**Abstract:** The growing risk of antimicrobial resistance besides the continuous increase in the number of cancer patients represents a great threat to global health, which requires intensified efforts to discover new bioactive compounds to use as antimicrobial and anticancer agents. Thus, a new set of pyridothienopyrimidine derivatives \(2a,b-9a,b\) was synthesized via cyclization reactions of 3-amino-thieno[2,3-\(b\)]pyridine-2-carboxamides \(1a,b\) with different reagents. All new compounds were evaluated against five bacterial and five fungal strains. Many of the target compounds showed significant antimicrobial activity. In addition, the new derivatives were further subjected to cytotoxicity evaluation against HepG-2 and MCF-7 cancer cell lines. The most potent cytotoxic candidates (3a, 4a, 5a, 6b, 8b and 9b) were examined as EGFR kinase inhibitors. Molecular docking study was also performed to explore the binding modes of these derivatives at the active site of EGFR-PK. Compounds 3a, 5a and 9b displayed broad spectrum antimicrobial activity with MIC ranges of 4–16 \(\mu\)g/mL and potent cytotoxic activity with IC\(_{50}\) ranges of 1.17–2.79 \(\mu\)M. In addition, they provided suppressing activity against EGFR with IC\(_{50}\) ranges of 7.27–17.29 nM, higher than that of erlotinib, IC\(_{50}\) = 27.01 nM.

**Keywords:** thieno[2,3-\(b\)]pyridine; cyclization reactions; pyridothienopyrimidines; antimicrobial activity; HepG-2 cells; MCF-7 cells; EGFR-PK inhibition; molecular docking

1. **Introduction**

In the past twenty years, the incidence of microbial infections associated with increased antimicrobial resistance has increased by alarming levels worldwide, endangering the possibility of curing many infectious diseases, and representing a global threat to population health [1–3]. One of the potential approaches to combat this resistance problem is based on designing innovative new molecules with different modes of action to overcome cross-resistance to present therapeutics [4,5]. Efforts in developing broad-spectrum as well as specific and targeted drugs against various microbes are continuous in all directions [5]. One of the recent strategies used for developing new antimicrobial agents is hybridization of different pharmacophores binding diverse biomolecular targets to exert synergistic effects against drug-resistant infectious diseases [6].

On the other hand, despite large advances in the diagnosis and treatment of various types of cancers, the survival of cancer patients remains poor because of the widespread side effects of anticancer therapeutics as well as the acquisition of multiple drug resistance
by the cancer cells [7]. Breast and liver cancers are among the most medically significant
cancers due to their high incidence and morbidity [8]. Therefore, obtaining new, effective,
more selective, and less toxic anticancer agents remains one of the most urgent demands [9].
In addition, cancer patients are at higher risk of drug-resistant infectious diseases because
of the weakness and immunosuppression caused by anticancer drug therapy [10], which
necessitates the development of new drugs that have potent dual activity against cancer
and pathogenic microbes [11].

Heterocyclic ring systems are considered key molecular structures in medicinal chem-
istry. In addition to their presence in the skeleton of many biological molecules such as
hemoglobin, DNA, RNA, vitamins, hormones, and heterocyclic compounds [12–14], they
produce a wide range of biological activities [15,16]. Accordingly, the structural diversity
and biological prominence of heterocyclic compounds have made them attractive syn-
thetic targets in drug design and discovery for many years [12,13]. Thieno[2,3-b]pyridines
constitute a set of heterocyclic compounds that have beneficial effects in the treatment of
many diseases. Recently, many studies have described various substituted thienopyridine
compounds as significant antiproliferative agents against a wide range of human cancer
cell lines [17–19] and as antimicrobial [20–22], anti-Alzheimer’s [23], anti-platelet [24],
antiviral [25,26] and anti-inflammatory [27] candidates.

Furthermore, the pyrimidine structure is closely related to the nucleobases—uracil,
thymine, and cytosine—which makes pyrimidine molecules important building blocks
in all living cells [28,29]. Pyrimidine-based compounds exhibit a broad spectrum of phar-
macological activity, such as antimalarial [30], antiabetic [31], antimicrobial [32,33], and
anticancer activities [34–36]. Therefore, combining thienopyridine and pyrimidine cores
in the same molecular architecture, forming a pyridothienopyrimidine nucleus, serves as
an attractive strategy for designing a novel scaffold with more favorable pharmacological
effects [37]. Recently, various pyridothienopyrimidine derivatives were reported to produce
significant antimicrobial [38,39] and anticancer activities [40,41], as well as to suppress
protein kinases such as serine/threonine kinase and vascular endothelial growth factor
receptor (VEGFR-2) [42,43].

Prompted by the abovementioned issues, the strategy of this work was focused on
the design of new tricyclic pyrido[3′,2′,4,5][thieno[3,2-d]pyrimidine compounds with struc-
tures that hybridize features of the reported thienopyridine and pyrimidine antimicrobial
and/or anticancer agents [21,22,33,34] Figure 1. The resulting new pyridothienopyrimi-
dines 2a,b–9a,b were synthesized via different cyclization reactions of the key starting
compounds, 3-aminothieno[2,3-b]pyridine-2-carboxamides 1a,b. The tricyclic ring system
of the target pyridothienopyrimidine compounds was linked at position-2 and/or position-4
to different privileged structural motifs such as morpholine, piprazine, spiro cycloalkane,
oxirane, and acetamide, which are renowned for their valuable and diverse pharmacolog-
ical activity [44–48]. All synthesized derivatives were evaluated as antimicrobial agents
against a panel of Gram-positive and Gram-negative bacterial and fungal pathogens. Then,
they were evaluated as cytotoxic agents against human liver carcinoma cells (HepG2) and
human breast cancer cells (MCF-7) in an effort to gain new compounds possessing dual
antimicrobial and anticancer activities. Since epidermal growth factor receptor (EGFR)
is a key mediator in the regulation of different important cellular processes [49,50] and
its overexpression displays a significant role in the development of many human cancers,
including hepatic cancer and breast cancer [51,52], it was of interest to examine the EGFR
inhibition activities of the compounds that revealed the most potent cytotoxic activities, as
one of their cytotoxic mechanisms of action. Furthermore, molecular docking study was
carried out to detect the compounds’ binding modes at the active site of EGFR-TK.
The synthetic pathways utilized for the synthesis of the target pyridothienopyrimidine compounds 2a,b–9a,b are depicted in Schemes 1 and 2. The structures of all new compounds were confirmed using $^{1}H$-NMR, $^{13}C$-NMR (Supplementary Materials), IR, and mass spectral data alongside the elemental microanalyses. Heating 3-amino-4,6-dimethyl-6-phenyl-4-[(p-tolyl)thieno[2,3-b]pyridine-2-carboxamides 1a,b, the starting compounds, with urea at 180 °C for 1 h led to the formation of the corresponding pyridothienopyrimidine-2,4-diones 2a,b, which were further refluxed with a mixture of phosphorus oxychloride and phosphorus pentachloride to give the corresponding 2,4-dichloro derivatives 3a,b, respectively. On the other hand, the treatment of the starting compounds 1a,b with 2-chloroacetyl chloride in cold acetonitrile resulted in cyclization via substitution reaction followed by intramolecular elimination to give the 2-(chloromethyl)-pyridothienopyrimidin-4(3H)-ones 4a,b, respectively. The subsequent treatment of 4b with different amines, namely, morpholine and 1-methylpiperazine, resulted in the corresponding 2-(morpholinomethyl)-/2-(4-methylpiperazin-1-yl)methyl)-pyridothienopyrimidin-4(3H)-ones 5a,b (Scheme 1). IR spectra of compounds 2a,b found two bands in the region 3394–3112 cm$^{-1}$ ascribed to 2NH and two bands in the region 1728–1640 cm$^{-1}$ related to the 2C=O groups of the pyrimidine-2,4(1H,3H)-dione ring. By comparison, IR spectra of the 2,4-dichloro derivatives 3a,b revealed the absence of the previous bands; instead, they showed two bands in the region 825–768 cm$^{-1}$ referring to the newly formed C-Cl groups. In addition, $^{1}H$-NMR spectra of compounds 2a,b revealed the two 2NH groups to be two D$_2$O exchangeable singlets at $\delta$ 10.42–11.71 ppm, which vanished in the $^{1}H$-NMR spectra of 2,4-dichloro derivatives 3a,b. Furthermore, the 2CH$_3$ of 2a, 3a and CH$_3$ of the p-tolyl moiety of 2b, 3b exhibited as singlet signals in the range 2.43–2.79 ppm along with aromatic protons in the range $\delta$ 7.21–8.24 ppm. The $^{13}C$ NMR spectra of compounds 2a,b and 3a,b revealed CH$_3$ moieties at $\delta$ 20.40–24.36 ppm in addition to the aromatic carbons.

