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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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TITLE RUNNING HEAD: Anti-HPV activity of ethyl 1,8-naphthyridone-3-carboxylates

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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\textsuperscript{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.\textsuperscript{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.\textsuperscript{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,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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,\textsuperscript{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\textsubscript{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.
According to the IC₅₀ 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-benzoazole, 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 was reacted with variously substituted arylalkylamines in Et₂O/EtOH mixture, to give intermediates 51-63, which were then cyclized in the presence of K₂CO₃ 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, and 68-76, differently functionalized at the N-1 position, with 2-(piperazin-1-yl)benzothiazole 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\textsubscript{50} value was determined for 14 derivatives. They reduced the LCR activity in a concentration-dependent fashion (data not shown) with IC\textsubscript{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\textsubscript{50} = 0.26 μM that, coupled with the lack of cytotoxicity (CC\textsubscript{50} > 100 μM ; CC\textsubscript{50} is the concentration of compound that reduced cellular viability by 50%) led to a selectivity index (SI, ratio of CC\textsubscript{50} to IC\textsubscript{50}) > 385. A similar behaviour was exhibited by its 4-benzoxazol bioisoster 26. A comparable potency was shown by compound 24 (IC\textsubscript{50} = 0.21 μM), even if coupled with some cytotoxicity (CC\textsubscript{50} = 59.3 μM). The thiazolpiperazine appeared a less suitable C-7 substituent, since compound 25 showed the same activity (IC\textsubscript{50} = 1.21 μM) but a markedly higher antiproliferative effect (CC\textsubscript{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\textsubscript{50} = 0.23 μM) of compound 23 (IC\textsubscript{50} = 0.26 μM) although coupled with a higher toxicity (CC\textsubscript{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 \( \mu\)M) and even more in the \( p \)-trifluoromethyl derivative 38 (IC\(_{50}\) = 10.90 \( \mu\)M).

