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Short communication

Synthesis and antiproliferative activity of indolizine derivatives incorporating a cyclopropylcarbonyl group against *Hep-G2* cancer cell line

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ABSTRACT

Indolizine and annulated indolizine derivatives incorporating a cyclopropylcarbonyl group were synthesized in a one pot procedure by the tanden reactions of [3 + 2] cycloaddition of the corresponding *N*-ylide with electron deficient alkene. Seventeen indolizine derivatives were reported for the first time. All the compounds were examined for their antiproliferative activity against the human hepatocellular liver carcinoma (*Hep-G2*) cell line by MTT method. Among the compounds tested, **5a**, **5d**, **5g** and **5j** showed the most favorable activities with IC_{50} values of 0.39, 0.48, 0.29 and 0.20 μg/mL. Especially, compound **5j** displayed potent antiproliferative activities with IC_{50} value of 0.20 μg/mL, and showed significant EGFR kinase inhibitory activity with IC_{50} value of 0.085 μM. Docking simulations of **5j** were carried out to illustrate the binding mode of the molecular into the EGFR active site.

1. Introduction

The indolizine and hydrogenated indolizine structures are found in many alkaloids such as amorine, crythraline, swainsonine, cryptaustoline, cryptowoline [1,2], camptothesin [3], nuevamine [4,5], etc. These natural and many synthetic indolizine derivatives have been found to have a variety of biological activity, such as anti-inflammatory [6,7], antiviral [8], aromatase inhibitory [9], analgestic [10], antitumor [11,12] activities. Besides, some of them are potent antioxidants inhibiting lipid peroxidation *in vitro* [13–15]. As a result, indolizine derivatives have now become important target compounds in developing new pharmaceuticals for the treatment of cancer, cardiovascular diseases [16], and HIV infections [17,18].

Meanwhile, the cyclopropane is a noteworthy structural motif because of its own biological activity and its effect to modify the bioactivity of the parent compounds [19,20]. As a rigid yet chemically relatively stable structural unit, cyclopropane is sterically smaller than isopropyl and less sterically demanding than germinal dimethyl. Because of these characteristics, cyclopropyl was more and more to be taken into consideration in designing new pharmaceuticals [21]. The introduction of a cyclopropyl or its replacement of an alicyclic chain or a larger alicyclic ring often showed beneficial effect to the bioactivity of the original targets. However, there were only a few scatter report on indolizine derivatives containing a cyclopropyl group and their biological activity are rarely investigated [22,23]. The hepatocellular carcinoma (HCC) is the most often seen histological type of primary liver carcinoma and is one of the cancer types of highest incidence in the world [24]. Although there were several reports on the antitumor activity of indolizines, their bioactivity against HCC have not been investigated. With the aim of searching for new pharmaceuticals against HCC, we designed and synthesized a series of indolizines and annulated indolizines incorporating a cyclopropylcarbonyl group and examined their activity against the *Hep-G2* cell line.

Receptor protein tyrosine kinases play a key role in signal transduction pathways that regulate cell division and differentiation. Among the growth factor receptor kinases, EGFR kinase (also known as erb-B1 or HER-1) is important in cancer deregulation of growth-factor signaling due to hyperactivation of EGFR is seen in several cancer types. Compounds that inhibit the kinase activity of EGFR after binding of its cognate ligand are of potential interest as new therapeutic antitumor agents [25,26]. Docking simulations of compound **5j** were carried out to give structural insights into the binding mode with the epidermal growth factor receptor tyrosine kinase (EGFR TK), to illustrate the antiproliferative activities against the *Hep-G2* cancer cell line. In the binding model, compound **5j** is

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nicely bound to the amino hydrogen of Met 769, with the −CN forming a more optimal H-bond interaction, and carbonyl group of compound **5j** also forms hydrogen bond with the side chain amino hydrogen of Lys 721. This molecular docking result, along with the data of EGFR inhibition assay, suggesting that compound **5j** is a potential inhibitor of EGFR with potent antiproliferative activity.

2. Result and discussion

2.1. Synthesis of 3-Cyclopropylcarbonylindolizines

Reaction of cyclopropylbromomethylmethanone with the pyridine derivatives in ethyl acetate or under solvent free conditions afforded the pyridinium salts in moderate to high yield. (Scheme 1 and Table 1).

The [3 + 2] cycloadditions of the in situ prepared pyridinium ylide with the electron deficient alkene were carried out in DMF solution in the presence of triethylamine as a base and the oxidant tetrakispyridinecobalt(II) dichromate [$Py_4Co(HCrO4)_2$] (TPCD) [27] as a dehedrogenative reagent. The primary cycloadduct tetrahydroindolizine was oxidatively dehydrogenated in situ by TPCD to give the final product. By these one pot tandem reactions, 3-cyclopropylcarbonylindolizines (**5a**–**5p**) and the yet unknown imidazo[2,1-*f*]pyrrolo[1,2-*b*]pyridazine derivatives(**6a, 6b**) were prepared (Schemes 2 and 3, Table 2). The structures of the products are based on their spectral (IR, ^1H and ^{13}C NMR, MS) and elemental analysis data and are further confirmed by an X-ray crystallographic analysis of product **5e** (Fig. 1).

It is seen in Table 2 that when R_2 is a cyano group, the yields of the products (**5a, 5e, 5h, 5j, 5l, 5n** and **6a**) are significantly higher than those when R_2 is an ester group (**5b, 5c, 5f, 5g, 5i, 5k, 5m, 5o, 5p** and **6b**). This is because the dipolarophiles methyl acrylate and dimethyl maleate are prone to polymerize under heating. As a matter of fact, a trimer of the dimethyl maleate (trimethyl 1,3,5-benzene tricarboxylate) was isolated from the reaction mixture after column chromatography.