2. Results and Discussion

2.1. Chemistry
The synthesis of spiro cycloalkane-pyridothienopyrimidin-4′-ones 6a–d in good yields was achieved via cyclocondensation reactions of the starting 1a,b with the appropriate cyclic ketones, cyclopentanone and/or cyclohexanone, in DMF containing zinc chloride anhydrous. Treating the latter compounds (6a–d) with phosphorus pentasulfide in refluxing pyridine yielded the corresponding 1'H-spiro[cycloalkane-1,2′-pyridothienopyrimidine]-
4′(3′H)-thiones 7a–d (Scheme 2). IR spectra of 6a–d and 7a–d revealed the presence of different absorption bands in the regions 3427–3157 cm\(^{-1}\) related to 2NH groups, 1660–1644 cm\(^{-1}\) related to the C=O groups of 6a–d and 1247–1232 cm\(^{-1}\) related to the C=S groups of compounds 7a–d. \(^1\)H-NMR spectra of 6a–d and 7a–d represented the eight protons of the spiro-cyclopentane and the ten protons of the spiro-cyclohexane substituents as multiplet signals in the range \(\delta 1.21–2.11\) ppm alongside one D\(_2\)O exchangeable singlet in the range \(\delta 4.59–6.49\) ppm, corresponding to the NH at position-1, and another D\(_2\)O exchangeable singlet appeared downfield in the range \(\delta 7.81–9.85\) ppm, related to the NH group at position-3. \(^{13}\)C-NMR spectra of 6a–d and 7a–d revealed various singlets in the ranges \(\delta 19.26–38.33\) ppm assignable to the cyclopentane and cyclohexane carbons, \(\delta 69.49–70.81\) ppm related to the spiro carbons, \(\delta 164.40–167.80\) ppm assigned to the C=O groups of 6a–d and \(\delta 181.33–183.35\) ppm ascribed to C=S moieties of compounds 7a–d, as well as the signals of the parent carbons, which appeared in their correct regions.

Compounds 7c,b were treated with 2-chloroacetamide in refluxing DMF containing sodium carbonate anhydrous to obtain the corresponding 4-sulfanyl acetamide derivatives 8a,b, while the treatment of 7c,b with epichlorohydrin in refluxing acetone containing a catalytic amount of triethyl amine resulted in the formation of the corresponding 4-(oxiran-2-ylmethyl)sulfanyl derivatives 9a,b (Scheme 2). The S-alkylation at position-4 was supported by \(^1\)H NMR and \(^{13}\)C-NMR spectra of 8a,b and 9a,b due to the vanishing of both the signal related to one of the two NH protons and that of the C=S carbon. Moreover, the \(^1\)H NMR spectra of compounds 8a,b exhibited singlet signals at \(\delta 4.05\) and \(4.19\) ppm due to SCH\(_2\) protons of the newly formed acetamide side chain. \(^1\)H NMR spectra of compounds 9a,b represented the protons of the oxirane ring as two multiplets in the range \(\delta 2.86–2.94\) ppm, related to the methylene protons, and a third multiplet at \(\delta 3.72–3.95\) ppm, due to the methine proton, while the methylene protons of SCH\(_2\) appeared as a doublet signal at \(\delta 3.51\) and \(3.67\) ppm. Additionally, the \(^{13}\)C-NMR spectra 9a,b showed three signals at the range \(\delta 31.88–53.45\) ppm referred to SCH\(_2\), OCH\(_2\), and OCH moieties.

Confirmation of the molecular structures of the new compounds was also supported by their mass spectra, which represented the correct molecular ion peaks.

### 2.2. Biological Activity

#### 2.2.1. Antimicrobial Activity

The newly synthesized pyridothienopyrimidine compounds 2a,b–9a,b were investigated for their antimicrobial activities against a panel of microbial strains, three Gram-positive bacteria viz. *Staphylococcus aureus* 25923, *Bacillus subtilis* 6633, *Bacillus cereus* 33018, two Gram-negative bacteria viz. *Escherichia coli* 8739, *Salmonella typhimurium* 14028, three yeasts viz. *Candida albicans* 10231, *Candida tropicalis* 750, *Saccharomyces cerevisiae* and two fungi viz. *Aspergillus flavus*, *Aspergillus niger* EM77 (KF774181). Their activities were expressed in terms of minimal inhibitory concentration (MIC) (µg/mL). Additionally, amoxicillin trihydrate and clotrimazole were utilized as positive antibiotic and antifungal controls. The obtained MIC results are represented in Tables 1 and 2 and Figures 2 and 3.

Based on the MIC values in Table 1, the antibacterial activity of the target compounds 2a,b–9a,b was compared with that of amoxicillin, whose MICs ranged between 4–16 µg/mL against the five bacterial strains. It is evident that pyridothienopyrimidine-2,4(1H,3H)-dione derivatives 2a,b showed antibacterial activity varying from weak to inactive against the tested strains, with MICs ranging from 64 to >128 µg/mL. However, the conversion of 2a,b to 2,4-dichloro derivatives 3a,b led to a significant increase in antibacterial activity, especially for 7,9-dimethyl derivative 3a, which exhibited more potent activity than amoxicillin against *B. subtilis* and *B. cereus* and equalized with it against the other strains. In addition, 2-chloromethyl derivatives 4a,b revealed significant antibacterial activity, particularly 7,9-dimethyl derivative 4a, which showed the most potent activity against *B. cereus* with MIC = 4 µg/mL and had equipotent activity to amoxicillin against the other strains. While 7-Phenyl-9-(p-tolyl) analogue 4b revealed potent activity against *S. aureus* and *B. cereus*, it showed weak activity against other bacterial strains with MIC = 128 µg/mL.
Further reaction of 2-chloromethyl derivative 4b with amines led to enhanced antibacterial activity, where 2-morpholinomethyl derivative 5a showed potent activity equal to amoxicillin against all the tested bacterial strains except S. aureus, with MICs ranging from 8 to 16 µg/mL. In addition, 2-(4-methylpiperazin-1-yl) derivative 5b revealed increasing activity against B. subtilis, E. coli and S. typhimurium.

Table 1. In vitro antibacterial activities of the synthesized compounds expressed as MICs (µg/mL) against the tested pathogenic bacteria.

| Compd. No. | Gram +ve Bacteria | Gram −ve Bacteria |
|------------|------------------|------------------|
|            | S. aureus        | B. subtilis      |
|            | B. cereus        | E. coli          |
|            | S. typhimurium   |
| 2a         | NA               | 64               |
| 2b         | 64               | 64               |
| 3a         | 4                | 4                |
| 3b         | 32               | 8                |
| 4a         | 4                | 8                |
| 4b         | 8                | 128              |
| 5a         | 8                | 16               |
| 5b         | 8                | 8                |
| 6a         | 32               | 16               |
| 6b         | 4                | 8                |
| 6c         | 4                | 4                |
| 6d         | 8                | 32               |
| 7a         | 4                | NA               |
| 7b         | 64               | 64               |
| 7c         | 32               | 32               |
| 7d         | 4                | 64               |
| 8a         | 8                | 16               |
| 8b         | 4                | 8                |
| 9a         | 16               | 32               |
| 9b         | 4                | 4                |
| Amoxicillin| 4                | 8                |

NA = No Activity (MIC > 128 µg/mL).

Table 2. In vitro antifungal activities of the synthesized compounds expressed as MICs (µg/mL) against the tested pathogenic yeasts and fungi.

| Compd. No. | Yeasts | Fungi |
|------------|-------|-------|
|            | C. albicans | C. tropicals | S. cerevisiae | A. flavus | A. niger |
| 2a         | 128    | 128   | 16          | NA        | NA       |
| 2b         | 64     | 32    | 32          | 32        | 32       |
| 3a         | 16     | 8     | 8           | 8         | 8        |
| 3b         | 32     | 16    | 64          | 32        | 16       |
| 4a         | 16     | 16    | 8           | 8         | 8        |
| 4b         | 8      | 16    | 8           | 16        | 16       |
| 5a         | 16     | 8     | 8           | 8         | 16       |
| 5b         | 64     | 16    | 32          | 32        | 64       |
| 6a         | 64     | 16    | 32          | 64        | 32       |
| 6b         | 8      | 8     | 8           | 16        | 8        |
| 6c         | 16     | 4     | 16          | 4         | 8        |
| 6d         | 64     | 64    | 32          | 32        | 16       |
| 7a         | 16     | 16    | 8           | 16        | 16       |
| 7b         | 64     | 64    | 32          | 64        | 128      |
| 7c         | 16     | 8     | 8           | 4         | 16       |
| 7d         | 32     | 64    | 32          | 64        | 64       |
| 8a         | 64     | 16    | 16          | 16        | 16       |
| 8b         | 8      | 16    | 16          | 8         | 8        |
| 9a         | 16     | 32    | 32          | 16        | 64       |
| 9b         | 4      | 4     | 8           | 8         | 8        |
| Clotrimazole| 16   | 8     | 8           | 8         | 8        |

NA = No Activity (MIC > 128 µg/mL).
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| MIC in µg/mL | 100 | 200 | 300 | 400 | 500 | 600 | 700 | 800 |
|--------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Compound 2a,b |     |     |     |     |     |     |     |     |
| Compound 4a,b |     |     |     |     |     |     |     |     |
| Compound 7a,b |     |     |     |     |     |     |     |     |

Figure 2. Antibacterial activities (MIC in µg/mL) of the new pyridothienopyrimidine compounds 2a,b–9a,b and the reference antibiotic (amoxicillin trihydrate).