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 \( \mu\)M, 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.\(^\text{47}\) 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 \( \mu\)M 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 Mg²⁺ 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\textsuperscript{51} with few exceptions, such as certain carboxylate esters which are converted in vivo back to a carboxylic acid.\textsuperscript{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, 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 60F254 (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%). 1H NMR and 13C 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 Na2SO4, 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)\textsuperscript{44} (1.0 g, 3.1 mmol) and (3-chloro-2-fluorophenyl)methanamine (0.6 g, 3.8 mmol) in Et\textsubscript{2}O/EtOH (4:1, 15 mL), was stirred at room temperature for 2 h. The reaction mixture was then evaporated to dryness to give a residue, which after treatment with cyclohexane gave a solid, which was filtered to give 51 (1.27 g, 95%): mp 155-156 °C; \textsuperscript{1}H-NMR (DMSO-\textit{d}_6) δ 0.80 (t, \textit{J} = 7.1 Hz, 3H, CH\textsubscript{2}CH\textsubscript{3}), 3.75 (q, \textit{J} = 7.1 Hz, 2H, CH\textsubscript{2}CH\textsubscript{3}), 4.75 (bs, 2H, CH\textsubscript{2}), 7.20-7.45 (m, 2H, aromatic CH), 7.50-7.60 (m, 2H, H-5' and aromatic CH), 7.75 (d, \textit{J} = 7.9 Hz, 1H, H-4'), 8.35 (d, \textit{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\textsuperscript{44} through method A (20 min), using (2-fluorobenzyl)amine, in 75% yield: mp 161-162 °C; \textsuperscript{1}H-NMR (DMSO-\textit{d}_6) 0.68 and 0.85 (each t, \textit{J} = 7.1 Hz, 0.65 and 2.35 H, CH\textsubscript{2}CH\textsubscript{3}), 3.80 (q, \textit{J} = 7.1 Hz, 2H, CH\textsubscript{2}CH\textsubscript{3}), 4.60 (bd, \textit{J} = 6.0 Hz, 2H, CH\textsubscript{2}), 7.10-7.45 (m, 4H, aromatic CH), 7.51 (d, \textit{J} = 8.0 Hz, 1H, H-4’), 7.75 (d, \textit{J} = 8.0 Hz, 1H, H-5’), 8.30 (d, \textit{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\textsuperscript{44} through method A (40 min), using (3-chlorobenzyl)amine, in 77% yield: mp 156-157 °C; \textsuperscript{1}H-NMR (DMSO-\textit{d}_6) 0.75 and 0.90 (each t, \textit{J} = 7.1 Hz, 0.7 and 2.3 H, CH\textsubscript{2}CH\textsubscript{3}), 3.80-3.90 (m, 2H, CH\textsubscript{2}CH\textsubscript{3}), 4.70-4.75 (m, 2H, CH\textsubscript{2}), 7.32-7.50 (m, 4H, aromatic CH), 7.55 (d, \textit{J} = 8.0 Hz, 1H, H-5’), 7.80 (d, \textit{J} = 8.0 Hz, 1H, H-4’), 8.40 (d, \textit{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\textsuperscript{44} through Method A (40 min), using 1-phenylmethanamine, in 70% yield: mp 123-124 °C; \textsuperscript{1}H-NMR (DMSO-\textit{d}_6) 0.65 and 0.80 (each t, \textit{J} = 7.1 Hz, 0.77 and 2.23 H, CH\textsubscript{2}CH\textsubscript{3}), 3.75-3.80 (m, 2H, CH\textsubscript{2}CH\textsubscript{3}), 4.65-4.75 (m, 2H, CH\textsubscript{2}), 7.25-7.40 (m, 5H, aromatic CH), 7.55 (d, \textit{J} = 8.0 Hz, 1H, H-5’), 7.75 (d, \textit{J} = 8.0 Hz, 1H, H-4’), 8.30 (d, \textit{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 through Method A (1 h), using (4-chlorobenzyl)amine hydrochloride, in 66% yield: mp 135-136 °C; 1H-NMR (DMSO-d6) 0.65 and 0.85 (each t, J = 7.1, 1 and 2H, CH2CH3), 3.75-3.90 (m, 2H, CH2CH3), 4.65 (bd, J = 6.0 Hz, 2H, CH2), 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 through Method A (6 h), using [4-(trifluoromethyl)benzyl]amine, in 90% yield: mp 110-112 °C; 1H-NMR (DMSO-d6) 0.75 and 0.90 (each t, J = 7.1 Hz, 0.8 and 2.2H, CH2CH3), 3.85-3.90 (m, 2H, CH2CH3), 4.80 (bd, J = 5.8 Hz, 2H, CH2), 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 through Method A (4 h), using (pyridin-2-ylmethyl)amine, in 91% yield: mp 157-158 °C; 1H-NMR (DMSO-d6) δ 0.75 and 0.90 (each t, J = 7.0 Hz, 1 and 2H, CH2CH3), 3.80 (q, J = 7.0 Hz, 2H, CH2CH3), 4.80 (bs, 2H, CH2), 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 through Method A (30 min), using (2-furylmethyl)amine, in 87% yield: mp 132-134 °C; 1H-NMR (DMSO-d6) 0.85 (t, J = 7.0 Hz, 3H, CH2CH3), 3.80 (q, J = 7.0 Hz, 2H, CH2CH3), 4.70 (bs, 2H, CH2), 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-[(1H-indol-5-ylmethyl)amino]acrylate (59). The title compound was prepared starting from 50 through Method A (1.2 h), using (1H-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-[(1H-benzimidazol-2-ylmethyl)amino]-2-[(2,6-dichloropyridin-3-yl)carbonyl]acrylate (60). The title compound was prepared starting from 50 through Method A (12 h), using (1H-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 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 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, CH₂CH₃), 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 through Method A (4 h) using (2-pyridin-2-ylethyl)amine, in 74% yield: mp 118-122 °C; ¹H-NMR (CDCl₃) δ 0.80-1.00 (m, 3H, CH₂CH₃), 3.10-3.20 (m, 2H, CH₂), 3.90-4.00 (m, 4H, CH₂CH₃ and NCH₂), 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-chlоро-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 K₂CO₃ (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 Et₂O, to give 64 (1.1 g, 96%); mp 191-192 °C; ¹H-NMR (CDCl₃) δ 1.45 (t, J = 6.8 Hz, 3H, CH₂CH₃), 4.45 (q, J = 6.8 Hz, 2H, CH₂CH₃), 5.60 (bs, 2H, CH₂), 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; ¹H-NMR (DMSO-d₆) δ 1.20 (t, J =7.0 Hz, 3H, CH₂CH₃), 4.20 (q, J =7.0 Hz, 2H, CH₂CH₃), 5.60 (s, 2H, CH₂), 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; ¹H-NMR (DMSO-d₆) δ 1.28 (t, J = 7.0 Hz, 3H, CH₂CH₃), 4.25 (q, J = 7.0 Hz, 2H, CH₂CH₃), 5.60 (s, 1H, CH₂), 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). 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-(1H-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/CHCl₃ (1%), in 40% yield: mp 222-224 °C; ¹H-NMR (CDCl₃) δ 1.35 (t, J = 7.0 Hz, 3H, CH₂CH₃), 4.35 (q, J = 7.0 Hz, 2H, CH₂CH₃), 5.60 (bs, 2H, CH₂), 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-(1H-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; ¹H-NMR (CDCl₃) δ 1.25 (t, J = 7.0 Hz, 3H, CH₂CH₃), 4.25 (q, J = 7.0 Hz, 2H, CH₂CH₃), 5.80 (bs, 2H, CH₂), 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; ¹H-NMR (DMSO-d₆) δ 1.15 (t, J = 7.1 Hz, 3H, CH₂CH₃), 3.05 (t, J = 6.5 Hz, 2H, CH₂), 4.10 (q, J = 7.1 Hz, 2H, CH₂CH₃), 4.55 (t, J = 6.5 Hz, 2H, NCH₂), 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; ¹H-NMR (CDCl₃) δ 1.30 (t, J = 7.1 Hz, 3H, CH₂CH₃), 3.10 (t, J = 7.2 Hz, 2H, CH₂), 4.30 (q, J = 7.1 Hz, 2H, CH₂CH₃), 4.50 (t, J = 7.2 Hz, 2H, NCH₂), 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; ¹H-NMR (DMSO-d₆) δ 1.25 (t, J = 7.0 Hz, 3H, CH₂CH₃), 3.25 (t, J = 6.7 Hz, 2H, NCH₂CH₂), 4.20
(q, J = 7.0 Hz, 2H, CH₂CH₃), 4.75 (t, J = 6.7 Hz, 2H, NCH₂), 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; ¹H-NMR (DMSO-d₆) δ 1.25 (t, J = 7.0 Hz, 3H, CH₂CH₃), 3.40-3.50 and 3.65-3.75 (m, each 4H, piperazine CH₂), 4.45 (q, J = 7.0 Hz, 2H, CH₂CH₃), 5.65 (s, 2H, CH₂), 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. (C₂₇H₂₅ClFN₅O₃) 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; ¹H NMR (DMSO-d₆) δ 1.30 (t, J = 6.9 Hz, 3H, CH₂CH₃), 3.55-3.65 and 3.80-3.90 (m, each 4H, piperazine CH₂), 4.25 (q, J = 6.9 Hz, 2H, CH₂CH₃), 5.70 (s, 2H, CH₂), 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. (C₂₉H₂₅ClFN₅O₃S) 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; ¹H-NMR (CDCl₃) δ 1.45 (t, J = 7.1 Hz, 3H, CH₂CH₃), 3.80 (bs, 8H, piperazine CH₂), 4.40 (q, J =
7.1, 2H, CH₂CH₃) 5.50 (s, 2H, CH₂), 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. (C₃₁H₂₇ClFN₅O₃) 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; ¹H-NMR (CDCl₃) δ 1.35 (t, J = 7.1 Hz, 3H, CH₂CH₃), 3.45-3.55 and 3.70-3.80 (m, each 4H, piperazine CH₂), 4.45 (q, J = 7.1 Hz, 2H, CH₂CH₃), 5.50 (s, 2H, CH₂), 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. (C₂₅H₂₃ClFN₅O₃) 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; ¹H-NMR (DMSO-d₆) δ 1.35 (t, J = 7.1 Hz, 3H, CH₂CH₃), 3.50-3.60 and 3.80-3.90 (m, each 4H, piperazine CH₂), 4.30 (q, J = 7.1 Hz, 2H, CH₂CH₃), 5.50 (s, 1H, CH₂), 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. (C₂₀H₂₃ClFN₅O₄) 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; ¹H-NMR (DMSO-d₆) δ 1.30 (t, J = 7.0 Hz, 3H, CH₂CH₃), 3.20-3.40 and 3.60-3.80 (m, each 4H, piperazine CH₂), 4.15 (q, J = 7.0 Hz, 2H, CH₂CH₃), 5.10 (s, 2H, CH₂), 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. (C₂₉H₂₅ClF₃N₅O₃) C, H, N.
Preparation of 1-(3-chloro-2-fluorobenzyl)-4-oxo-7-(4-pyridin-2-yl)piperazin-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-$d_6$) $\delta$ 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$_2$FN$_3$O$_3$) 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-$d_6$) $\delta$ 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$_3$FN$_5$O$_3$) C, H, N.