The cycloadditions are regioselective, with the electron withdrawing group (the cyano or the ester group) bonded to the C_{ortho} atom in the pyridinium ylide to give the 1-cyano (1-alkoxycarbonyl) 3-cyclopropylcarbonyl indolizines and their annulated products. This regioselectivity can be rationalized by considering the frontier molecular orbital (FMO) interactions [28] in the two reactants as shown in Fig. 2. It can be seen that in these 1,3-dipolar cycloadditions, in the predominant $HOMO_{(ylide)}$-$LUMO_{(alkene)}$ interactions, maximum positive FMO overlap indeed result in the actually observed regioselectivity.

It is also noted that by using maleimide as the dipolarophile, beside the normal product **5d**, the indolizinecarboxamide product **5d′** (Scheme 4) was also obtained. This may derive from decarboxylative hydrolysis of **5d** in the basic reaction media under heating. It is known that the indolizine carboxylic acid derivatives are difficult to undergo aminolysis reaction [29], therefore, our finding of formation of **5d′** from **5d** might provide a route for the access of the indolizinecarboxamides after the reaction conditions are further improved and optimized.

Table 1
Yield and melting point of the pyridinium salts.

Iminium salts	R	m.p. (°C)	Yield (%)
2a	H	160–162	97
2b	4-CH_3	172–173	98
2c	2-Br	174–175	49
2d	2-CH_3,5-Br	166–168	62
2e	2,3-benzo	180–181	61
2f	3,4-benzo	187–188	99
3	–	208–209	98

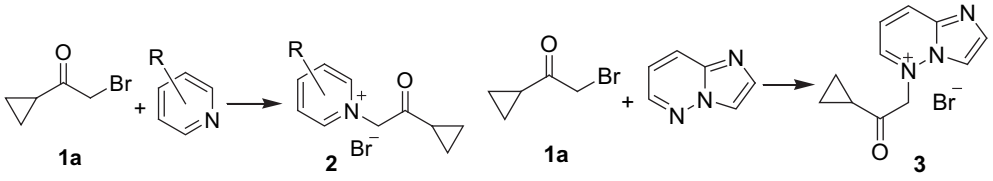
2.2. Biological assay

The *in vitro* antiproliferative activities of the above synthesized indolizines were studied on the human liver cancer cell line *Hep-G2* by applying the MTT colorimetric assay. Compounds were tested over a range of concentrations from 0.1 to 40 μg/mL, and the calculated IC_{50} values, that is, the concentration (μg/mL) of a compound able to cause 50% of cell death with respect to the control culture, are reported in Table 3.

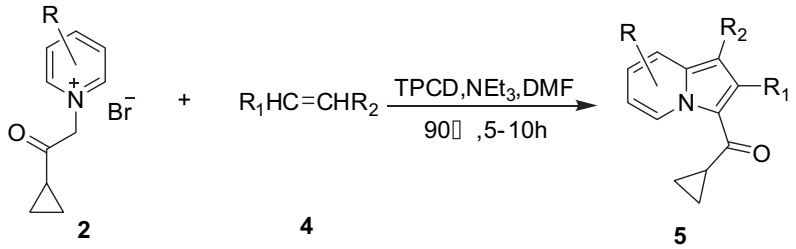
The results in Table 3 show that several of the products (**5a**, **5d**, **5g** and **5j**) strongly exhibit the most potent activity with IC_{50} values of 0.39, 0.48, 0.29 and 0.2 μg/mL. It is also seen that as a general trend, the indolizines with a 1−CN group usually have better antiproliferative activity than the corresponding analog with a 1-ester functionality (eg. **5a** vs. **5b**, **5e** vs. **5f**, **5h vs 5i, 5l** vs. **5m**, **6a** vs. **6b**). The presence of methyl group and halo atom has significant influence on the anticancer activity. Furthermore, the carboxamide group (**5d** $IC_{50} = 0.48$ μg/mL and **5d′** $IC_{50} = 0.56$ μg/mL) can significantly increase the activity. The benzo fusion of the indolizines at either 5,6- (**5l** and **5m**) or 7,8- (**5n** an **5o**) positions showed no better antiproliferative activities against *Hep-G2* and these annulated indolizines turned out to be rather weaker antiproliferative agents. Compounds **6a** and **6b** show the same trend. Furthermore, compound **5j** was evaluated for their ability to inhibit EGFR tyrosine kinases. The results (Table 4) indicated that compound **5j** exhibited potent activity with IC_{50} values of 0.085 μM, which was comparable to the positive control Iressa.

2.3. Binding model of compound 5j into EGFR

Molecular docking of the most potent antiproliferative compound **5j** into ATP binding site of EGFR kinase was performed on the binding model based on the EGFR complex structure (1M17.pdb). The binding model of compound **5j** and EGFR is depicted in Fig. 3. In the binding model, compound **5j** is nicely bound to the region with the −CN group project toward the amino hydrogen of Met 769, with the −CN forming a more optimal H-bond interaction, and carbonyl group of compound **5j** also forms hydrogen bond with the side chain amino hydrogen of Lys 721. This molecular docking result suggesting that compound **5j** is a potential inhibitor of EGFR with potent antiproliferative activity.



Scheme 1. Synthesis of pyridinium salts.



5a R=H R_1=H R_2=CN
5b R=H R_1=H R_2=CO_2CH_3
5c R=H R_1=CO_2Et R_2=CO_2Et
5d R=H R_1=1,2-N-phenylmaleimide
5e R=7-CH_3 R_1=H R_2=CN
5f R=7-CH_3 R_1=H R_2=CO_2CH_3
5g R=7-CH_3 R_1=CO_2Et R_2=CO_2Et
5h R=5-Br R_1=H R_2=CN
5i R=5-Br R_1=H R_2=CO_2CH_3
5j R=5-CH_3,8-Br R_1=H R_2=CN
5k R=5-CH_3,8-Br R_1=H R_2=CO_2CH_3
5l R=5,6-benzo R_1=H R_2=CN
5m R=5,6-benzo R_1=H R_2=CO_2CH_3
5n R=7,8-benzo R_1=H R_2=CN
5o R=7,8-benzo R_1=H R_2=CO_2CH_3
5p R=7,8-benzo R_1=CO_2CH_3 R_2=CO_2CH_3

Scheme 2. Synthesis of compounds **5a**–**5p**.