Figure 3. The antifungal activities (MIC in µg/mL) of the new pyridothienopyrimidine compounds 2a,b–9a,b and the reference drug (clotrimazole).

Spiro cycloalkane-1,2′-pyridothienopyrimidin]-4′(3′H)-ones 6a–d exhibited antibacterial activity varying from potent to moderate against the tested strains, with MICs ranging from 4 to 32 µg/mL. Compound 6b showed an activity equal to that of the reference drug against the five bacterial strains, while 6c exceeded the potency of amoxicillin against B. subtilis and B. cereus and gave equipotent activity against the other strains. The other derivatives, 6a and 6d, showed activity varying from potent to moderate with MICs ranging 8–32 µg/mL. The conversion of 6a–d to 4′(3′H)-thiones analogues 7a–d resulted in an obvious weakening in the activity against the most of the tested strains, particularly derivative 7b, which showed weak activity against the five strains with MICs in the range 64–128 µg/mL. However, the S-alkylation of 7b and 7c at position-4 afforded increases in the antibacterial activity. The spiro cyclopentane 4-sulfanylacetamide derivative 8b revealed potent activity similar to that of amoxicillin, and the spiro cyclohexane analogue 8a showed potent to moderate activity with MICs ranging from 8 to 32 µg/mL. Spiro cyclopentane 4-(oxiran-2-ylmethyl)sulfanyl derivative 9b showed the most potent antibacterial activity against all tested strains, especially against E. coli, while spiro cyclohexane...
analogue 9a showed antibacterial activity ranging from potent to moderate with MICs in the range 8–32 μg/mL.

The antifungal activity of the new compounds 2a,b–9a,b was evaluated according to their MIC values in comparison to the MIC values of the reference antifungal drug clotrimazole, listed in Table 2. The target compounds (3a, 4a, 4b, 5a, 6b, 6c, 7a, 7c, 8b and 9b) appeared to be potent antifungal candidates, representing MIC values ranging from 4 to 16 μg/mL against all the tested yeasts and fungi strains, similar to the range of clotrimazole (MICs: 4–16 μg/mL). Furthermore, 4-(oxiran-2-ylmethyl)sulfanyl derivative 9b represented more potent antifungal activity than that of clotrimazole against the yeast pathogens C. albicans and C. tropicalis, with MICs of 4 and 4 μg/mL, respectively. The rest of the target compounds displayed moderate to weak activity against the tested yeasts and fungi strains. The obtained results suggested that the conjugation of chloroine, chloromethyl, morpholinomethyl and spiro cyclopentane/cyclohexane at position-2 of the parent pyridothienopyrimid-4(3H)-one (3a, 4a,b, 5a, 6b,c, 7c) as well as the attachment of (oxiran-2-ylmethyl)sulfanyl and/or sulfanylacetamide side chains at position-4 of the pyridothenopyrimidine scaffold (8b, 9b) produced a beneficial impact for antimicrobial activity, resulting in promising broad-spectrum growth inhibition activity against the examined Gram-positive and Gram-negative microbes as well as fungal pathogens.

2.2.2. Cytotoxic Activity

The newly synthesized pyridothenopyrimidine derivatives 2a,b–9a,b were subjected to antiproliferative activity evaluation against two cancer cell lines, hepatocellular carcinoma (HepG2) and breast cancer (MCF-7), by using the MTT colorimetric assay[53]. The cytotoxic activity of the target compounds was compared with that of doxorubicin, utilized as a positive control. The concentrations of the examined derivatives that induced 50% inhibition of cell viability (IC_{50}, μM) were detected and are listed in Table 3.

Based on IC_{50} values from Table 3, the examined compounds displayed versatile anti-proliferative activities against the tested cell lines, producing more potent growth inhibitory activity against HepG2 cells than MCF-7 cells with IC_{50} ranges of 1.17–56.18 μM and 1.52–77.41 μM, respectively, compared to doxorubicin’s IC_{50} values of 2.85 and 3.58 μM, respectively. The most active cytotoxic activity was exhibited by the target compounds (9b, 5a, 3a, 6b, 8b and 4a), listed in descending order according to their activity against the two cell lines, as represented in Figure 4. Interestingly, the attachment of the sulfanyl methyl-oxirane side chain at position-4 of the spiro cyclopenane-1,2′-pyridothenopyrimidine nucleus in compound 9b produced the most potent growth inhibition activity against both HepG2 and MCF-7 cancer cells, approximately 2–3 fold higher than doxorubicin, representing IC_{50} values of 1.17 and 1.52 μM, respectively. However, the activity slightly decreased against HepG2 cells and detectably decreased against MCF-7 for the spiro cyclohexane sulfanyl methyl oxirane analogue 9a compared to doxorubicin, with IC_{50} values of 4.88 and 23.56 μM, respectively. Furthermore, the incorporation of the morpholine nucleus into the pyridothenopyrimidine scaffold at position-2 in compound 5a led to a significant cytotoxic potency against both HepG2 and MCF-2, greater than that obtained from the reference drug, at IC_{50} values of 1.99 and 2.79 μM, respectively. However, the 4-methylpiperazinyl analogue 5b exhibited an observable reduction in growth inhibition activity towards both tested cancer cell lines with IC_{50} values of 10.16 and 21.06 μM, respectively. A comparable growth inhibitory activity to doxorubicin was displayed against HepG2 cancer cells by the 2,4-dichloro-pyridothenopyrimidine derivative 3a at an IC_{50} value of 2.31 μM, but its activity was less against MCF-7 with an IC_{50} value of 7.24 μM, while its 7-phenyl-9-p-tolyl analogue 3b represented a detectable drop in potency, with IC_{50} values of 11.34 and 24.72 μM against HepG2 and MCF-7, respectively. The spirocyclopentane-pyridothenopyrimidine-4-one derivative 6b was nearly equipotent to doxorubicin in inhibiting the growth of HepG2 cancer cells, with an IC_{50} value of 2.75 μM, but produced a mild decrease in activity against MCF-7 with IC_{50} of 9.89 μM; however, the displacement of the spiro pentane moiety with spirocyclohexane in the analogue 6d led to a twofold decrease in cytotoxic activity against
HepG2 cells and a significant drop in potency against MCF-7 cancer cells with IC\(_{50}\) values of 4.45 and 21.67 \(\mu\)M, respectively. The other members in this series, 6a, c, appeared to be significantly less potent candidates than the reference drug, with IC\(_{50}\) ranges of 12.11–77.41 \(\mu\)M.

**Table 3.** Cytotoxic activities (IC\(_{50}\) \(\mu\)M) of the new compounds and doxorubicin against HepG2, MCF7 and WISH cells.

| Compd. No. | HepG2 (\(\mu\)M) | MCF7 (\(\mu\)M) | WISH     |
|------------|------------------|-----------------|----------|
| 2a         | 56.57 ± 3.64     | 64.34 ± 2.91    |          |
| 2b         | 33.21 ± 1.79     | 42.39 ± 2.24    |          |
| 3a         | 2.31 ± 0.35      | 7.24 ± 0.64     | 416.83 ± 15.17 |
| 3b         | 11.34 ± 0.65     | 24.72 ± 1.32    |          |
| 4a         | 2.99 ± 0.15      | 15.42 ± 0.45    | 460.23 ± 11.08 |
| 4b         | 36.52 ± 1.82     | 43.27 ± 2.28    |          |
| 5a         | 1.99 ± 0.09      | 2.79 ± 0.18     | 408.48 ± 15.93 |
| 5b         | 10.16 ± 0.29     | 21.06 ± 1.16    |          |
| 6a         | 52.18 ± 1.45     | 77.41 ± 3.62    |          |
| 6b         | 2.75 ± 0.13      | 9.89 ± 0.55     | 394.98 ± 10.20 |
| 6c         | 12.11 ± 0.33     | 22.24 ± 0.67    |          |
| 6d         | 4.45 ± 0.22      | 21.67 ± 0.70    |          |
| 7a         | 26.05 ± 1.89     | 28.11 ± 0.92    |          |
| 7b         | 39.74 ± 1.89     | 41.62 ± 2.20    |          |
| 7c         | 10.35 ± 0.31     | 20.9 ± 0.93     |          |
| 7d         | 23.25 ± 0.35     | 26.55 ± 1.62    |          |
| 8a         | 6.78 ± 0.73      | 20.88 ± 1.46    |          |
| 8b         | 2.79 ± 0.08      | 13.54 ± 0.76    | 401.37 ± 17.32 |
| 9a         | 4.88 ± 0.65      | 23.56 ± 1.24    |          |
| 9b         | 1.17 ± 0.09      | 1.52 ± 0.08     | 417.55 ± 14.1 |
| Doxorubicin | 2.85 ± 0.21     | 3.58 ± 0.33     | 432.10 ± 19.30 |