1-(3-Chloro-2-fluorobenzyl)-4-oxo-7-(4-quinolin-2-yl)piperazin-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-$d_6$) $\delta$ 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$_5$O$_3$) 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-$d_6$) $\delta$ 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_{3}O_{3}) 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-\(d_6\)) \(\delta\) 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\(_5\)O\(_4\)) 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% yield: mp 210-212 °C; \(^{1}\)H-NMR (DMSO-\(d_6\)) \(\delta\) 3.20-3.30 and 3.75-3.85 (m, each 4H, piperazine CH\(_2\)), 4.15 (q, \(J = 7.1\) Hz, 2H, 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\(_{26}\)F\(_2\)N\(_4\)O\(_3\)) C, H, N.

Ethyl 7-[4-(1,3-benothiazol-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-benothiazol-2-yl)piperazine,\(^{36}\) in 86% yield: mp 270-272 °C; \(^{1}\)H-NMR (DMSO-\(d_6\)) \(\delta\) 1.20 (t, \(J = 7.1\) Hz, 3H, CH\(_2\)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, CH\(_2\)CH\(_3\)), 5.55 (s, 2H, CH\(_2\)), 6.90-7.30 (m, 7H, H-6, benothiazole CH, and aromatic CH), 7.40 (bd, \(J = 7.6\) Hz, 1H, benothiazole CH), 7.70 (bd, \(J = 7.6\) Hz, 1H, benothiazole CH), 8.15 (d, \(J = 9.0\) Hz, 1H, H-5), 8.75 (s, 1H, H-2). Anal. (C\(_{29}\)H\(_{28}\)F\(_2\)N\(_3\)O\(_3\)) 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\(_2\)CO\(_3\) (2 equiv), in 60% yield: mp 251-252 °C; \(^1\)H-NMR (DMSO-\(d_6\)) \(\delta\) 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, \(J = 7.0\) Hz, 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\(_3\)O\(_3\)S) 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\(_2\)CO\(_3\) (2 equiv.), in 16% yield: mp 246-247 °C; \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 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\(_3\)O\(_3\)S) 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-\(d_6\)) \(\delta\) 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\(_3\)O\(_3\)S) 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$_3$N$_6$O$_3$S) 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,$^4$ adding K$_2$CO$_3$ (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$_6$O$_3$S) 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,$^4$ 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$_{26}$N$_6$O$_3$S) C, H, N.

