemented with 10% fetal calf serum.

Compounds. Human TGF-β1 was obtained from Peprotech (RockyHill, NJ), IL-4 was purchased from Bender MedSystems (Burlingame, CA). All quinolones were solubilized in 100% dimethyl sulfoxide (DMSO), aliquoted and stored at – 20 °C.

Luciferase Assay. P21 clones were seeded in 24-well culture plates at a density of 6×10^4/well. After 24 h, they were incubated with different concentrations of the compounds in triplicate or treated with equal volumes of DMSO. The next day, monolayers were washed twice with PBS 1X and lysed on ice with 40 μL of the reporter lysis buffer (Promega, Madison, WI). The lysate was centrifuged at 13000 rpm for 10 min at 4 °C. Supernatants (soluble proteins) were quantified for protein concentration and assayed for luciferase activity with the Promega Luciferase Assay System using a VICTOR Light Luminescence counter (Perkin Elmer, Massachusetts, USA).[25,61] The end-point of the assay was the inhibitory concentration of drug which reduced luciferase activity by 50% (IC_{50}) in comparison to the DMSO - treated control. The IC_{50} and the 95% confidence interval (95%CI) values for inhibition curves were calculated by using the program PRISM 4 (GraphPad Software, San Diego, California, U.S.A.) to fit a variable slope-sigmoidal dose–response curve.

Cell viability assay. To test the cytotoxic effect of compounds on P21 and CaSki cultures, cells were seeded at a density of 1 x 10^4/well in 96-well plates. The next day, cells were either incubated with different concentrations of the compounds in triplicate or treated with equal volumes of DMSO. After a 24-h treatment, cell viability was measured by the CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions, as previously described.[62] Absorbances were measured at 490 nm using the microplate reader Multiskan FC (Thermo Fisher Scientific, MA, USA). The effect on cell viability of each test compound at different concentrations was expressed as a percentage, by comparing absorbances of compound-treated cells with that of DMSO-treated cells. The 50% cytotoxic concentrations (CC_{50}s) and 95% confidence intervals (CIs) were determined using Prism software (Graph-Pad Software, San Diego, CA). The selectivity index (SI) value of each compound was calculated by the ratio of CC_{50} to IC_{50}.

Real-time Quantitative RT-PCR Analysis. CaSki cells were seeded at a density of 1,5 x 10^6/well in 100mm-plates. The next day, cells were treated with 10 μM of each compounds or TGF-β_1 (50 ng/mL) for 24 h. Total RNA isolation was performed using a NucleoSpin II kit (Macherey-Nagel, Düren, Germany), and 2 μg of RNA were retrotranscribed with a Transcriptor First Strand cDNA Synthesis kit (Roche, Mannheim, Germany). The resulting cDNAs were quantitated by real-time PCR using the following primers: HPV-16 E6 forward primer 5′-GCAAGCAACAGTTACTGAGACGT-3′, HPV-16 E6 reverse primer 5′-GCAACAAGACATACATCGACCGG-3′, HPV-16 E7 forward primer 5′-GATGGTCCAGCTGGACAAGC-3′, HPV-16 E7 reverse primer 5′-GTGCCCATTAACAGGTCTTC-3′, β-actin forward primer 5′-GTTGCTATCCAGGCTGTG-3′, β-actin reverse primer 5′-TGTCCACGTCACACTTCA-3′.[33] DNA amplifications were carried out by the SYBR Green real-time PCR method in a 96-well reaction plate format in a Light Cycle 480 Real-Time Machine PCR System (Roche, Mannheim, Germany). The results were normalized to the β-actin transcript levels and fold increase (mean ± SD) was compared with the DMSO-treated controls. Results are expressed as the mean of three independent experiments.

Statistical Analysis. Each data point is the mean ± standard deviation of the mean values for three determinations performed in duplicate. Statistical analysis was performed by analysis of variance (ANOVA) followed by a Bonferroni test if *P* values showed significant differences (P value < 0.05), using GraphPad Prism 5.00 (GraphPad Software).

In Vitro Uptake Studies in CaSki cells. The quantitative determination of 1,8-naphthyridone derivatives was achieved by reverse-phase HPLC method using a UV detector, developed for this study. A preliminary evaluation of UV spectra of the series of ester/acid pairs (compounds **23-25** and **29-31**) was carried out by spectrophotometric analysis to identify the value of λ_{max} for each compound. For this purpose a weighted amount of each 1,8-naphthyridone derivatives was dissolved with a methanol-chloroform mixture (1:1, v/v). The solutions were then diluted with

methanol and analyzed using a spectrophotometer (Beckman Coulter DU 730), collecting the data in the range between 200 and 300 nm to identify the absorbance maximum (λ_{max}).

Table 3 reports the λ_{max} values for each compound, subsequently used in the HPLC quantitative procedure.

Table 3. UV λ_{max} values

Compound	λ_{max} (nm)
23	278
24	277
25	275
29	274
30	276
31	280

For the quantitative chromatographic analyses of the 1,8-naphthyridone derivatives a HPLC system consisting 2of a binary pump (Perkin Elmer PUMP 250B) equipped with a spectrophotometer detector (Perkin Elmer Flexar UV-VIS Detector) was used. The mobile phase was a mixture of TFA (0,1%), methanol and acetonitrile (60:20:20, v/v), degassed and pumped through a Microsorb-MV 100-5 C_{18} column (250 mm × 4.6 mm, 5 µm) with a flow rate of 1,2 mL/min. Ultraviolet detection was set to a different wavelength (λ_{max}) for each compound (see Table 3). The external standard method was used to calculate the compound concentrations.

For this purpose, one milligram of each 1,8-naphthyridone derivatives was weighed, placed in a volumetric flask and dissolved with a methanol-chloroform mixture (1:1 v/v) to obtain stock standard solutions. The stock solutions of each compound were diluted using the mobile phase, providing a series of calibration solutions for each ester/acid pair subsequently injected in the HPLC system.

Linear calibration curves were obtained for each compound reported in Table 3 over the concentration range of 0.5 - 20 µg/mL with a regression coefficient of 0.998.