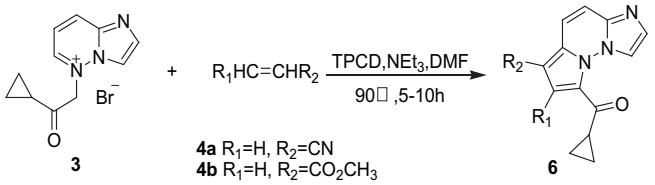
3. Conclusions

In summary, indolizine and annulated indolizine derivatives incorporating a cyclopropylcarbonyl group are synthesized in a one pot procedure by the tanden reactions of [3 + 2] cycloaddition of the corresponding *N*-ylide with electron deficient alkene and the dehydroaromatization of the primary cycloadduct under the action of the mild oxidant TPCD. Antiproliferative experiment against the human hepatocellular liver carcinoma (*Hep-G2*) cell line were examined with 5-fluorouracil as a reference compound. Several of the synthesized indolizines (**5a**, **5d**, **5g** and **5j**) show strong anticancer activity comparable with 5-fluorouracil. Therefore, the cyclopropyl incorporated indolizines turned out to be promising candidates in developing new agents against the hepatocellular carcinoma (HCC). Molecular docking result suggesting that compound **5j** is a potential inhibitor of EGFR with potent antiproliferative activity. Further investigation on the structure-activity relationship on this new type of HCC inhibiting compounds is being undertaken.

4. Experimental section

4.1. Chemistry

Melting points were measured on an X-4 (Taike Corp., Beijing, China) microscopic melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker ACF-400 spectrometer with $CDCl_3$ as solvent unless otherwise specified. ^{13}C NMR spectra were measured on a Bruker ACF-400 spectrometer at 100 MHz with $CDCl_3$ as solvent. The chemical shifts (δ) are reported in ppm relative to the residual undeuterated solvent signal, and coupling constants (J) are given in Hz. IR spectra were taken with a Nicolet FT-IR 5DX spectrometer for samples were characterized by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy. Mass spectra (EI) were recorded with a VG ZAB-HS spectrometer. Elemental analyses were obtained using a Heraeus CHN-O-Rapid analyzer. For X-ray Crystallographic analysis, the X-ray diffraction intensities and the unit cell parameters were determined on a Brucker SMART APEXII CCD diffractometer employing graphite-monochromated (Mo-Kα) radiation ($\lambda = 0.71073$ Å) and operating in the $\omega/2\theta$ scan mode. Data collection and cell refinement were performed with APEX2 Software. Structures were solved by direct methods and refined by full-matrix least-squares on F_2 with SHELXTL. Non-hydrogen atoms were refined by anisotropic displacement parameters, and the positions of all H-atoms were fixed geometrically and included in estimated positions using a riding model.



4a R_1=H, R_2=CN
4b R_1=H, R_2=CO_2CH_3

Scheme 3. Synthesis of compounds **6a** and **6b**.

4.2. General procedure for the synthesis of the pyridinium salts

A mixture of pyridine (7.9 g, 100 mmol) and cyclopropylbromomethylmethanone (12 g, 100 mmol) in EtOAc (50 mL) was stirred at room temperature for 0.5 h. After it stood for another 48 h, the precipitated solid was collected and rinsed with EtOAc (50 mL) to give **2a**. Salts **2b**–**2f**, **3** were prepared by the same procedure and they were directly used in the next step without any further purification.

Table 2
Synthesis of the 3-Cyclopropylcarbonylindolizines and 6-Cyclopropylcarbonyl-imidazo[2,1-f]pyrrolo[1,2-b]pyridazines.

Compd.	R	R_1	R_2	Yield (%)
5a	H	H	CN	73
5b	H	H	CO_2CH_3	42
5c	H	CO_2Et	CO_2Et	58
5d	H	1,2-*N*-phenylmaleimide		51[a]
5e	7-CH_3	H	CN	70
5f	7-CH_3	H	CO_2CH_3	35
5g	7-CH_3	CO_2Et	CO_2Et	50
5h	5-Br	H	CN	48
5i	5-Br	H	CO_2CH_3	46
5j	5-CH_3,8-Br	H	CN	75
5k	5-CH_3,8-Br	H	CO_2CH_3	50
5l	5,6-benzo	H	CN	57
5m	5,6-benzo	H	CO_2CH_3	55
5n	7,8-benzo	H	CN	93
5o	7,8-benzo	H	CO_2CH_3	66
5p	7,8-benzo	CO_2CH_3	CO_2CH_3	76
6a	–	H	CN	50
6b	–	H	CO_2CH_3	40

[a] Another product **5d′** was also obtained in 26% yield (vide infra).

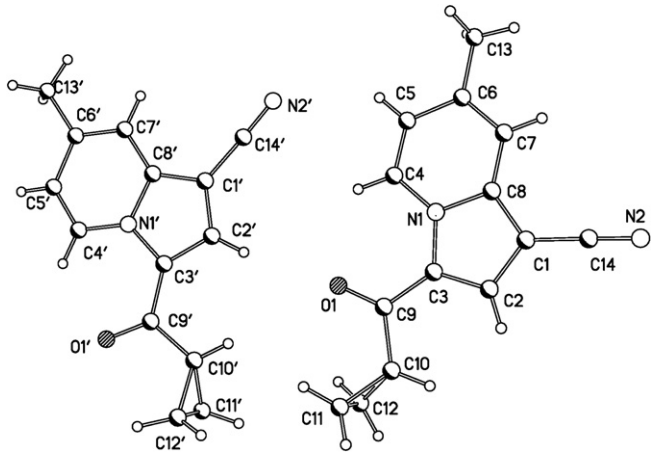


Fig. 1. ORTEP drawing of compound **5e**.