**Ethyl 7-[4-(1,3-benzothiazol-2-yl)piperazin-1-yl]-1-(1H-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,$^4$ 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
Ethyl 1-(1H-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-\(d_6\)) \(\delta\) 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\(_{7}\)O\(_{3}\)) 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-\(d_6\)) \(\delta\) 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, \(J = 7.1\) Hz, 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\(_{25}\)FN\(_{3}\)O\(_{3}\)) 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-\(d_6\)) \(\delta\) 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, NCH₂), 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. (C₃₀H₂₈ClN₅O₃S) 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)piperazine⁴⁶ and adding K₂CO₃ (2 equiv.), in 96% yield: mp 138-139; ¹H-NMR (DMSO-d₆) δ 1.25 (t, J = 7.0 Hz, 3H, CH₂CH₃), 3.25 (t, J = 6.7 Hz, 2H, CH₂), 3.65-3.75 and 3.85-3.95 (m, each 4H, piperazine CH₂), 4.15 (q, J = 7.0 Hz, 2H, CH₂CH₃), 4.70 (t, J = 6.7 Hz, 2H, NCH₂), 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. (C₂₉H₂₈N₁₀O₃S) 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; ¹H-NMR (DMSO-d₆) δ 3.60-3.70 and 3.90-4.00 (m, each 4H, piperazine CH₂), 5.70 (s, 2H CH₂), 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. (C₂₇H₂₃N₅O₃S) 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; ¹H-NMR (DMSO-d₆) δ 3.60-3.70 and 3.80-3.90 (m, each 4H, piperazine CH₂), 5.65 (s, 2H, CH₂), 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_{5}O_{3}S) 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; 1H-NMR (DMSO-\textit{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_{6}O_{3}S) 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; 1H-NMR (DMSO-\textit{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_{7}O_{3}S) 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.\textsuperscript{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 x10⁴/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). The endpoint of the assay was the inhibitory concentration of drug which reduced luciferase activity by 50% (IC₅₀) in comparison to the DMSO - treated control. The IC₅₀ 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 10⁴/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. 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 (CC₅₀s) 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 CC₅₀ to IC₅₀.
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-β1 (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′-GCAACAGACATACATCGACCG-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′-TGCCACGTCACACTTCA-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 ($\lambda_{\text{max}}$).

Table 3 reports the $\lambda_{\text{max}}$ values for each compound, subsequently used in the HPLC quantitative procedure.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
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<tr>
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</table>

For the quantitative chromatographic analyses of the 1,8-naphthyridone derivatives a HPLC system consisting of 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 ($\lambda_{\text{max}}$) 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.\textsuperscript{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.

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**Table 1. Structure of Selected Quinolone-based Derivatives.**

![Chemical Structure Diagram]

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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.

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<th>Compd</th>
<th>% of LCR inhibition (mean values ± SD)</th>
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<th>CC_{50} (µM)^d</th>
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<td>&gt; 89.2</td>
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<td>23</td>
<td>94.9 ± 1.7 82.8 ± 3.1 0.26 (0.16-0.42)</td>
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<td>&gt; 385</td>
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<td>93.7 ± 1.8 92.3 ± 4.5 0.21 (0.12-0.35)</td>
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<td>90 ± 1.7</td>
<td>2.37 (1.62-3.47)</td>
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<td>89.3 ± 1.8</td>
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<td>10.9 (1.61-73.8)</td>
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<sup>a</sup> IC<sub>50</sub>: Concentration of compound which reduced luciferase activity by 50%. <sup>b</sup> CC<sub>50</sub>: Concentration of compound that reduced cellular viability by 50%. <sup>c</sup> 95% CI: Confidence interval. <sup>d</sup> SI: ratio of CC<sub>50</sub>/IC<sub>50</sub>. <sup>e</sup> NT: Not Tested. The IC<sub>50</sub> and CC<sub>50</sub> values were not determined due to the low % of LCR inhibition. <sup>f</sup> 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 independent 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

\(^a\) Reagents and conditions: i) arylalkylamine, Et\(_2\)O/EtOH; ii) K\(_2\)CO\(_3\), 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.

**C-7 modifications**

**N-I modifications**

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Hit compound, 1

New hit compound, 23
Figure 3.

![Graph showing relative mRNA levels for compounds E6 and E7.](image1)

Figure 4.

![Bar chart showing percentage of cell uptake over time for compounds acid 29, ester 23, and acid 29 derived from ester 23.](image2)
Acid derived from ester 31

Ester 25

Acid 30 derived from ester 24

Acid 31

Ester 25

Acid 31 derived from ester 25

% of cell uptake

Time (hours)

1 h 2 h 4 h 16 h
Scheme 1

\[
\begin{align*}
\text{50} & \xrightarrow{i} \text{51-63} \xrightarrow{ii} \text{64-76} \\
\text{from 64} & \xrightarrow{iii} \text{from 65-76} \\
\text{1, 23-27 R}^3 = \text{Et} & \xrightarrow{iv} \text{28-33 R}^2 = \text{H} \\
\text{34-45 R}^3 = \text{Et} & \xrightarrow{iv} \text{36,37,39,45} \\
\text{46,49 R}^3 = \text{H} & 
\end{align*}
\]

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