**Figure 4.** The cytotoxic activity of the most potent compounds.

Additionally, the spiro cyclopentane sulfanylacetamide 8b produced an equivalent cytotoxic potency to that obtained by doxorubicin against HepG2 cells, with an IC\(_{50}\) value of 2.79 \(\mu\)M, but less potency than doxorubicin against MCF7, with an IC\(_{50}\) value of 13.54 \(\mu\)M. The spiro cyclohexane sulfanylacetamide analogue 8a appeared to be a less potent growth inhibitor towards both HepG2 and MCF-7 cancer cells, with IC\(_{50}\) values of 6.78 and 20.88 \(\mu\)M, respectively. Finally, the 2-chloromethyl-7,9-dimethyl derivative 4a, the last among the most
potent compounds, showed significant growth inhibitory activity against HepG2 cancer cells with IC$_{50}$ = 2.99 µM, but its potency decreased against MCF-7 cells with an IC$_{50}$ value of 15.42 µM, whereas a high drop in activity was shown by the 7-phenyl-9-p-tolyl analogue 4b towards the two cancer cell lines with IC$_{50}$ values of 36.52 and 43.27 µM, respectively. The rest of the target compounds, the spiro[cycloalkane-1,2′-pyridothienopyrimidine-4′(3′H)]thiones 7a–d and the pyridothienopyrimidine-2,4(1H,3H)-diones 2a,b, showed moderate to weak cytotoxic activities in the IC$_{50}$ range of 10.35–41.62 µM, compared to doxorubicin.

The frequency and severity of undesirable effects on normal cells at therapeutic doses are considered among the most important characteristics that differentiate anticancer drugs from each other. Accordingly, the cytotoxic activity of the most active compounds (3a, 4a, 5a, 6b, 8b, 9b) was evaluated against the normal WISH cell line (Human amnion normal Liver cells) via MTT assay as IC$_{50}$ values in µM, listed in Table 3. The obtained results revealed that the tested compounds had IC$_{50}$ values in the range 394.98–460.23 µM, nearly equal to that obtained by the reference drug doxorubicin, IC$_{50}$ doxorubicin = 432.10 µM, which confirmed the high safety of the most potent compounds towards normal cells.

2.2.3. In Vitro EGFR Enzyme Inhibition Assay

The most potent cytotoxic compounds (3a, 4a, 5a, 6b, 8b, 9b) were subjected to further investigations of their inhibiting profiles against EGFR, in order to study one of their proposed modes of action as potent cytotoxic agents [54]. Accordingly, these derivatives were assessed as EGFR kinase inhibitors, using erlotinib as a reference drug as it is one of the most potent EGFR inhibitors [55]. The obtained results were expressed as IC$_{50}$ values (nM) (Table 4). Interestingly, the most cytotoxic derivatives, spiro cyclopentane-1,2′ pyridothienopyrimidine–oxirane 9b and pyridothienopyrimidine–morpholine 5a, appeared to be 3–2 times more potent than erlotinib, representing IC$_{50}$ values of 7.27, 9.66 and 27.01 nM, respectively. The 2-chloromethyl-pyridothienopyrimidin-4-one derivative 4a displayed a slight decrease in inhibition activity, but remained 1.5-fold more potent than erlotinib. An evident drop in EGFR inhibition activity was observed for the rest of the examined derivatives (3a, 6b, 8b) representing an IC$_{50}$ range of 38.44–53.57 nM.

Table 4. In vitro enzymatic inhibitory activity against EGFR kinase.

| Compound No. | EGFR IC$_{50}$ (nM) |
|--------------|---------------------|
| 3a           | 17.29 ± 0.24        |
| 4a           | 53.57 ± 0.41        |
| 5a           | 9.66 ± 0.08         |
| 6b           | 53.19 ± 0.46        |
| 8b           | 38.44 ± 0.25        |
| 9b           | 7.27 ± 0.11         |
| Erlotinib    | 27.01 ± 0.16        |

2.3. Molecular Docking Studies

Molecular docking studies were performed to study the binding modes of the most potent cytotoxic compounds (3a, 4a, 5a, 6b, 8b, 9b) to the active sites of the target EGFR compared with erlotinib (ERL) as EGFR inhibitor. Docking setup was first validated through self-docking of the co-crystallized ligand (ERL) in the vicinity of the binding site of the enzyme. The docking score (S) was −10.48 kcal/mol. and root mean square deviation (RMSD) was 1.03 Å (Figure 5). The calculated RMSD value confirms the validity of the docking procedure.

Examination of the binding interactions of erlotinib to the active site of the EGFR kinase domain showed several conventional hydrogen bond interactions with the Met769, Leu768, Val702 and Leu820 amino acids (Figure 6).
Figure 5. 3D representation of the superimposition of the co-crystallized ligand (purple) and the docking pose (dark grey) of ERL at the active site of the EGFR enzyme.

Figure 6. (a) 2D interactions and (b) 3D interactions of erlotinib within the EGFR kinase domain’s active site.

From the docking results of the examined compounds (Table 5), it was noticed that all compounds showed binding interactions within the active site of EGFR kinase domain with binding scores ranging from –12.01 to –8.94 kcal/mol. The spiro cyclopentane 4-((oxiran-2-ylmethyl)sulfanyl derivative 9b, which showed the highest biological activity, also showed the highest binding score of –12.01 kcal/mol, through binding with the key amino acids, Met769, Leu768, and Leu820, via the S atom of thiophene ring using a hydrogen bond acceptor, with further interactions with Thr766 via σ-hole bonding with the S atom of the S-methyl side chain. In addition, it bound to the amino acid Val702 through a hydrogen bond acceptor with the O atom of the oxirane moiety and showed high fitting in the vicinity of the active site, contributing to its high biological activity (Figure 7).
Table 5. Molecular docking results of the most active pyridothienopyrimidine compounds.

| Compound | S (kcal/mol) | Amino Acids | Interacting Groups | Type of Bond | Length (Å) |
|----------|-------------|-------------|-------------------|--------------|------------|
| 3a       | –11.42      | Val702      | N (Pyrimidine)    | H-bond acceptor | 4.12       |
|          |             | Lys721      | N (Pyridine)      | H-bond acceptor | 3.25       |
|          |             | Met769      | Cl (Pyrimidine)   | Halogen bond   | 3.47       |
|          |             | Thr830      | Cl (Pyrimidine)   | Halogen bond   | 4.16       |
|          |             | Thr766      | Cl (Pyrimidine)   | Halogen bond   | 3.26       |
|          |             | Thr830      | Cl (Pyrimidine)   | Halogen bond   | 4.13       |
|          |             | Thr766      | Cl (Pyrimidine)   | Halogen bond   | 3.03       |
|          |             | Thr830      | Cl (Pyrimidine)   | Halogen bond   | 3.79       |
| 4a       | –8.94       | Lys721      | Cl                | Halogen bond   | 3.69       |
|          |             | Cys751      | O (C=O)           | σ-hole bond    | 3.78       |
|          |             | Thr766      | S                 | σ-hole bond    | 4.21       |
|          |             | Met769      | S and N (Pyridine)| H-bond acceptor| 3.41/3.55  |
|          |             | Leu768      | N (Pyridine)      | H-bond acceptor| 3.84       |
| 5a       | –11.48      | Asp831      | NH+               | Ionic interaction | 3.71       |
|          |             | Ly5721      | O (Morpholine)    | H-bond acceptor | 3.51       |
|          |             | Cys773      | O (C=O)           | σ-hole bond    | 3.46       |
|          |             | Asp776      | S                 | σ-hole bond    | 4.18       |
| 6b       | –10.06      | Leu820      | NH                | H-bond acceptor | 3.78       |
|          |             | Cys751      | O (C=O)           | H-bond acceptor | 3.49       |
|          |             | Thr766      | S                 | σ-hole bond    | 3.12       |
|          |             | Met769      | S                 | σ-hole bond    | 3.87       |
|          |             | Leu768      | S (thiophene)     | H-bond acceptor | 3.83       |
| 8b       | –9.11       | Leu694      | S (Side chain)    | σ-hole bond    | 4.38       |
|          |             | Thr766      | N (Pyridine)      | H-bond acceptor | 3.79       |
|          |             | Leu820      | N and NH (Pyrimidine)| H-bond acceptor| 3.73/3.74  |
|          |             | Val702      | O (Oxirane)       | H-bond acceptor | 3.64       |
| erlotinib| –10.48      | Leu768      | N (Pyrimidine)    | H-bond acceptor | 3.64       |
|          |             | Met769      | N (Pyrimidine)    | H-bond acceptor | 2.70       |

Figure 7. (a) 2D interactions and (b) 3D interactions of compound 9b within the EGFR kinase domain’s active site.
2-morpholinomethyl derivative 5a and 2,4-dichloro derivative 3a also showed higher binding scores than the co-crystallized ligand, erlotinib, at –11.48 and –11.42 kcal/mol, respectively; they showed a good binding pattern with the key amino acids, and compound 5a showed an ionic interaction with Asp831 revealing its high biological activity (Figures 8 and 9). Other tested derivatives showed a lower binding score than erlotinib, with less binding interaction with the key amino acids.