The concentration of the ester/acid pairs in CaSki cells was investigated as a measure of the intracellular accumulation of the 1,8-naphthyridone derivatives. After incubation with the six compounds solubilized in DMSO (at a concentration = 10 μM), cells were washed twice with PBS at pH 7.4, lysed with a solution containing an excess of ammonium sulfate and the centrifuged at 4 °C for 10 min at 10000 rpm to collect the supernatants and separate cell pellets. The use of ammonium sulfate was reported in the literature for cell lysis.[63,64] Cell lysates (supernatants) were frozen and stored at −80°C. Immediately prior to their HPLC analysis, cell lysates were thawed and centrifuged at 8000 rpm for 10 min at 10 °C. The supernatants were collected and diluted with the mobile phase, vortexed for 2 min and injected into the HPLC system, as described above, for the quantitative determination of 1,8-naphthyridone derivatives. The amount of compounds present inside the cells was calculated from the standard calibration curve. Cell uptake of each 1,8-naphthyridone derivative was expressed as a percentage of the total compound administered.

Before to perform the experiments, the stability of the esters in the experimental conditions was evaluated by incubating the compounds with a saturated solution of ammonium sulfate for 4 h and determining spectrophotometrically the ester concentration. No change in ester concentration was observed.

ASSOCIATED CONTENT

Supporting Information Available: Table S1 reporting the effect of the 22 selected quinolone-based derivatives on HPV-16 LCR activity. Table S2 reporting the antiviral activity of the hit compound **23** against a panel of DNA and RNA viruses. Chemistry and experimental procedures for the synthesis of the target compounds **10-13**, **16**, **17**, **19**, and **20**. Table S3 containing Elemental analysis data for target compounds **1**, **10-13**, **16**, **17**, **19**, **20**, and **23-49**. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

6-DFQs, 6-desfluoroquinolones; CC, cervical cancer; HCMV, human cytomegalovirus; HPV, human papillomavirus; LCR, long control region; RT-PCR, real time-polymerase chain reaction; TGF-β1, transforming growth factor beta 1.

REFERENCES

1. Tommasino, M. The human papillomavirus family and its role in carcinogenesis. *Semin. Cancer Biol.* **2014**, *26*, 13-21.
2. Bosch, F.X.; de Sanjosé, S. Chapter 1: Human papillomavirus and cervical cancer–burden and assessment of causality. *J. Natl. Cancer Inst. Monogr.* **2003**, *31*, 3-13.
3. IARC CANCER Mondial website (http://www-dep.iarc.fr/) consulted on January 2014.
4. CDC website: 2012 Sexually transmitted diseases surveillance (http:// www.cdc.gov) consulted on January 2014.
5. Hong, D.; Lu W.; Ye, F.; Hu, Y.; Xie, X. Gene silencing of HPV16 E6/E7 induced by promoter-targeting siRNA in SiHa cells *Br. J. Cancer* **2009**, *101*, 1798-1804.
6. Garland, S.M.; Hernandez-Avila, M.; Wheeler, C.M.; Perez, G.; Harper, D.M.; Leodolter, S.; Tang, G.W.; Ferris, D.G.; Steben, M.; Bryan, J.; Taddeo, F.J.; Railkar, R.; Esser, M.T.; Sings,

H.L.; Nelson, M.; Boslego, J.; Sattler, C.; Barr, E.; Koutsky, L.A. Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. Females United to Unilaterally Reduce Endo/Ectocervical Disease (FUTURE) I Investigators. *N. Engl. J. Med.* **2007**, *356*, 1928-1943.

7. Serkies, K.; Jassem, J. Concurrent weekly cisplatin and radiotherapy in routine management of cervical cancer: a report on patient compliance and acute toxicity. *Int. J. Radiat. Oncol. Biol. Phys.* **2004**, *60*, 814-821.
8. Eifel, P. J. Chemoradiotherapy in the treatment of cervical cancer. *Semin. Radiat. Oncol.* **2006**, *16*, 177-185.
9. Doorbar, J.; Quint, W.; Banks, L.; Bravo, I.G.; Stoler, M.; Broker, T.R.; Stanley, M.A. The biology and life-cycle of human papillomaviruses. *Vaccine* **2012**, *30S*, F55–F70.
10. Goodwin, E.C.; DiMaio, D. Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 12513-12518.
11. Münger, K.; Baldwin, A.; Edwards, K.M.; Hayakawa, H.; Nguyen, C.L.; Owens, M.; Grace, M.; Huh, K. Mechanisms of human papillomavirus-induced oncogenesis. *J. Virol.* **2004**, *78*, 11451-1160.
12. Zur Hausen, H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat. Rev. Cancer* **2002**, *2*, 342-350.
13. Finzer, P.; Aguilar-Lemarroy, A.; Rosl, F. The role of human papillomavirus oncoproteins E6 and E7 in apoptosis. *Cancer Lett.* **2002**, *15*, 15-24.
14. Longworth, M. S.; Laimins, L.A. Pathogenesis of human papillomaviruses in differentiating epithelia. *Microbiol. Mol. Biol. Rev.* **2004**, *68*, 362-372.
15. Alvarez-Salas, L.M.; Cullinan, A.E.; Siwkowski, A.; Hampel, A.; DiPaolo, J.A. Inhibition of HPV-16 E6/E7 immortalization of normal keratinocytes by hairpin ribozymes. *Proc. Natl. Acad. Sci. U S A* **1998**, *95*, 1189-1194.