4.3. General procedure for the synthesis and purification of the indolizines

A mixture of the pyridinium salt (10 mmol), acrylonitrile (40 mmol), triethylamine (2 ml) and TPCD (4 g) in DMF (40 ml) was heated at 90 °C for 5 hours. After cooling, the reaction mixture was poured into an aqueous hydrochloric acid solution (5%, 100 ml), the precipitated crude product was collected by filtration and further purified by silica gel column chromatography with petroleum ether (bp 60–90 °C)-ethyl acetate as eluents.

4.3.1. 3-Cyclopropylcarbonyl-1-indolizinecarbonitrile (**5a**)

White powder. m.p. 163–164 °C. IR (ATR) ν: 3114, 2924, 2213, 1637, 1628, 942, 758 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.04(s, 2H, CH_2), 1.25(s, 2H, CH_2), 2.51(s, 1H, CH), 7.06(s, 1H, py-H), 7.42(s, 1H, py-H), 7.79(s, 1H, pyrrole-H), 7.94(s, 1H, py-H), 9.90(s, 1H, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 10.7, 18.2, 84.4, 115.3, 115.6, 117.4, 123.8, 125.7, 127.2, 129.5, 140.7, 189.5. MS m/z (%) = 210 (M^+, 100), 169 (46), 141 (15), 114 (8). $C_{13}H_{10}N_2O$ (210.23): calcd. C 74.25, H 4.80, N 13.30; Found C 74.27, H 4.79, N 13.33.

4.3.2. Methyl 3-cyclopropylcarbonylindolizine-1-carboxylate (**5b**)

Yellow powder. m.p.135–136 °C. IR (ATR) ν: 3113, 2951, 1697, 1618, 756 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.97–1.02(m, 2H, CH_2), 1.21–1.25(m, 2H, CH_2), 2.56–2.62(m, 1H, CH), 3.94(s, 3H, OCH_3), 6.99(t, 1H, J = 7.0 Hz, py-H), 7.38(t, 1H, J = 7.8 Hz, py-H), 8.17 (s, 1H, pyrrole-H), 8.34(d, 1H, J = 9.2 Hz, py-H), 9.90(d, 1H, J = 6.8 Hz, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 10.3, 18.0, 51.3, 105.4, 115.0, 119.2, 123.3, 125.4, 127.0, 129.087, 139.2, 164.5, 189.8. MS m/z (%) = 243 (M^+, 100), 212 (10), 202 (19). $C_{14}H_{13}NO_3$ (243.26): calcd. C 69.07, H 5.43, N 19.77; Found C 69.12, H 5.39, N 5.76.

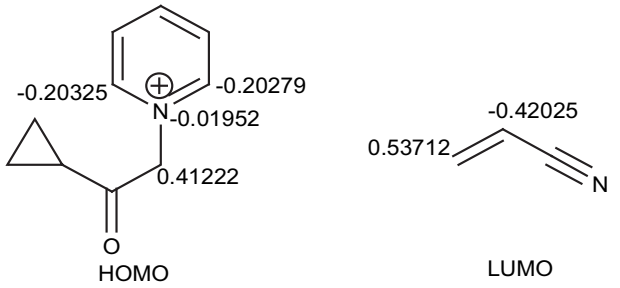
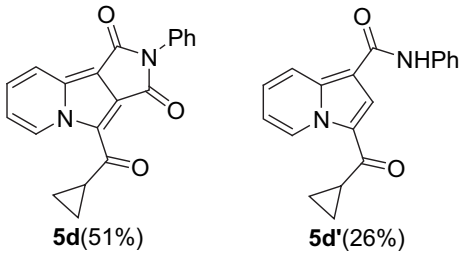


Fig. 2. FMOs in the pyridinium ylide and in acrylonitrile.



Scheme 4. Chemical structures of compounds **5d** and **5d′**.

4.3.3. Diethyl 3-cyclopropylcarbonylindolizine-1,2-dicarboxylate (**5c**)

White crystal. m.p. 69–70 °C.IR ν: 2988, 1728, 1690, 1622, 1207, 963, 784, 756 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.95–0.99(m, 2H, CH_2), 1.25–1.29(m, 2H, CH_2), 1.39–1.44(m, 6H, CH_3), 2.34–2.41 (m, 1H, CH), 4.40(q, 2H, J = 7.1 Hz, OCH_2), 4.48(q, 2H, J = 7.2 Hz, OCH_2), 7.03(t, 1H, J = 7.0 Hz, py-H), 7.41(t, 1H, J = 7.8 Hz, py-H), 8.36 (d, 1H, J = 8.8 Hz, py-H), 9.82(d, 1H, J = 7.2 Hz, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 11.0, 14.0, 14.4, 19.1, 60.5, 62.3, 103.8, 115.8, 119.7, 127.7, 129.036, 130.8, 133.6, 137.6, 163.1, 167.0, 190.1. MS m/z (%) = 329 (M^+, 100), 227 (12). $C_{18}H_{19}NO_5$ (329.35): calcd. C 65.60, H 5.79, N 4.27; Found C 65.64, H 5.81, N, 4.25.