Figure 8. (a) 2D interactions and (b) 3D interactions of compound 5a within the EGFR kinase domain’s active site.

Figure 9. (a) 2D interactions and (b) 3D interactions of compound 3a within the EGFR kinase domain’s active site.
3. Materials and Methods

3.1. Chemistry

3.1.1. General Information

The melting points were obtained in open capillary tubes using an Electrothermal IA9100 digital melting point apparatus. Elemental microanalyses were carried out at the Micro Analytical Unit at Cairo University. $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker High Performance Digital FT-NMR Spectrometer Advance III (400/100 MHz) in the presence of TMS as the internal standard at Ain Shams University, Cairo, Egypt. Infrared spectra were measured using the KBr disc technique on a Jasco FT/IR-6100 Fourier transform IR spectrometer (Japan) at the scale of 400–4000 cm$^{-1}$. ESI-mass spectra were determined using an Advion Compact Mass Spectrometer (CMS), NY, USA. TLC on silica gel-precoated aluminum sheets (Type 60, F 254, Merck, Darmstadt, Germany) was used for following up the reactions and checking the purity of the chemical compounds using chloroform/methanol (3:1, $\nu/\nu$), and spots were detected with iodine vapor or through exposure to a UV lamp at $\lambda$ 254 nm for several seconds. The nomenclature of the compounds is according to the IUPAC system. The starting compounds, 3-amino-thieno[2,3-$b$]pyridine-2-carboxamides (1a,b), were prepared using the reported method [56].

3.1.2. Synthesis of 2,4-Dichloropyrido[3′,2′:4,5]thieno[3,2-$d$]pyrimidine-2,4(1H,3H)-diones 2a,b

A mixture of compounds 1a,b (5 mmol) and urea (0.42 g, 7 mmol) was heated at 180$^\circ$C for 1 h. The solidified residue was treated with hot water and the obtained solid was separated by filtration. The solid was washed with hot water several times and recrystallized from acetone to yield compounds 2a,b.

7,9-Dimethylpyrido[3′,2′:4,5]thieno[3,2-$d$]pyrimidine-2,4(1H,3H)-dione (2a) was obtained from 1a (1.11 g, 5 mmol) in 85% yield (1.05 g) as a brown solid, m.p. 325–326$^\circ$C. IR (KBr, $\nu_{max}$/cm$^{-1}$): 3394, 3112 (2NH), 3010 (CH-aromatic), 2828 (CH-aliphatic), 1728, 1662 (2C=O); ESI-MS: $m/z$ = 319.07, 319.20 (M$^+$). Anal. Calcd. for C$^{11}$H$_{12}$N$_{2}$O$_{2}$: C, 53.12; H, 3.33; N, 16.62; S, 13.31% Found: C, 53.12, H, 3.31; N, 16.62, S, 13.31%.

7-Phenyl-9-(p-tolyl)pyrido[3′,2′:4,5]thieno[3,2-$d$]pyrimidine-2,4(1H,3H)-dione (2b) was obtained from 1b (0.25 g, 1 mmol) in 67% (0.19 g) yield as a buff solid, m.p. 250–251$^\circ$C. IR (KBr, $\nu_{max}$/cm$^{-1}$): 3370, 3154 (2NH), 3024 (CH-aromatic), 2925, 2855 (CH-aliphatic), 1728, 1662 (2C=O); ESI-MS: $m/z$ = 339.07, 339.20 (M$^+$). Anal. Calcd. for C$^{11}$H$_{13}$N$_{2}$O$_{2}$S: C, 53.11; H, 3.67; N, 16.99; S, 12.97% Found: C, 53.12, H, 3.91; N, 16.62; S, 13.13%.

3.1.3. Synthesis of 2,4-Dichloropyrido[3′,2′:4,5]thieno[3,2-$d$]pyrimidines 3a,b

To a solution of compounds 2a,b (1 mmol) in phosphorus oxychloride (20 mL), phosphorus pentachloride (0.41 g, 2 mmol) was added. The reaction mixture was refluxed for 15 h, then left to cool. The mixture was poured slowly onto crushed ice and the formed solid was separated by filtration, washed with water several times, and recrystallized from ethanol to yield the 2,4-dichloro compounds 3a,b.

2,4-Dichloro-7,9-dimethylpyrido[3′,2′:4,5]thieno[3,2-$d$]pyrimidine (3a) was obtained from 2a (0.25 g, 1 mmol) in 67% (0.19 g) yield as a buff solid, m.p. 250–251$^\circ$C. IR (KBr, $\nu_{max}$/cm$^{-1}$): 3015 (CH-aromatic), 2920, 2853 (CH-aliphatic), 825, 768 (C=C); $^1$H-NMR (DMSO-d$_6$, 400 MHz): $\delta$ = 2.54 (s, 3H, CH$_3$), 2.79 (s, 3H, CH$_3$), 7.21 (s, 1H, Ar-H), 10.59, 11.65 (2s, 2H, 2NH, D$_2$O exchangeable); $^{13}$C-NMR (DMSO-d$_6$, 100 MHz): $\delta$ = 20.40 (CH$_3$), 24.36 (CH$_3$), 108.64, 120.72, 122.85, 140.22, 145.63, 152.01, 160.07, 160.41, 161.48 (Ar-C, 2 C=O); ESI-MS: $m/z$ = 247.33 [M – H$^+$]. Anal. Calcd. for C$^{11}$H$_{13}$N$_{2}$O$_{2}$(SO$_2$): C, 53.43; H, 3.67; N, 16.99; S, 12.97% Found: C, 53.12, H, 3.91; N, 16.62; S, 13.13%.

3.1.4. Synthesis of 2,4-Dichloropyrido[3′,2′:4,5]thieno[3,2-$d$]pyrimidines 3a,b

To a solution of compounds 2a,b (1 mmol) in phosphorus oxychloride (20 mL), phosphorus pentachloride (0.41 g, 2 mmol) was added. The reaction mixture was refluxed for 15 h, then left to cool. The mixture was poured slowly onto crushed ice and the formed solid was separated by filtration, washed with water several times, and recrystallized from ethanol to yield the 2,4-dichloro compounds 3a,b.

7,9-Dimethylpyrido[3′,2′:4,5]thieno[3,2-$d$]pyrimidine-2,4(1H,3H)-diones 2a,b were prepared using the reported method [56].
2.4-Dichloro-7-phenyl-9-(p-tolyl)pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidine (3b) was obtained from 2b (0.39 g, 1 mmol) in 78% yield (0.31 g) as a yellow solid, m.p. 220 °C. IR (KBr, v_{\text{max}}/\text{cm}^{-1}): 3094 (CH-aromatic), 2922 (CH-aliphatic), 1621 (C=N), 1326, 1399, 1402, 1507, 1572, 1592, 1601, 1612 (Ar-C); ESI-MS: m/z = 421.31 [M−H^+]. Anal. Calcd. for C_{22}H_{13}ClN_{3}S (422.33): C, 62.57; H, 3.10; N, 9.95; S, 7.59% Found: C, 62.76, H, 3.35; N, 9.73; S, 7.78%.

3.1.4. Synthesis of 2-(Chloromethyl)pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin-4(3H)-ones 4a,b

To a cold solution of 1a,b (5 mmol) in acetonitrile (30 mL) at 0−5 °C, 2-chloroacetyl chloride (0.56 g, 5 mmol) was added dropwise while stirring. After addition, the reaction mixture was stirred for 1 h at room temperature and the solution was evaporated until dryness under reduced pressure, and then the oily residue was treated with hot petroleum ether 40−60. Then the formed solid was collected by filtration and recrystallized from ethanol to yield 4a,b.

2-(Chloromethyl)-7,9-dimethylpyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin-4(3H)-one (4a) was obtained from 1a (1.11 g, 5 mmol) in 78% yield (1.09 g) as a yellow solid, m.p. 330–331 °C. IR (KBr, v_{\text{max}}/\text{cm}^{-1}): 3387 (NH), 3097 (CH-aromatic), 2821 (CH-aliphatic), 1669 (C=O), 1772 (C-Cl); 1H-NMR (DMSO-d6, 400 MHz): δ = 2.59 (s, 3H, CH3), 2.88 (s, 3H, CH3), 4.66 (s, 2H, CH2Cl), 7.28 (s, 1H, Ar-H), 13.20 (s, 1H, NH, D2O exchangeable); 13C-NMR (DMSO-d6, 100 MHz): δ = 19.17, 24.37 (2CH2), 52.80 (CH2Cl), 123.27, 124.88, 144.56, 147.42, 152.87, 158.65, 159.42, 160.22 (Ar-C), 161.93 (C=O); ESI-MS: m/z = 278.69 [M−H^+]. Anal. Calcd. for C_{12}H_{10}ClN_{2}O_{3}S (279.74): C, 51.52; H, 3.60; N, 15.02; S, 11.46% Found: C, 51.28; H, 3.35; N, 14.82; S, 11.20%.