16. Chang, J.T.; Kuo, T.F.; Chen, Y.J.; Chiu, C.C.; Lu, Y.C.; Li, H.F.; Shen, C.R.; Cheng, A.J. Highly potent and specific siRNAs against E6 or E7 genes of HPV16- or HPV18-infected cervical cancers *Cancer Gene Therapy* **2010**, *17*, 827–836.
17. De Filippis, R.A.; Goodwin, E.C.; Wu, L.; DiMaio, D. Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells. *J. Virol.* **2003**, *77*, 1551–1563.
18. Goodwin, E.C.; Yang, E.; Lee, C.J.; Lee, H.W.; DiMaio, D.; Hwang, E.S. Rapid induction of senescence in human cervical carcinoma cells. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 10978–10983.
19. Wells, S. I.; Francis, D.A.; Karpova, A.Y.; Dowhanick, J.J.; Benson, J.D.; Howley, P.M. Papillomavirus E2 induces senescence in HPV-positive cells via pRB- and p21(CIP)-dependent pathways. *EMBO J.* **2000**, *19*, 5762–5771.
20. Tan, S.; Hougardy, B.M.; Meersma, G.J.; Schaap, B.; de Vries, E.G.; van der Zee, A.G.; de Jong, S. Human papilloma virus 16 E6 RNA interference enhances cisplatin and death receptor-mediated apoptosis in human cervical carcinoma cells. *Mol. Pharmacol.* **2012**, *81*, 701-709.
21. Dueñas-Gonzalez, A.; Cetina, L.; Coronel, J.; Cervantes-Madrid, D. Emerging drugs for cervical cancer. *Expert Opin. Emerg. Drugs.* **2012**, *17*, 203-218.
22. Tan, S.; de Vries, E.G.; van der Zee, A.G.; de Jong, S. Anticancer drugs aimed at E6 and E7 activity in HPV-positive cervical cancer. *Curr. Cancer Drug Targets* **2012**, *12*, 170-184.
23. Fera, D.; Schultz, D.C.; Hodawadekar, S.; Reichman, M.; Donover, P.S.; Melvin, J.; Troutman, S.; Kissil, J.L.; Huryn, D.M.; Marmorstein, R. Identification and characterization of small molecule antagonists of pRb inactivation by viral oncoproteins. *Chem. Biol.* **2012**, *19*, 518-528.
24. Howley, P.M.; Lowy, D.R. Papillomaviruses. In Fields virology (Knipe, D.M., Howley, P.M., 5th edn), Lippincott Williams & Wilkins, Philadelphia, **2007**, pp. 2299-2354.

25. Lembo, D.; Donalisio, M.; De Andrea, M.; Cornaglia, M.; Scutera, S.; Musso, T.; Landolfo, S. A cell-based high-throughput assay for screening inhibitors of human papillomavirus-16 long control region activity. *FASEB J.* **2006**, *20*, 148-150.

26. Tabarrini, O.; Massari, S.; Cecchetti, V. 6-Desfluoroquinolones as HIV-1 Tat-mediated transcription inhibitors. *Future Med. Chem.* **2010**, *2*, 1161-1180.

27. Massari, S.; Sabatini, S.; Tabarrini, O. Blocking HIV-1 replication by targeting the Tat-hijacked transcriptional machinery. *Curr. Pharm. Des.* **2013**, *19*, 1860-1879.

28. Massari, S.; Daelemans, D.; Barreca, M. L.; Knezevich, A.; Sabatini, S.; Cecchetti, V.; Marcello, A.; Pannecouque, C.; Tabarrini, O. A 1,8-naphthyridone derivative targets the HIV-1 Tat-mediated transcription and potently inhibits the HIV-1 replication. *J. Med. Chem.* **2010**, *53*, 641-648.

29. Tabarrini, O.; Massari, S.; Sancineto, L.; Daelemans, D.; Sabatini, S.; Manfroni, G.; Cecchetti, V.; Pannecouque, C. Structural investigation on naphthyridone scaffold: identification of 1,6-naphthyridone derivative with potent and selective anti-HIV activity. *ChemMedChem* **2011**, *6*, 1249-1257.

30. Loregian, A.; Mercorelli, B.; Muratore, G.; Sinigalia, E.; Pagni, S.; Massari, S.; Gribaudo, G.; Gatto, B.; Palumbo, M.; Tabarrini, O.; Cecchetti, V.; Palù, G. The 6-aminoquinolone WC5 inhibits human cytomegalovirus replication at an early stage by interfering with the transactivating activity of viral immediate-early 2 Protein. *Antimicrob. Agents Chemother.* **2010**, *54*, 1930-1940.

31. Massari, S.; Mercorelli, B.; Sancineto, L.; Sabatini, S.; Cecchetti, V.; Gribaudo, G.; Palù G, Pannecouque, C.; Loregian, A.; Tabarrini, O. Design, synthesis, and evaluation of WC5 analogues as inhibitors of human cytomegalovirus immediate-early 2 protein, a promising target for anti-HCMV treatment. *ChemMedChem* **2013**, *8*, 1403-1414.

32. Baldwin, A.; Pirisi, L.; Creek, K.E. NFI-Ski interactions mediate transforming growth factor beta modulation of human papillomavirus type 16 early gene expression. *J. Virol.* **2004**, *78*, 3953–3964.
33. Donalisio, M.; Cornaglia, M.; Landolfo, S.; Lembo, D. TGF-beta1 and IL-4 downregulate human papillomavirus-16 oncogene expression but have differential effects on the malignant phenotype of cervical carcinoma cells. *Virus Res.* **2008**, *132*, 253-256.
34. Emami, S.; Shafiee, A.; Foroumadi, A. Structural features of new quinolones and relationship to antibacterial activity against gram-positive bacteria. *Mini Rev. Med. Chem.* **2006**, *6*, 375-386.
35. Appelbaum, P.C.; Hunter, P.A. The fluoroquinolone antibacterials: past, present and future perspectives. *Int. J. Antimicrob. Agents* **2000**, *16*, 5-15.
36. Cecchetti, V.; Clementi, S.; Cruciani, G.; Fravolini, A.; Pagella, P.G.; Savino, A.; Tabarrini, O. 6-Aminoquinolones: a new class of quinolone antibacterials? *J. Med. Chem.* **1995**, *38*, 973-982.
37. Cecchetti, V.; Fravolini, A.; Palumbo, M.; Sissi, C.; Tabarrini, O.; Terni, P.; Xin, T. Potent 6-desfluoro-8-methylquinolones as new lead compounds in antibacterial chemotherapy. *J. Med. Chem.* **1996**, *39*, 4952-4957.
38. Cecchetti, V.; Fravolini, A.; Lorenzini, M.C.; Tabarrini, O.; Terni, P.; Xin, T. Studies on 6-aminoquinolones: synthesis and antibacterial evaluation of 6-amino-8-methylquinolones. *J. Med. Chem.* **1996**, *39*, 436-445.
39. Ledoussal, B.; Bouzard, D.; Coroneos, E. Potent non-6-fluoro-substituted quinolone antibacterials: synthesis and biological activity. *J. Med. Chem.* **1992**, *35*, 198-200.
40. Tabarrini, O.; Sabatini, S.; Massari, S.; Pieroni, M.; Franzblau, S.G.; Cecchetti, V. 6-Hydrogen-8-methylquinolones active against replicating and non-replicating mycobacterium tuberculosis. *Chem. Biol. Drug Des.* **2012**, *80*, 781-786.
41. Takiff, H.; Guerrero, E. Current prospects for the fluoroquinolones as first-line tuberculosis therapy. *Antimicrob. Agents Chemother.* **2011**, *55*, 5421-5429.