4.3.4. 4-Cyclopropylcarbonyl-2-methyl-1,3-dioxo-1H-pyrrolo[3,4-a]indolizine (**5d**)

Yellow powder. m.p.211–212 °C. IR (ATR) ν: 3124, 2961, 1760, 1699, 1628, 1358, 1062, 912, 808, 748, 696 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.10–1.15(m, 2H, CH_2), 1.26–1.30(m, 2H, CH_2), 3.81-3.87(m, 1H, CH), 7.10–7.13(m, 1H, py-H), 7.39–7.46(m, 3H, Ar-H), 7.47–7.53(m, 3H, Ar-H, py-H), 8.00(d, 1H, J = 8.8 Hz, py-H), 10.05 (d, 1H, J = 7.2 Hz, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 12.2, 19.9, 110.6, 116.7, 118.6, 118.8, 127.1, 128.0, 128.6, 129.0, 130.1, 130.6, 131.9, 132.3, 162.5, 163.9, 191.6. MS m/z (%) = 330 (M^+, 100), 245 (5), 238 (21). $C_{20}H_{14}N_2O_3$ (330.34): calcd. C 72.05, H 4.24, N 8.44; Found C 72.72, H 4.27, N 8.48.

4.3.5. 3-Cyclopropylcarbonyl-N-phenyl-1-indolizinecarboxamide (**5d′**)

Yellow powder. m.p.213–215 °C. IR (ATR) ν: 3354, 3107, 2926, 2359, 1625, 1594, 1536, 1526, 1487, 1442, 1215, 1109, 947, 777, 756, 695 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.99–1.04(m, 2H, CH_2), 1.26–1.29(m, 2H, CH_2), 2.58(q, 1H, J = 4.1 Hz, CH), 7.01–7.04(m, 1H, py-H), 7.13–7.17(m, 1H, py-H), 7.36–7.41(m, 3H, Ar-H), 7.66(dd, 2H, J = 8.2, 1 Hz, Ar-H), 7.69(s, 1H, py-H), 7.97(s, 1H, pyrrole-H), 8.54–8.57(m, 1H, N-H), 9.91(p, 1H, J = 2 Hz, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 10.3, 10.4, 18.1, 108.9, 115.4, 119.8, 120.2, 120.6, 122.9, 124.2, 126.8, 128.9, 129.1, 138.2, 139.2, 162.3, 189.4. MS m/z (%) = 304 (M^+, 6), 212 (100). $C_{19}H_{16}N_2O_2$ (304.34): calcd. C 74.93, H 5.34, N 9.23; Found C 74.98, H 5.30, N 9.20.

Table 3
The antiproliferative effect of the indolizines against *Hep-G2*.

Compd.	IC_{50}(μg/mL)	Compd.	IC_{50}(μg/mL)
5a	0.32 ± 0.9	**5j**	0.20 ± 0.09
5b	6.6 ± 1.6	**5k**	1.1 ± 0.6
5c	32 ± 0.9	**5l**	2.1 ± 0.9
5d	0.48 ± 0.05	**5m**	4.6 ± 1.6
5d′	0.56 ± 0.07	**5n**	4.3 ± 1.9
5e	21 ± 0.6	**5o**	9.3 ± 1.3
5f	25 ± 0.7	**5p**	27 ± 0.9
5g	0.29 ± 0.09	**6a**	15 ± 0.9
5h	1.8 ± 0.6	**6b**	25 ± 0.7
5i	2.5 ± 0.5		

Table 4
Inhibition of EGFR kinase compound **5j**.

Compound	EGFR Inhibition IC_{50} (μM)
5j	0.085 ± 0.007
Iressa	0.033 ± 0.001

*4.3.6. 3-Cyclopropylcarbonyl-7-methyl-1-indolizinecarbonitrile (**5e**)*

White powder. m.p.149–150 °C. IR (ATR) ν: 3114, 2924, 2213, 1637, 1628, 942, 758 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.02(d, 2H, $J = 3.6$ Hz, CH_2), 1.23(s, 2H, CH_2), 2.48(s, 4H, CH, CH_3), 6.88(d, 1H, $J = 6.4$ Hz, py-H), 7.54(s, 1H, py-H), 7.87(s, 1H, pyrrole-H), 9.75 (d, 1H, $J = 6.8$ Hz, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 10.6, 18.0, 21.4, 83.0, 115.7, 116.1, 118.1, 123.3, 125.832, 128.8, 139.0, 141.2, 189.2. MS m/z (%) = 224 (M^+, 100), 183 (32), 155 (19). $C_{14}H_{12}N_2O$ (224.26): calcd. C 74.91, H 5.38, N 12.52; Found C 74.98, H 5.39, N 12.49. X-ray structure analysis: $C_{14}H_{12}N_2O$, M = 224.26. Triclinic, space group P-1, a = 7.1397(8), b = 11.6103(13), c = 14.6499(15) Å, α = 76.201(7), β = 86.261(7), γ = 87.924(7), V = 1176.6(2) Å, Z = 4, Dc = 1.266 - g cm^{-3}, F(000) = 472.0, absorption coefficient 0.082 mm^{-1}, scan range for data collection $1.43 \leq \theta \leq 27.63°$, 17,716 measured reflections, 5390 independent reflections, 3088 reflections with $I > 2\sigma(I)$, Rint = 0.0308, 307 refinable parameters, R[F2 > 2σ(F2)] = 0.0450, wR2 (F2) = 0.1347.

*4.3.7. Methyl 3-cyclopropylcarbony-7-methyllindolizine-1-carboxylate (**5f**)*

White powder. m.p.154–155 °C. IR (ATR) ν: 2988, 1728, 1622, 962, 756 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.97(s, 2H, CH_2), 1.21 (s, 2H, CH_2), 2.46(s, 3H, CH_3), 2.56(s, 1H, CH), 3.93(s, 3H, OCH_3), 6.84 (s, 1H, py-H), 8.12(s, 2H, pyrrole-H, py-H), 9.78(s, 1H, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 10.1, 17.8, 21.5, 51.2, 104.2, 117.5, 118.0, 123,0, 125.7, 128.5, 138.7, 139.7, 164.7, 189.5. MS m/z (%) = 257 (M^+, 100), 226 (8), 216 (15). $C_{15}H_{15}NO_3$ (257.28):calcd. C 69.98, H 5.83, N 5.46; Found C 70.02, H 5.88, N 5.44.