2-(Chloromethyl)-7-phenyl-9-(p-tolyl)pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin-4(3H)-one (4b) was obtained from 1b (1.79 g, 5 mmol) in 82% yield (1.61 g) as a brown solid, m.p. 220 °C. IR (KBr, v_{\text{max}}/\text{cm}^{-1}): 3440 (NH), 3094 (CH-aromatic), 2821 (CH-aliphatic), 1669 (C=O), 1772 (C-Cl); 1H-NMR (DMSO-d6, 400 MHz): δ = 2.59 (s, 3H, CH3), 2.88 (s, 3H, CH3), 4.66 (s, 2H, CH2Cl), 7.32 (d, 2H, J = 6.4 Hz, Ar-H), 7.45–7.57 (m, 3H, Ar-H), 7.66 (d, 2H, J = 6.4 Hz, Ar-H), 7.99 (s, 1H, Ar-H), 8.21 (d, 2H, J = 8.4 Hz, Ar-H), 9.79 (s, 1H, NH, D2O exchangeable); 13C-NMR (DMSO-d6, 100 MHz): δ = 16.32 (CH2), 53.71 (CH2Cl), 119.37, 126.63, 127.15, 127.94, 129.16, 129.51, 130.08, 132.64, 139.94, 140.57, 147.84, 148.12, 149.61, 155.69, 157.27, 158.65, 159.42, 160.22 (Ar-C), 161.93 (C=O); ESI-MS: m/z = 416.87 [M−H^+]. Anal. Calcd. for C_{23}H_{18}ClN_{3}OS (417.91): C, 66.10; H, 3.86; N, 10.06; S, 7.67% Found: C, 66.41, H, 4.06; N, 9.86; S, 7.88%.

3.1.5. Synthesis of 2-Substituted-7-phenyl-9-(p-tolyl)pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin-4(3H)-ones 5a,b

A mixture of 4b (0.42 g, 1 mmol) and the appropriate amine (1 mmol) in DMF (15 mL) was refluxed for 1 hr, and then the reaction mixture was poured onto cold water. The obtained precipitate was separated by filtration, washed with water, and recrystallized from ethanol to yield 5a,b.

2-(Morpholinomethyl)-7-phenyl-9-(p-tolyl)pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin-4(3H)-one (5a) was obtained by reaction of 4b with morpholine (0.087 g, 1 mmol) in 79% yield (0.07 g) as a pale yellow solid, m.p. 245 °C. IR (KBr, v_{\text{max}}/\text{cm}^{-1}): 3440 (NH), 3094 (CH-aromatic), 2919, 2854 (CH-aliphatic), 1662 (C=O); 1H-NMR (DMSO-d6, 400 MHz): δ = 2.38 (s, 4H, 2CH2N-morpholine), 2.39 (s, 3H, CH3), 3.29 (s, 2H, CH2N), 3.54 (s, 4H, 2CH2O), 7.28 (d, 2H, J = 10.4 Hz, Ar-H), 7.49–7.60 (m, 3H, Ar-H), 7.63 (d, 2H, J = 10.4 Hz, Ar-H), 7.97 (s, 1H, Ar-H), 8.27 (d, 2H, J = 8.4 Hz, Ar-H), 12.66 (s, 1H, NH, D2O exchangeable); 13C-NMR (DMSO-d6, 100 MHz): δ = 21.44 (CH3), 53.37 (2CH2N-morpholine), 60.65 (CH2N), 66.56 (2CH2O), 119.80, 127.49, 127.82, 128.47, 129.49, 130.45, 130.83, 132.14, 139.71, 140.50, 150.02, 150.40, 154.58, 157.49, 158.22, 160.01, 161.26 (Ar-C); ESI-MS: m/z = 421.31 [M−H^+]. Anal. Calcd. for C_{22}H_{13}ClN_{3}S (422.33): C, 62.57; H, 3.10; N, 9.95; S, 7.59% Found: C, 62.76, H, 3.35; N, 9.73; S, 7.78%.
151.48, 155.65, 157.06, 158.75 (Ar-C), 163.50 (C=O); ESI-MS: m/z = 467.50 [M − H+]∗. Anal. Calcd. for C_{27}H_{24}N_{3}O_{2}S (468.58): C, 69.21; H, 5.16; N, 11.96; S, 6.84 % Found: C, 69.48; H, 5.39; N, 11.72; S, 6.51%.

2-((4-Methylpiprazin-1-yl)methyl)-7-phenyl-9-(p-tolyl)pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidine-4(3H)-one (5b) was obtained by reaction of 4b with 4-methylpiprazine (0.10 g, 1 mmol) in 73% yield (0.35 g) as a yellowish white solid, m.p. 260–261 °C IR (KBr, v_{max} cm^{−1}): 3433 (NH), 3032 (CH-aromatic), 2920 (CH-aliphatic), 1655 (C=O); 1H-NMR (CDCl_{3}, 400 MHz): δ = 2.48 (s, 3H, CH_{3}), 2.54 (s, 3H, NCH_{3}), 2.81–3.01 (m, 8H, 4CH_{2}N-piprazine), 3.57 (s, 2H, NCH_{2}), 7.32 (d, 2H, J = 8 Hz, Ar-H), 7.48–7.54 (m, 3H, Ar-H), 7.64 (d, 2H, J = 8.4 Hz, Ar-H), 7.81 (1H, Ar-H), 8.17 (d, 2H, J = 6.8 Hz, Ar-H), 11.32 (s, 1H, NH, D_{2}O exchangeable); 13C-NMR (DMSO-d_{6}, 100 MHz): δ = 21.34 (CH_{3}), 48.22 (CH_{3}N), 56.13, 57.56 (4CH_{2}N-piprazine), 61.49 (CH_{2}N), 119.11, 126.96, 127.21, 127.79, 128.90, 129.01, 129.44, 130.08, 132.56, 139.31, 140.21, 145.01, 149.47, 150.17, 155.56, 157.15, 158.44 (Ar-C); ESI-MS: m/z = 480.64 [M − H^{+}]∗. Anal. Calcd. for C_{29}H_{27}N_{3}O_{5} (481.62): C, 69.83; H, 5.65; N, 14.54; S, 6.66 % Found: C, 69.62, H, 5.90; N, 14.21; S, 6.79%.

3.1.6. Synthesis of 1′H-Spiro[cycloalkane-1,2′-pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin]-4′(3′H)-ones 6a–d

A mixture of 1a,b (0.02 mol) and the appropriate cyclic ketone (0.03 mol) in DMF (50 mL) containing zinc chloride anhydrous (2.72 g, 0.02 mol) was heated under reflux for 6 h, and then the reaction mixture was poured onto cold water. The obtained precipitate was separated by filtration, washed with water, and recrystallized from DMF/H_{2}O to yield 6a–d.

7′,9′-Dimethyl-1′H-spirocyclopentane-1,2′-pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin]-4′(3′H)-one (6a) was obtained by reaction of 1a (4.42 g, 0.02 mol) with cyclopentanone (2.52 g, 0.03 mol) in 75% yield (4.31 g) as a brown solid, m.p. 245–246 °C IR (KBr, v_{max} cm^{−1}): 3319, 3210 (2NH), 3070 (CH-aromatic), 2921, 2828 (CH-aliphatic), 1650 (C=O); 1H-NMR (DMSO-d_{6}, 400 MHz): δ = 1.72–2.07 (m, 8H, 4CH_{2}), 2.51 (s, 3H, CH_{3}), 2.66s (3H, CH_{3}), 6.48 (s, 1H, NH, D_{2}O exchangeable), 7.04 (s, 1H, Ar-H), 9.82 (s, 1H, NH, D_{2}O exchangeable); 13C-NMR (DMSO-d_{6}, 100 MHz): δ = 20.41 (CH_{3}), 22.65, (2CH_{2}), 24.33 (CH_{3}), 37.55 (2CH_{2}), 70.15 (spiro C), 122.54, 123.29, 135.88, 145.63, 148.71, 159.30, 161.57, 167.65 (C=O); ESI-MS: m/z = 286.41 [M − H^{+}]∗. Anal. Calcd. for C_{15}H_{17}N_{3}O_{5} (287.38): C, 62.69; H, 5.96; N, 14.62; S, 11.16 % Found: C, 62.52, H, 5.74; N, 14.41; S, 10.98%.