42. Ziganshina, L.E.; Titarenko, A.F.; Davies, G.R. Fluoroquinolones for treating tuberculosis (presumed drug-sensitive). *Cochrane Database Syst. Rev.* **2013**, *6*, CD004795.
43. Mercorelli, B.; Muratore, G.; Sinigalia, E.; Tabarrini, O.; Biasolo, M.A.; Cecchetti, V.; Palù, G.; Loregian, A. A 6-aminoquinolone compound, WC5, with potent and selective anti-human cytomegalovirus activity. *Antimicrob. Agents Chemother.* **2009**, *53*, 312-315.
44. Anderson, D.; Beutel, B.; Bosse, T. D.; Clark, R.; Cooper, C.; Dandliker, P.; David, C.; Yu-Gui, H.; Todd, M.; Hinman, M.; Kalvin, D.; Larson, D. P.; Lynch, L.; Ma, Z.; Motter, C.; Palazzo, F.; Rosenberg, T.; Rehm, T.; Sanders, W.; Tufano, M.; Wagner, R.; Weitzberg, M.; Yong, H.; Zhang, T. Preparation of naphthyridines as antibacterial compounds. 2003, US 2003232818.
45. Hirose, T.; Mishio, S.; Matsumoto, J.; Minami, S. Pyridone-carboxylic acids as antibacterial agents. I. Synthesis and antibacterial activity of 1-alkyl-1,4-dihydro-4-oxo-1,8- and -1,6-naphthyridine-3-carboxylic acids. *Chem. Pharm. Bull.* **1982**, *30*, 2399-2409.
46. Verderame, M. 1,4-Disubstituted piperazines. 3-Piperazinylbenzothiazoles. *J. Med. Chem.* **1972**, *15*, 693-694.
47. Heitman, L. H.; van Veldhoven, J. P. D.; Zweemer, A. M.; Ye, K.; Brussee, J.; Ijzerman, A. P. False Positives in a Reporter Gene Assay: Identification and synthesis of substituted N-pyridin-2-ylbenzamides as competitive inhibitors of firefly luciferase. *J. Med. Chem.* **2008**, *51*, 4724-4729.
48. Ahmed, A.; Daneshtalab, M. Nonclassical biological activities of quinolone derivatives. *J. Pharm. Pharm. Sci.* **2012**, *15*, 52-72.
49. Palumbo, M.; Gatto, B.; Zagotto, G.; Palù, G. On the mechanism of action of quinolone drugs. *Trends Microbiol.* **1993**, *1*, 232-235.
50. Palù, G.; Valisena, S.; Ciarrocchi, G.; Gatto, B.; Palumbo, M. Quinolone binding to DNA is mediated by magnesium ions. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 9671-9675.

51. Tillotson, G.S. Quinolones: structure-activity relationships and future predictions. *J. Med. Microbiol.* **1996**, *44*, 320-324.

52. Pesson, M.; De Lajudie, P.; Antoine, M. Synthesis based on 3-acetyl-4-hydroxy quinolones. *C.R. Acad. Sci. Ser.* **1971**, *273*, 907-910.

53. Manfroni, G.; Cannalire, R.; Barreca, M.L.; Kaushik-Basu, N.; Leyssen, P.; Winquist, J.; Iraci, N.; Manvar, D.; Paeshuyse, J.; Guhamazumder, R.; Basu, A.; Sabatini, S.; Tabarrini, O.; Danielson, U.H.; Neyts, J.; Cecchetti, V. The Versatile nature of the 6-aminoquinolone scaffold: identification of submicromolar hepatitis C virus NS5B inhibitors. *J. Med. Chem.* **2014**, *57*, 1952-1963.

54. Kumar, D.V.; Rai, R.; Brameld, K.A.; Somoza, J.R.; Rajagopalan, R.; Janc, J.W.; Xia, Y.M.; Ton, T.L.; Shaghafi, M.B.; Hu, H.; Lehoux, I.; To, N.; Young, W.B.; Green, M.J. Quinolones as HCV NS5B polymerase inhibitors. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 82-87.

55. Sultana, N.; Arayne, M.S.; Naz, A.; Mesaik, M.A. Identification of anti-inflammatory and other biological activities of 3-carboxamide, 3-carbohydrazide and ester derivatives of gatifloxacin. *Chem. Cent. J.* **2013**, *7*, 1-6.

56. Pieroni, M.; Dimovska, M.; Brincat, J.P.; Sabatini, S.; Carosati, E.; Massari, S.; Kaatz, G.W.; Fravolini A. From 6-aminoquinolone antibacterials to 6-amino-7-thiopyranopyridinylquinolone ethyl esters as inhibitors of Staphylococcus aureus multidrug efflux pumps. *J. Med. Chem.* **2010**, *53*, 4466-4480.

57. Chandrasekhar, S.D.; Radhakrishna, P. Antitumor action of Curcumin in human papillomavirus associated cells involves downregulation of viral oncogenes, prevention of NFkB and AP-1 translocation and modulation of apoptosis. *Mol. Carcinogen.* **2006**, *45*, 320-332.

58. Rodriguez, R. Process for producing an anti-depressant effect with piperazine quinolines. 1971, USP 3629418.

59. Kimura, T.; Katsube, T. Preparation of aminoquinolone derivatives as anti-HIV agents. 1994, EP 572259 A1.

60. Liu, K.G.; Robichaud, A.J. A general and convenient synthesis of N-aryl piperazines, *Tetahedron lett.* **2005**, *46*, 7921-7922.

61. Donalisio, M.; Poli, A.; Civra, A.; Landolfo, S.; Lembo, D. Effects of cytokines on long control region transcriptional activity in high-risk cutaneous human papillomavirus types 5 and 8. *Arch. Virol.* **2010**, *155*, 583-587.