*4.3.8. Diethyl 3-Cyclopropylcarbonyl-7-methyl-indolizine-1,2-dicarboxylate (**5g**)*

White powder. m.p.65–66 °C. IR (ATR) ν: 2985, 1733, 1688, 1613, 1202, 920, 806, 783 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.93–0.97 (m, 2H, CH_2), 1.23–1.27(m, 2H, CH_2), 1.41(q, 6H, $J = 6.9$ Hz, CH_3), 2.31–2.38(m, 1H, CH), 2.46(s, 3H, CH_3), 4.38(q, 2H, $J = 7.2$ Hz, CH_2), 4.46(q, 2H, $J = 7.2$, CH_2), 6.86(dd, 1H, $J = 7.4$, 1.4 Hz, py-H), 8.15(s, 1H, py-H), 9.71(d, 1H, $J = 7.2$ Hz, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 10.8, 14.0, 14.4, 18.9, 21.6, 60.3, 62.3, 102.7, 118.3, 120.3, 128.5, 130.9, 133.6, 138.2, 139.4, 163.2, 167.1, 189.8. MS m/z (%) = 343 (M^+, 100), 241 (5), 184 (3). $C_{19}H_{21}NO_5$ (343.37): calcd. C 66.40, H 6.15, N 4.10; Found C 66.46, H 6.16; N 4.08.

*4.3.9. 5-Bromo-3-cyclopropylcarbonyl-1-indolizinecarbonitrile (**5h**)*

Yellow powder. m.p.105–106 °C. IR (ATR) ν: 3076, 2223, 1660, 949, 788 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.08–1.14(m, 2H, CH_2), 1.30–1.34(m, 2H, CH_2), 2.47–2.53(m, 1H, CH), 7.24(dd, 1H, $J = 8.6$, 7.4 Hz, py-H), 7.31(dd, 1H, $J = 7.2$, 0.8 Hz, py-H), 7.80(dd, 1H, $J = 8.4$, 1.2 Hz, py-H), 7.83(s, 1H, pyrrole-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 11.6, 20.4, 85.0, 114.9, 116.7, 118.5, 122.1, 125.4, 126.4, 129.1, 143.8, 188.8. MS m/z (%) = 290 (M^+, 2), 209 (100), 179 (13), 169 (15), 140 (18). $C_{13}H_9BrN_2O$ (289.13): calcd. C 53.03, H 3.16, N 9.71; Found C 54.00, H 3.14, N 9.69.

*4.3.10. Methyl 5-bromo-3-cyclopropylcarbonyl-indolizine-1-carboxylate (**5i**)*

White powder. m.p.85–86 °C. IR (ATR) ν: 3111, 2947, 1704, 1655, 946, 786, 729 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.05–1.09(m, 2H, CH_2), 1.27–1.31(m, 2H, CH_2), 2.56(m, 1H, CH), 3.94(s, 3H, CH_3), 7.20 (t, 1H, $J = 8.0$ Hz, py-H), 7.25(s, 1H, py-H), 8.08(s, 1H, pyrrole-H), 8.41(d, 1H, $J = 8.4$ Hz, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 11.1, 20.0, 51.4, 105.7, 118.0, 118.5, 121.7, 125.7, 126.4, 128.5, 142.6, 164.1, 188.8. MS m/z (%) = 321 (M^+, 0.5), 243 (63), 242 (100), 202 (14). $C_{14}H_{12}BrNO_3$ (322.15): calcd. C 52.17, H 3.71, N 4.37; Found C 52.20, H 3.75, N 4.35.

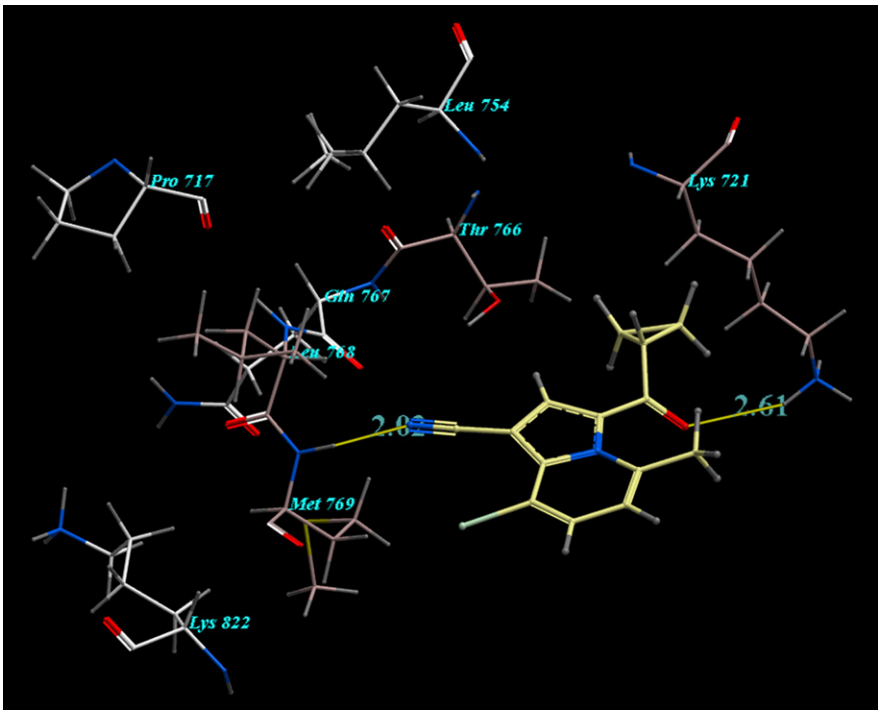


Fig. 3. Molecular docking modeling of compound **5j** with EGFR kinase.