7′-Phenyl-9′-(p-tolyl)-1′H-spirocyclopentane-1,2′-pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin]-4′(3′H)-one (6b) was obtained by reaction of 1b (7.19 g, 0.02 mol) with cyclopentanone (2.52 g, 0.03 mol) in 71% yield (6.04 g) as a yellow solid, m.p. 210 °C IR (KBr, v_{max} cm^{−1}): 3410, 3200 (2NH), 3055 (CH-aromatic), 2919 (CH-aliphatic), 1644 (C=O); 1H-NMR (DMSO-d_{6}, 400 MHz): δ = 1.21–1.85 (m, 8H, 4CH_{2}), 2.43 (s, 3H, CH_{3}), 5.94 (s, 1H, NH, D_{2}O exchangeable), 7.29 (d, 2H, J = 7.4 Hz, Ar-H), 7.41–7.62 (m, 5H, Ar-H), 7.81s (1H, NH, D_{2}O exchangeable), 7.96 (s, 1H, Ar-H), 8.22 (d, 2H, J = 10.2 Hz, Ar-H); 13C-NMR (DMSO-d_{6}, 100 MHz): δ = 21.36, 21.54 (2CH_{2}, CH_{3}), 38.33 (2CH_{2}), 70.81 (spiro C), 118.26, 121.25, 127.54, 127.65, 129.39, 129.79, 130.52, 132.83, 139.15, 139.31, 148.17, 150.62, 155.15, 163.25 (Ar-C); 13C-NMR (DMSO-d_{6}, 100 MHz): δ = 19.36, 20.16, 22.27, 24.32 (2CH_{2}, 2CH_{3}), 36.24 (2CH_{2}), 69.49 (spiro C), 122.11, 123.58, 134.96, 145.31, 148.44, 159.14, 161.51 (Ar-C); ESI-MS: m/z = 300.46 [M − H^{+}]∗. Anal. Calcd. for C_{16}H_{19}N_{3}O (301.41): C, 63.76; H, 6.35; N, 13.94; S, 10.64 % Found: C, 63.49, H, 6.17; N, 13.69; S, 10.41%.
7'-Phenyl-9'-(p-tolyl)-1'H-spiro[cyclohexane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidin]-4'(3'H)-one (6d) was obtained by reaction of 1b (7.19 g, 0.02 mol) with cyclohexanone (2.94 g, 0.03 mol) in 77% yield (6.77 g) as a yellow solid, m.p. 263 °C. IR (KBr, νmax/cm⁻¹): 3415, 3166 (2NH), 3053 (CH-aliphatic), 2924, 2854 (CH-aliphatic), 1660 (C=O); ¹H-NMR (DMSO-d6, 400 MHz): δ = 1.34–1.87 (m, 10H, 5CH₂), 2.42 (s, 3H, CH₃), 4.59 (s, 1H, NH, D₂O exchangeable), 7.38 (d, 2H, J = 6.8 Hz, Ar-H), 7.49–7.54 (m, 5H, Ar-H), 7.83 (s, 1H, Ar-H), 8.06 (s, 1H, NH, D₂O exchangeable), 8.21 (d, 2H, J = 6 Hz, Ar-H); ¹³C-NMR (DMSO-d6, 100 MHz): δ = 19.96, 21.38, 22.54 (3CH₂, CH₃), 35.86 (2CH₂), 70.67 (spiro C), 118.52, 121.27, 127.48, 127.84, 129.34, 129.67, 129.96, 130.86, 133.48, 137.29, 139.20, 139.50, 148.67, 155.50, 156.14 (Ar-C), 167.73 (C=O); ESI-MS: m/z = 438.52 [M – H⁺]. Anal. Calcd. for C₂₇H₂₂N₅O₃ (439.58): C, 73.77; H, 5.73; N, 9.56; S, 7.29% Found: C, 73.54, H, 5.49; N, 9.69; S, 7.59%.

3.1.7. Synthesis of 1'H-Spiro[cycloalkane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidine]-4'(3'H)-thiones 7a–d

A mixture of 6a–d (5 mmol) and phosphorus pentasulfide (1.11 g, 5 mmol) in pyridine (40 mL) was heated under reflux for 12 h. The reaction mixture was poured onto cold water and left overnight. The formed solid was separated by filtration, washed with water, and recrystallized from 1,4-dioxane to yield compounds 7a–d.

7',9'-Dimethyl-1'H-spiro[cyclopane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidine]-4'(3'H)-thione (7a) was obtained from 6a (1.44 g, 5 mmol) in 63% yield (0.95 g) as a burnt orange solid, m.p. 220–221 °C. IR (KBr, νmax/cm⁻¹): 3372, 3157 (2NH), 3063 (CH-aliphatic), 2922 (CH-aliphatic), 1247 (C=O); ¹H-NMR (DMSO-d6, 400 MHz): δ = 1.36–2.03 (m, 8H, 4CH₂), 2.57 (s, 3H, CH₃), 2.73 (s, 3H, CH₃), 6.49 (s, 1H, NH, D₂O exchangeable), 7.03 (s, 1H, Ar-H), 9.85 (s, 1H, NH, D₂O exchangeable); ¹³C-NMR (DMSO-d6, 100 MHz): δ = 19.56, 22.53 (CH₃), 24.43 (CH₃), 37.67 (2CH₂), 78.59 (spiro C), 117.93, 122.11, 123.15, 139.87, 146.17, 159.21, 162.86 (Ar-C), 182.25 (C=O); ESI-MS: m/z = 302.40 [M – H⁺]. Anal. Calcd. for C₁₃H₁₅N₂S₂ (303.44): C, 59.37; H, 5.65; N, 13.85; S, 21.13% Found: C, 59.09, H, 5.47; N, 13.56, S, 20.89%.

7'-Phenyl-9'-(p-tolyl)-1'H-spiro[cyclohexane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidine]-4'(3'H)-thione (7b) was obtained from 6b (2.13 g, 5 mmol) in 68% yield (1.50 g) as a brown solid, m.p. 180 °C. IR (KBr, νmax/cm⁻¹): 3402, 3186 (2NH), 3055 (CH-aliphatic), 2929, 2852 (CH-aliphatic), 1232 (C=O); ¹H-NMR (DMSO-d6, 400 MHz): δ = 1.34–2.01 (m, 8H, 4CH₂), 2.42 (s, 3H, CH₃), 4.67 (s, 1H, NH, D₂O exchangeable), 7.42 (d, 2H, J = 7.2 Hz, Ar-H), 7.48–7.53 (m, 5H, Ar-H), 7.83 (s, 1H, Ar-H), 8.22 (d, 2H, J = 7.2 Hz, Ar-H), 9.68 (s, 1H, NH, D₂O exchangeable); ¹³C-NMR (DMSO-d6, 100 MHz): δ = 21.47, 21.96 (2CH₂, CH₃), 36.50 (2CH₂), 77.79 (spiro C), 118.42, 121.20, 124.67, 127.47, 127.71, 129.11, 129.74, 130.20, 133.56, 139.21, 139.34, 148.17, 149.62, 155.16, 163.25 (Ar-C), 183.35 (C=O); ESI-MS: m/z = 440.69 [M – H⁺]. Anal. Calcd. for C₂₆H₂₃N₅S₂ (441.61): C, 70.72; H, 5.25; N, 9.52; S, 14.52% Found: C, 70.53, H, 5.01; N, 9.37; S, 14.38%.

7',9'-Dimethyl-1'H-spiro[cyclohexane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidine]-4'(3'H)-thione (7c) was obtained from 6c (1.51 g, 5 mmol) in 71% yield (1.13 g) as a greenish grey solid, m.p. 201 °C. IR (KBr, νmax/cm⁻¹): 3427, 3190 (2NH), 3048 (CH-aliphatic), 2929, 2853 (CH-aliphatic), 1234 (C=O); ¹H-NMR (DMSO-d6, 400 MHz): δ = 1.21–2.11 (m, 10H, 5CH₂), 2.51 (s, 3H, CH₃), 2.71 (s, 3H, CH₃), 6.22 (s, 1H, NH, D₂O exchangeable), 7.07 (s, 1H, Ar-H), 9.68 (s, 1H, NH, D₂O exchangeable); ¹³C-NMR (DMSO-d6, 100 MHz): δ = 19.26, 22.02, 24.32, 25.15 (3CH₂, 2CH₃), 34.46 (2CH₂), 70.16 (spiro C), 118.38,122.29, 123.61, 138.66, 146.42, 159.31, 162.71 (Ar-C), 181.31 (C=O); ESI-MS: m/z = 316.40 [M – H⁺]. Anal. Calcd. for C₁₅H₁₉N₅S₂ (317.47): C, 60.53; H, 6.03; N, 13.24; S, 20.20% Found: C, 60.79, H, 6.17; N, 13.49; S, 20.44%.