62. Donalisio. M.; Rusnati, M.; Cagno, V.; Civra, A.; Bugatti, A.; Giuliani, A.; Pirri, G.; Volante, M.; Papotti, M.; Landolfo, S.; Lembo, D. Inhibition of human respiratory syncytial virus infectivity by a dendrimeric heparan sulfate-binding peptide. *Antimicrob. Agents Chemother.* **2012**, *56*, 5278-5288.

63. Yang, J.; Liu, Y.; Wang, H.; Liu, L.; Wang, W.; Wang, C.; Wang, Q.; Liu, W. The biocompatibility of fatty acid modified dextran-agmatine bioconjugate gene delivery vector. *Biomaterials* **2012**, *33*, 604-613.

64. Lembo, D.; Swaminathan, S.; Donalisio, M.; Civra. A.; Pastero, L.; Aquilano, D.; Vavia, P.; Trotta, F.; Cavalli, R. Encapsulation of acyclovir in new carboxylated cyclodextrin-based nanosponges improves the agent's antiviral efficacy. *Int. J. Pharm.* **2013**, *443*, 262-272.

65. Cecchetti, V.; Parolin, C.; Moro, S.; Pecere, T.; Filipponi, E.; Calistri, A.; Tabarrini, O.; Gatto, B.; Palumbo, M.; Fravolini, A.; Palù, G. 6-Aminoquinolones as new potential anti-HIV agents. *J. Med. Chem.* **2000**, *43*, 3799-3802.

66. Tabarrini, O.; Massari, S.; Daelemans, D.; Meschini, F.; Manfroni, G.; Bottega, L.; Gatto, B.; Palumbo, M.; Pannecouque, C.; Cecchetti, V. Studies on anti-HIV transcription inhibitor quinolones: identification of potent N-1-vinyl derivatives. *ChemMedChem* **2010**, *5*, 1880-1892.

67. Tabarrini, O.; Massari, S.; Daelemans, D.; Stevens, M.; Manfroni, G.; Sabatini, S.; Balzarini, J.; Cecchetti, V.; Pannecouque, C.; Fravolini, A. Structure-Activity Relationship Study on Anti-HIV 6-Desfluoroquinolones. *J. Med. Chem.* **2008**, *51*, 5454-5458.

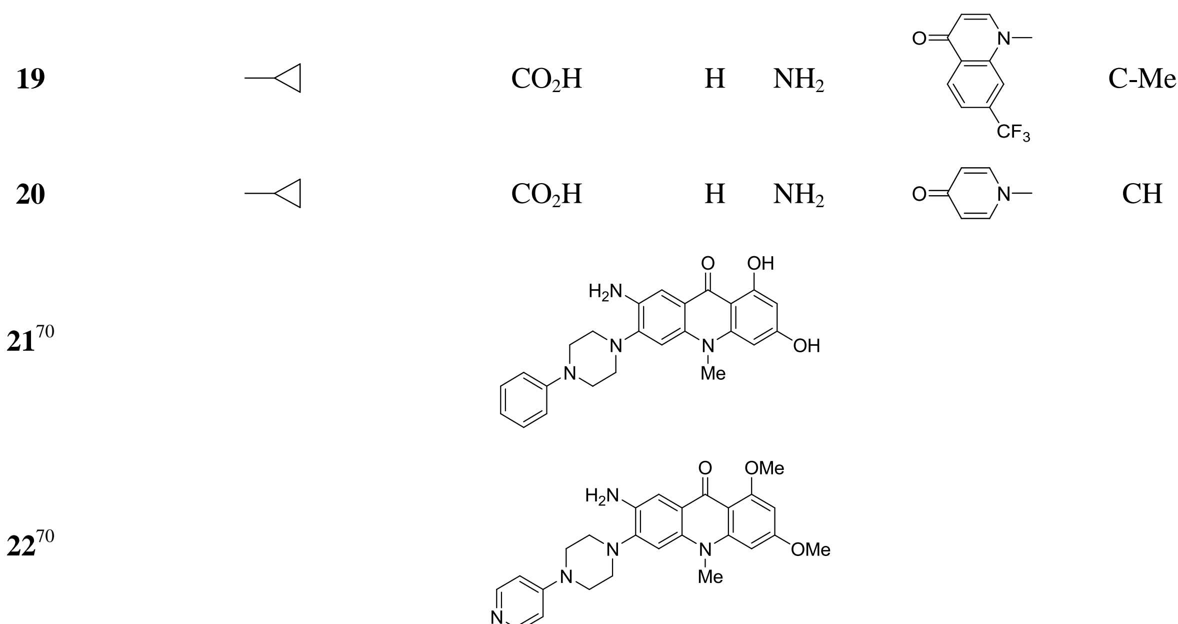
68. Massari, S.; Daelemans, D.; Manfroni, G.; Sabatini, S.; Tabarrini, O.; Pannecouque, C.; Cecchetti, V. Studies on anti-HIV quinolones: new insights on the C-6 Position. *Bioorg. Med. Chem.* **2009**, *17*, 667-674.

69. Natalini, B.; Sardella, R.; Massari, S.; Ianni, F.; Tabarrini, O.; Cecchetti, V. Synthesis and chromatographic enantioresolution of anti-HIV quinolone derivatives. *Talanta* **2011**, *85*, 1392-1397.

70. Manfroni, G.; Paeshuyse, J.; Massari, S.; Zanoli, S.; Gatto, B.; Maga, G.; Tabarrini, O.; Cecchetti, V.; Fravolini, A.; Neyts, J. Potential NS3 helicase-mediated inhibition of subgenomic HCV RNA replication by acridone Derivatives. *J. Med. Chem.* **2009**, *52*, 3354-3365.

Table 1. Structure of Selected Quinolone-based Derivatives.