4.3.11. 8-Bromo-3-cyclopropylcarbonyl-5-methyl-1-indolizinecarbonitrile (**5j**)

Yellow powder. m.p.133–135 °C.IR (ATR) ν: 3100, 2214, 1647, 957, 746 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.09–1.14(m, 2H, CH_2), 1.25–1.29(m, 2H, CH_2), 2.48(s, 3H, CH_3), 2.54–2.59(m, 1H, CH), 6.72 (d, 1H, $J = 7.6$ Hz, py-H), 7.52(d, 1H, $J = 7.6$ Hz, py-H), 7.98(s, 1H, pyrrole-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 11.7, 20.0, 22.9, 86.3, 109.9, 115.9, 117.0, 28.1, 128.8, 130.5, 138.8, 139.5, 189.2. MS m/z (%) = 304 (M^+, 80), 302 (100), 287 (75), 285 (67), 273 (66), 261 (21), 127 (20). $C_{14}H_{11}BrN_2O$ (303.15): calcd. C 55.35, H 3.68, N 9.42; Found C 55.47, H 3.66, N 9.24.

4.3.12. Methyl 8-bromo-3-cyclopropylcarbonyl-5-methyl-indolizine-1-carboxylate (**5k**)

Yellow crystal. m.p.107–108 °C. IR (ATR) ν: 2951, 1714, 1652, 960, 777 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.03–1.08(m, 2H, CH_2), 1.21–1.25(m, 2H, CH_2), 2.48(s, 3H, CH_3), 2.61(m, 1H, CH), 3.94(s, 3H, OCH_3), 6.66(d, 1H, $J = 8.0$ Hz, py-H), 7.53(d, 1H, $J = 7.6$ Hz, py-H), 8.08(s, 1H, pyrrole-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 11.1, 19.7, 23.0, 51.9, 108.8, 109.2, 116.2, 126.8, 127.7, 131.1, 136.7, 138.9, 164.5, 188.9. MS m/z (%) = 337(M^+, 74), 335(100), 318(53), 172(22). $C_{15}H_{14}BrNO_3$ (336.18): calcd. C 53.48, H 4.25, N 4.21; Found C, 53.59; H, 4.20; N, 4.17.

4.3.13. 1-CyclopropylcarbonylPyrrolo[1,2-a]quinoline-3-carbonitrile (**5l**)

Yellow powder. m.p.161–162 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.14(d, 2H, $J = 4.0$ Hz, CH_2), 1.36(s, 2H, CH_2), 2.61(s, 1H, CH), 7.52 (t, 1H, $J = 6.8$ Hz, Ar-H), 7.61–7.69(m, 3H, Ar-H), 7.80(d, 1H, $J = 6.4$ Hz, py-H), 7.89(s, 1H, py-H), 8.16(d, 1H, $J = 8.0$ Hz, pyrrole-H).

4.3.14. Methyl 1-cyclopropylcarbonylPyrrolo[1,2-a]quinoline-3-carboxylate (**5m**)

Yellow powder. m.p.174–175 °C. IR (ATR) ν: 2943, 1698, 1644, 940, 815, 742 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.91(s, 2H, CH_2), 1.32(s, 2H, CH_2), 2.68(s, 1H, CH), 3.95(s, 3H, OCH_3), 7.47(s, 1H, Ar-H), 7.58(s, 1H, Ar-H), 7.64(s, 1H, Ar-H), 7.77(s, 1H, Ar-H), 8.13(s, 2H, pyrrole-H), 8.29(s, 1H, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 11.2, 19.6, 51.4, 107.3, 117.6, 120.5, 125.4, 127.0, 128.4, 128.6, 128.8, 129.8, 133.5, 140.0, 164.5, 190.5. MS m/z (%) = 293 (M^+, 100), 252 (25), 194 (5). $C_{18}H_{15}NO_3$ (293.32): calcd. C 73.66, H 5.24, N 4.76; Found C 73.71, H 5.15, N 4.78.

4.3.15. 3-Cyclopropylcarbonylpyrrolo[1,2-a]isoquinoline-1-carbonitrile (**5n**)

Pink powder. m.p.190–191 °C.IR (ATR) ν: 3110, 2925, 2218, 1625, 796, 685 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.05–1.09(m, 2H, CH_2), 1.26–1.30(m, 2H, CH_2), 2.53–2.59(m, 1H, CH), 7.22(d, 1H, $J = 7.6$ Hz, Ar-H), 7.66–7.73(m, 2H, Ar-H), 7.76–7.79(m, 1H, Ar-H), 7.91(s, 1H, pyrrole-H), 8.97(dd, 1H, $J = 6.8$, 2.0 Hz, py-H), 9.59(d, 1H, $J = 7.2$ Hz, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 11.0, 18.5, 85.5, 115.6, 117.3, 123.6, 124.0, 125.3, 125.4, 127.1, 128.7, 129.5, 129.8, 137.2, 190.1. MS m/z (%) = 260 (M^+, 100), 219 (36), 191 (21), 164 (17). $C_{17}H_{12}N_2O$ (260.29): calcd. C 78.40, H 4.63, N 10.78; Found C 78.44, H 4.65, N 10.76.