7'-Phenyl-9'-(p-tolyl)-1'H-spiro[cyclohexane-1,2'-pyrido[3',2':4,5]thieno[3,2-d]pyrimidine]-4'(3'H)-thione (7d) was obtained from 6d (2.19 g, 5 mmol) in 66% yield (1.50 g) as a yellow solid, m.p. 178–179 °C. IR (KBr, νmax/cm⁻¹): 3402, 3188 (NH), 3068 (CH-aliphatic), 2923, 2852 (CH-aliphatic), 1232 (C=O); ¹H-NMR (DMSO-d6, 400 MHz): δ = 1.2–2.07 (m, 10H, 5CH₂), 2.43 (s, 3H, CH₃), 4.79 (s, 1H, NH, D₂O exchangeable), 7.39 (d, 2H, J = 8.4 Hz,
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3.1.8. Synthesis of 2-((1'H-Spiro[cycloalkane-1,2'-pyrido][3'2',4:5']thieno[3,2-d]pyrimidin]-4'-yl)sulfonyl)acetamides 8a,b

A mixture of compounds 7c,b (2 mmol) and 2-chloroacetamide (0.187 g, 2 mmol) in DMF (30 mL) containing sodium carbonate anhydrous (0.5 g) was refluxed for 6 h. The reaction mixture was poured onto iced water and the medium was neutralized with 1N HCl to pH = 7. The formed precipitate was separated by filtration, washed with water, and recrystallized from chloroform to yield the acetamide derivatives 8a,b.

3.1.9. Synthesis of 4'-(oxiran-2-ylmethyl)sulfonyl)-1'H-spiro[cycloalkane-1,2'-pyrido][3'2',4:5']thieno[3,2-d]pyrimidin]-4'-yl]acetamides 9a,b

A mixture of compounds 7c,b (1 mmol) and epichlorohydrin (0.092 g, 1 mmol) in acetone (20 mL) containing triethyl amine (0.1 mL) was refluxed for 5 h. The solvent was evaporated until dryness and the oily residue was treated with hot petroleum ether (30 mL). The formed solid was separated by filtration and recrystallized from ethanol to yield 9a,b.
4′-((Oxiran-2-ylmethyl)sulfanyl)-7′-phenyl-9′-(p-tolyl)-1′H-spiro[cyclopentane-1,2′-pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidine] (9b) was obtained from 7b (0.44 g, 1 mmol) in 74% yield (0.37 g) as a pale yellow solid, m.p. 120 °C. IR (KBr, \( \nu_{\text{max}} \)/cm\(^{-1} \)): 3422 (NH), 3094 (CH-aromatic), 2919 (CH-aliphatic), (C=N); \(^1\)H-NMR (DMSO-d\(_6\), 400 MHz): \( \delta = 1.35–1.94 \) (m, 8H, 4CH\(_2\)), 2.43 (s, 3H, CH\(_3\)), 2.94–3.13 (2m, 2H, OCH\(_2\)-oxirane), 3.51 (d, 2H, \( J = 6.0 \) Hz, SCH\(_2\)), 3.91–3.95 (m, 1H, OCH-oxirane), 6.87 (s, 1H, NH, D\(_2\)O exchangeable), 7.32 (d, 2H, \( J = 7.2 \) Hz, Ar-H), 7.48–7.69 (m, 5H, Ar-H), 7.87 (s, 1H, Ar-H), 8.26 (d, 2H, \( J = 7.6 \) Hz, Ar-H); \(^1^3\)C-NMR (DMSO-d\(_6\), 100 MHz): \( \delta = 21.40 \) (CH\(_3\)), 29.15 (2CH\(_2\)), 31.88 (SCH\(_2\)), 36.33 (2CH\(_2\)), 46.33, 53.45 (OCH\(_2\), OCH, oxirane), 69.33 (spiro C), 118.49, 123.62, 127.81, 127.93, 128.78, 128.94, 129.40, 129.50, 130.53, 133.77, 139.17, 140.17, 148.73, 152.73, 156.17, 163.83 (Ar-C); ESI-MS: \( m/z = 496.70 \) [M−H\(^+\)]. Anal. Calcd. for C\(_{29}\)H\(_{27}\)N\(_3\)O\(_2\) (497.68): C, 69.99; H, 5.47; N, 8.44; S, 12.88%; Found: C, 69.81, H, 5.31; N, 8.20; S, 12.63%.

### 3.2. Antimicrobial Assay

All synthesized compounds (2a,b–9a,b) were screened for their in vitro antimicrobial activity against five bacterial strains (S. aureus 25923, B. subtilis 6633, B. cereus 33018, E. coli 8739, S. typhimurium 14028), three yeasts (C. albicans 10231, C. tropicals 750, S. cerevisiae) and two fungi (A. flavus, A. niger EM77). The MIC values (in \( \mu \)g/mL) of the tested compounds were determined using the broth dilution method and are listed in Tables 1 and 2 [57]. (More details are provided in the Supplementary Materials).

### 3.3. In Vitro Cytotoxicity Screening

The in vitro cytotoxic activity of the target compounds 2a,b–9a,b was screened against HepG-2 and MCF-7 cancer cell lines, as well as the WISH normal cell line for the most active compounds, using the MTT assay [53]. The cells used in the cytotoxicity assays were cultured in a RPMI 1640 medium supplemented with 10% fetal calf serum. The cytotoxicity was estimated as IC\(_{50}\) in \( \mu \)M for the tested compounds and the reference drug doxorubicin, and are listed in Table 3. (More details are provided in Supplementary Materials).

### 3.4. EGFR Kinase Inhibitory Assay

EGFR kinase inhibitory assays were performed for target compounds 3a, 4a, 5a, 6b, 8b and 9b with erlotinib as a reference inhibitor using the EGFR kinase assay kit (Cat. # 40321). The assay kit is designed to measure EGFR Kinase activity for screening applications using Kinase-Glo® MAX as a detection reagent. The luminescence was measured using a microplate reader (Infinite M200 microplate reader, Tecan, Männedorf, Switzerland) [54]. All assays were performed in triplicate and the relative inhibition (%) of inhibitors was then calculated via comparison with the control with no inhibitor. Then, the IC\(_{50}\) values (the concentration that provides 50% enzyme inhibition) and their standard deviation (SD) for the tested compounds and the reference drug were determined in \( \mu \)M and are listed in Table 4. (More details are provided in the Supplementary Materials).

### 3.5. Molecular Modeling Studies

To investigate molecular interactions between the most potent compounds and the active site of the epidermal growth factor receptor (EGFR), molecular docking study was performed using molecular operating environment software (MOE 2019.0102). Energy minimization was carried out until a RMSD gradient of 0.1 kcal·mol\(^{-1}\)Å\(^{-1}\) was achieved using a MMFF94x force field. The co-crystalized ligand (Erlotinib) was used to define the binding site for docking [58,59]. (More details are provided in the Supplementary Materials).

### 4. Conclusions

In conclusion, two novel series of pyridothienopyrimidine and spiro[cyclopentane-hexane-1,2′-pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidine] derivatives 2a,b–5a,b and 6a,b–9a,b, were synthesized and structurally elucidated. The new derivatives were subjected to
in vitro antimicrobial screening against a panel of bacterial and fungal pathogens. According to the MIC values, derivatives 3a, 4a, 5a, 6a, 6b, 7c, 8b, and 9b exhibited significant antibacterial and antifungal activity with MIC ranges of 4–16 µg/mL, compared to amoxicillin trihydrate and clotrimazole as reference drugs with MIC ranges of 4–16 µM.

Furthermore, all new derivatives were evaluated as cytotoxic agents against HepG2 and MCF7 cancer cell lines. Compounds 9b, 5a, 3a, 6b, 8b, 4a produced the most potent antiproliferative activity with IC₅₀ values ranging between 1.17–2.99 µM against HepG2 cells and IC₅₀ values ranging between 1.52–15.42 µM against MCF-7 cells, compared to doxorubicin as reference drug with IC₅₀ of 2.85 and 3.58 µM, respectively. In addition, these derivatives exhibited a promising safety profile when evaluated against the human normal WISH cell line.

Compounds 3a, 4a, 5a, 6b, 8b, 9b presented promising dual antimicrobial and antiproliferative activities, achieving the desired goal of this study.

The suppressing effect of compounds 3a, 4a, 5a, 6b, 8b, 9b against EGFR TK was also evaluated. It was detected that compounds 9b, 5a, 4a showed higher suppressing activity than erlotinib with IC₅₀ values of 7.27, 9.66 and 27.01 nM, respectively. In addition, molecular docking study was performed to determine the modes of interaction of the examined derivatives with amino acid residues at the active site of EGFR-PK. The docking results revealed that compounds 9a and 5a showed higher binding scores (–12.01 and –11.48 kcal/mol) than that of erlotinib (–10.48 kcal/mol).

Supplementary Materials: Figures S2–S34: NMR spectral data of the new compounds, S35: in vitro antimicrobial assay, S35: cytotoxicity MTT assay, S36: in vitro EGFR kinase inhibitory assay, S37: docking modeling evaluation.

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