Compd	R_1	R_3	R_5	R_6	R_7	X
1	F Cl	CO_2Et	H	H	N N N—	N
2[65]	*c*-Pro	CO_2H	H	NH_2	N N N—	CH
3[65]	Me	CO_2Et	H	NH_2	″	CH
4[66]	$CH{=}CH_2$	CO_2H	H	NH_2	″	CH
5[66]	NH_2	CO_2H	H	H	″	CH
6[67]	Me	CO_2H	H	H	N N N N—	CH
7[67]	Me	CO_2H	H	NH_2	N N N N—	CH
8[68]	Me	CO_2H	H	OMe	N N N—	CH
9[69]	OH Me	CO_2H	H	NH_2	″	CH
10		H_2N N–NH CO_2H Cl N N N				
11	$CH{=}CH_2$	CO_2H	Cl	NH_2	N N N—	CH
12	—$(CH_2)_2$-N N- N	CO_2H	H	NH_2	″	CH
13	H	COMe	H	NH_2	″	CH
14[67]	Me	$CONH_2$	H	NH_2	″	CH
15[67]	Me	$CONHCH_2$— —Cl	H	NH_2	″	CH
16	H	CN	H	NH_2	″	CH
17	Me	CO_2H	H	NH_2	H_2N O_2S–N N—	CH
18[69]	Me	CO_2H	H	NH_2	Me N N N—	CH



The synthetic procedure for the preparation of compounds **10-13**, **16**, **17**, **19**, and **20** is reported in the Supporting Information.

Table 2. Effect of Compound **1** and Analogues on HPV-16 LCR Activity and Cytotoxicity on P21 Reporter Cells.

	% of LCR inhibition (mean values ± SD)				
Compd	**100 µM**	**30 µM**	**IC_{50} (µM)[a] (95% C.I.)[c]**	**CC_{50} (µM)[b]**	**SI[d]**
1	93.1 ± 3.1	89.2 ± 1.8	1.12 (0.83-1.50)	> 100	> 89.2
23	94.9 ± 1.7	82.8 ± 3.1	0.26 (0.16-0.42)	> 100	> 385
24	93.7 ± 1.8	92.3 ± 4.5	0.21 (0.12-0.35)	59.3	282
25	99.7 ± 2.3	98.0 ± 2.1	1.21 (0.95- 1.53)	25.3	20.9
26	83.9 ± 1.8	88.1 ± 3.2	0.39 (0.02-7.74)	> 100	> 256
28	25.8 ± 3.7	30.5 ± 0.5	NT[e]	NT	
29	56.0 ± 0.7	38.3 ± 3.8	NT	NT	
30	100 ± 0.1	99.3 ± 0.7	2.86 (1.34-268)[f]	17.6	6.1
31	2.1 ± 0.7	7.39 ± 0.9	NT	NT	
34	75.6 ± 0.8	88.6 ± 1.4	4.18 (0.84-20.8)	> 100	> 23.9
35	96 ± 3.2	96 ± 1.9	2.62 (0.02-24.5)	> 100	> 38.1
36	98.1 ± 0.2	98.2 ± 4.1	0.23 (0.08-0.61)	61.7	268

37	90 ± 0.7	90 ± 1.7	2.37 (1.62-3.47)	> 100	> 42.1
38	89.3 ± 1.8	67.5 ± 2.3	10.9 (1.61-73.8)[f]	78.2	7.16
39	73.4 ± 1.3	63.1 ± 2.4	5.30 (0.15-182)[f]	34.6	6.5
41	90.2 ± 1.2	93.6 ± 3.0	0.13 (0.0 - 4.20)	> 100	> 769
42	45.5 ± 0.7	38.3 ± 2.2	NT	NT	
44	50.4 ± 2.0	49.6 ± 4.1	NT	NT	
45	100 ± 0.2	87 ± 2.4	1.05 (0.54-291)[f]	17.1	16.2
46	100 ± 1.9	100 ± 0.5	4.55 (3.56-5.80)	69.6	15.2
48	61.0 ± 2.9	24.0 ± 1.3	NT	NT	
49	0	0	NT	NT	

[a] IC_{50}: concentration of compound which reduced luciferase activity by 50%. [b] CC_{50}: Concentration of compound that reduced cellular viability by 50%. [c] 95% CI: Confidence interval. [d] SI: ratio of CC_{50}/IC_{50}. [e] NT: Not Tested. The IC_{50} and CC_{50} values were not determined due to the low % of LCR inhibition. [f] Wide 95% CI. All data represent mean value of three similar experiments performed in duplicate. Compounds **32** and **47** were not tested due to their low solubility in DMSO. Compounds **27**, **33**, **40**, and **43** caused a noticeable alteration of cultured cells not permitting the evaluation of LCR inhibition.

Figure legends

Figure 1. Dose-dependent reduction of LCR-driven transcription by compound **1**. P21 cells were seeded on 24-well plates and then treated for 24 h with increasing concentrations of compound . The results represent the mean ± SD of three indipendent experiments performed in duplicate.

Figure 2. From hit compound **1** to an enlarged series of analogues.

Figure 3. Effect of molecules on the steady-state levels of E6 and E7 mRNAs in CaSki cells. Cells were treated with 10 μM of compounds or 50 ng/mL of TGF-β1 for 24 h. mRNA was retrotranscribed, and the levels of HPV-16 E6 and E7 transcripts were determined by real-time PCR. Results shown are the mean of three independent experiments. * statistical significance for E6 or E7 (P<0.05).

Figure 4. Cell uptake percentages of 1,8-naphthyridone acids, esters counterparts, and amount of acids derived from esters, determined in cell lysate 1, 2, 4, and 16 h post-incubation.

Scheme Footnotes

Scheme 1[a]

[a] Reagents and conditions: i) arylalkylamine, Et_2O/EtOH; ii) K_2CO_3, DMF, 60°C; iii) 4-arylpiperazine, DMF, 80-90 °C; iv) 4% NaOH, reflux or 4% NaOH, dioxane, mw, 120 °C.

Figure 1

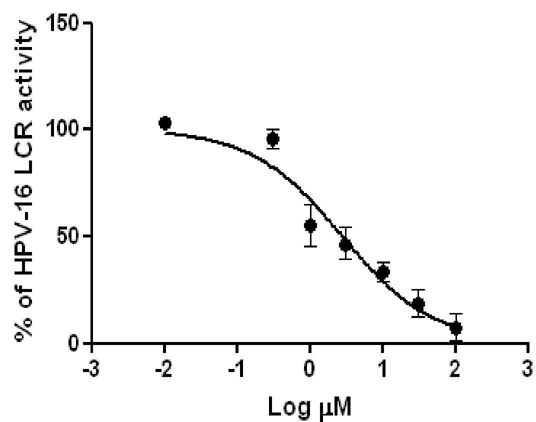
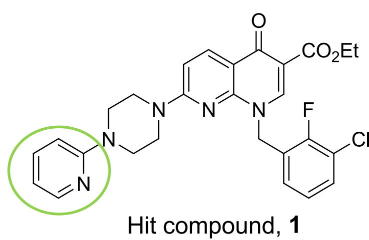
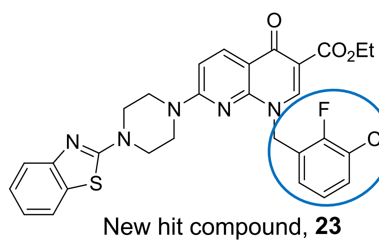


Figure 2.



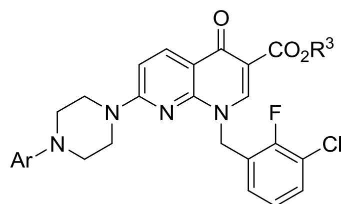
Hit compound, **1**



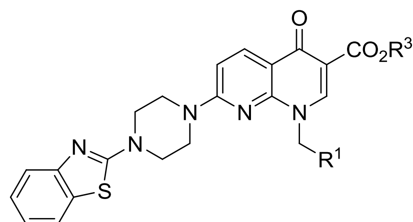
New hit compound, **23**

C-7 modifications

N-1 modifications



Compd		Ar
R^3 = Et	R^3 = H	
	28	
23	**29**	
24	**30**	
25	**31**	
26	**32**	
27	**33**	



Compd	R^1
R^3 = Et	
34	
35	
36	
37	
38	
39	

Compd	R^1
R^3 = Et	
40	
41	
42	
43	
44	
45	

Compd	R^1
R^3 = H	
46	
47	
48	
49	

Figure 3.

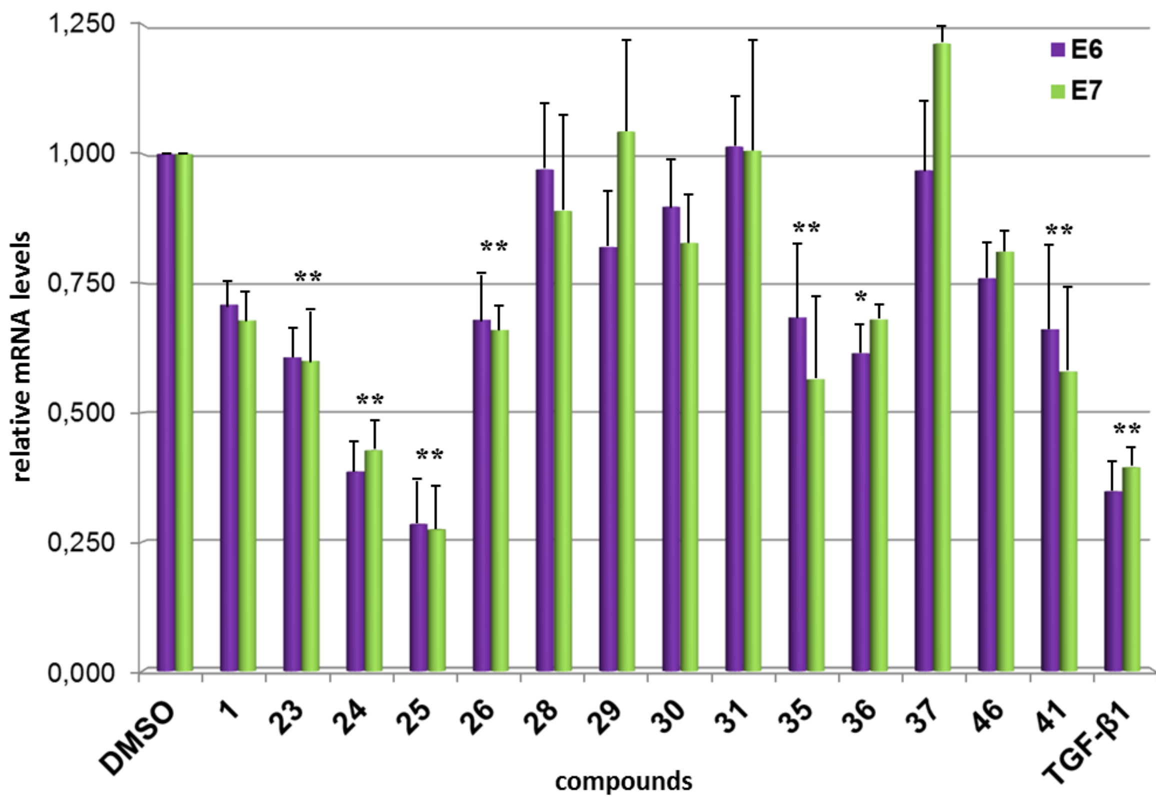
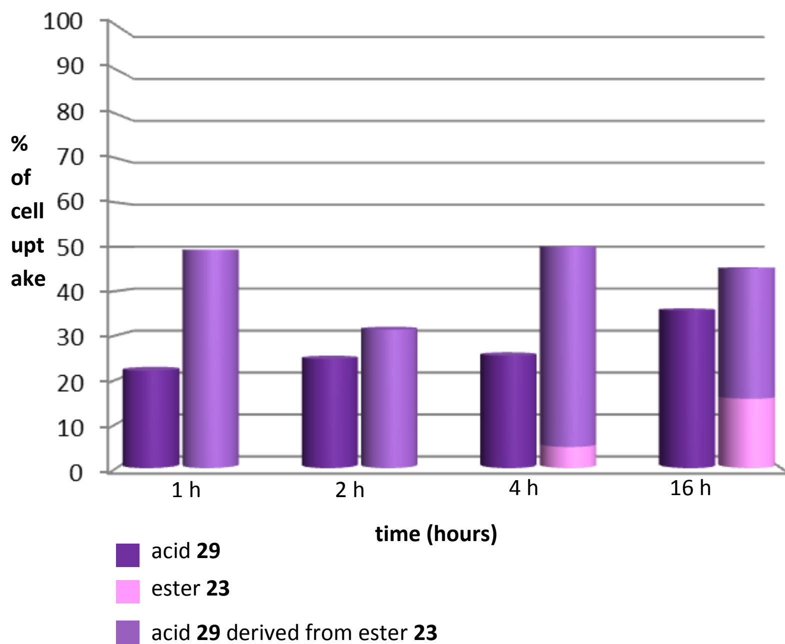
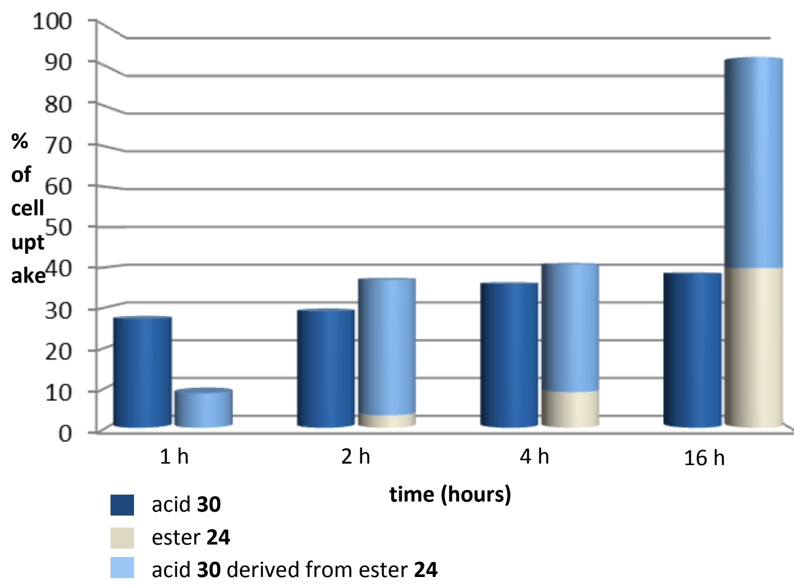
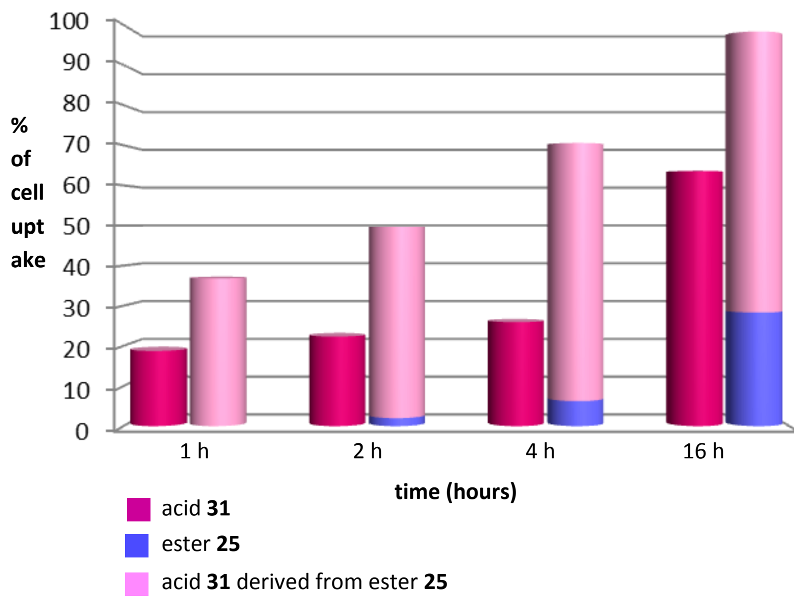


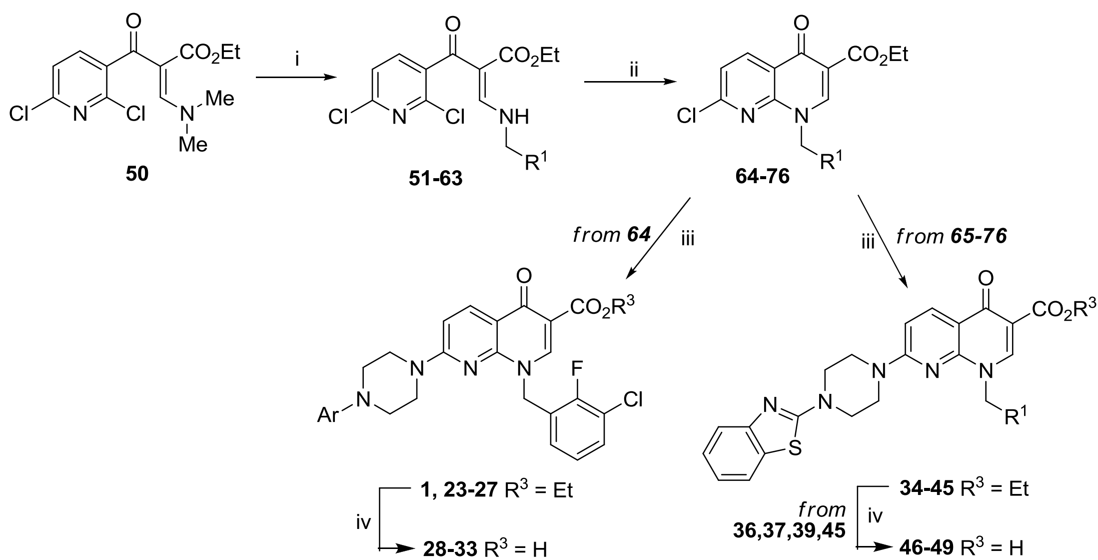
Figure 4.




100
90
80
70
60
50
40
30
20
10
0
% of cell uptake
1 h
2 h
4 h
16 h
time (hours)
acid **30**
ester **24**
acid **30** derived from ester **24**


100
90
80
70
60
50
40
30
20
10
0
% of cell uptake
1 h
2 h
4 h
16 h
time (hours)
acid **31**
ester **25**
acid **31** derived from ester **25**

Scheme 1[a]



Ar	Compd
	1,28
	23,29
	24,30
	25,31
	26,32
F_3C	27,33

R^1	Compd	R^1	Compd
Cl, F	51,64		58,71,40
F	52,65,34	HN	59,72,41
Cl	53,66,35	N, H	60,73,42
	54,67,36,46	F	61,74,43
Cl	55,68,37,47	Cl	62,75,44
F_3C	56,69,38	N	63,76,45,49
N	57,70,39,48		

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