4.3.16. Methyl 3-cyclopropylcarbonylpyrrolo[1,2-a]isoquinoline-1-carboxylate (**5o**)

Yellow powder. m.p.157–158 °C. IR (ATR) ν: 3133, 1708, 1634, 991, 756 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.03(s, 2H, CH_2), 1.26 (s, 2H, CH_2), 2.66(s, 1H, CH), 3.98(s, 3H, OCH_3), 7.21(d, 1H, $J = 5.6$ Hz, Ar-H), 7.69(d, 3H, $J = 32.4$ Hz, Ar-H), 8.25(s, 1H, pyrrole-H), 9.71 (d, 1H, $J = 5.6$ Hz, py-H), 9.86(d, 1H, $J = 5.6$ Hz, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 10.7, 18.4, 51.8, 109.5, 115.4, 124.1, 124.6, 125.3, 126.6, 127.7, 128.1, 129.2, 130.3, 136.3, 165.1, 190.4. MS m/z (%) = 293 (M^+, 100), 262 (8), 252 (11). $C_{18}H_{15}NO_3$ (293.32): calcd. C 73.64, H 5.10, N 4.81; Found C 73.71, H 5.15, N 4.78.

4.3.17. Dimethyl 3-cyclopropylcarbonylpyrrolo[1,2-a]isoquinoline-1,2- dicarboxylate (**5p**)

Yellow crystal. m.p.115–116 °C. IR (ATR) ν: 2954, 1739, 1707, 1644, 989, 799, 756 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.00–1.05 (m, 2H, CH_2), 1.30–1.34(m, 2H, CH_2), 2.39(m, 1H, CH), 3.97(d, 6H, $J = 3.2$, OCH_3), 7.16(d, 1H, $J = 7.6$ Hz, Ar-H), 7.60(q, 2H, $J = 3.1$ Hz, Ar-H), 7.70(q, 1H, $J = 3.1$ Hz, pyrrole-H), 9.17(q, 1H, $J = 3.2$ Hz, py-H), 9.23(d, 1H, $J = 7.6$ Hz, py-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 11.9, 20.6, 52.3, 52.9, 108.8, 115.9, 123.0, 124.2, 124.5, 126.8, 126.9, 128.0, 128.2, 129.2, 130.0, 133.3, 165.0, 166.9, 191.8. MS m/z (%) = 351 (M^+, 100), 310 (11), 296 (8). $C_{20}H_{17}NO_5$ (351.35): calcd. C 68.33, H 4.90, N 3.96; Found C 68.37, H 4.88, N 3.99.

4.3.18. 6-Cyclopropylcarbonyl-imidazo[2,1-f]pyrrolo[1,2-b] pyridazine-8-carboxylate-3-carbonitrile (**6a**)

Red powder. m.p.230–232 °C IR (ATR) ν: 3143, 2924, 2194, 1601, 1217, 791, 731, 681 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.84–0.94 (m, 2H, CH_2), 1.06–1.12(m, 2H, CH_2), 2.05–2.12(m, 1H, CH), 5.53(d, 1H, $J = 5.6$ Hz, imidazole-H), 7.24(s, 1H, pyrrole-H), 7.37(t, 1H, $J = 4.0$ Hz, imidazole-H), 7.46(d, 1H, $J = 5.6$ Hz, pyridazine-H), 8.79(d, 1H, $J = 2.0$ Hz, pyridazine-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 9.9, 15.1, 76.1, 96.5, 115.2, 117.6, 117.6, 121.3, 134.903, 136.3, 139.1, 152.3, 186.7. MS m/z (%) = 250 (M^+, 100), 221 (5), 181 (16). $C_{14}H_{10}N_4O$ (250.26): calcd. C 67.11, H 4.00, N 22.41; Found C, 67.19; H, 4.03; N, 22.39.

4.3.19. Methyl 6-Cyclopropylcarbonyl- imidazo[2,1-f]pyrrolo[1,2-b] pyridazine-8-carboxylate (**6b**)

Red powder. m.p.231–233 °C. IR (ATR) ν: 3197, 2923, 1684, 1599, 1212, 728 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.91(t, 2H, $J = 3.6$ Hz, CH_2), 1.08(s, 2H, CH_2), 2.28(m, 1H, CH), 3.80(s, 3H, OCH_3), 6.49 (d, 1H, $J = 5.2$ Hz, imidazole-H), 7.39(s, 1H, pyrrole-H), 7.53(s, 1H, imidazole-H), 7.89(s, 1H, pyridazine-H), 8.86(s, 1H, pyridazine-H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 9.8, 15.1, 51.1, 95.5, 99.5, 114.8, 117.1, 120.1, 134.4, 134.9, 150.9, 165.6, 187.5. MS m/z (%) = 283 (M^+, 100), 214 (4), 156 (3). $C_{15}H_{13}N_3O_3$ (283.28): calcd. C 63.56, H 4.66, N 14.80; Found C 63.60, H 4.63, N 14.83.

4.4. Antiproliferative activities assay

The antiproliferative activities of indolizine derivatives were determined using a standard (MTT)-based colorimetric assay (Sigma). Briefly, cell lines were seeded at a density of 7×10^3 cells/well in 96-well microtiter plates (Costar). After 12 h, exponentially growing cells were exposed to the indicated compounds at final concentrations ranging from 0.1 to 40 μg/mL. After 48 h, cell survival was determined by the addition of an MTT solution (25 μL of 4 mg/mL MTT in PBS). After 6 h, 100 μL of 10% SDS in 0.01 N HCl was added, and the plates were incubated at 37 °C for a further 12 h; optical absorbance was measured at 570 nm on an LX300 Epson Diagnostic microplate reader. Survival ratios are expressed in percentages with respect to untreated cells. IC_{50} values were determined from replicates of 6 wells from at least two independent experiments.

4.5. EGFR inhibitory assay

The EGFR kinase assay methods used are the same as our previous papers [25,26].

4.6. Molecular Docking modeling

Molecular docking of compound **5j** into the three-dimensional EGFR complex structure (1M17.pdb, downloaded from the PDB)

was carried out using the AutoDock software package (version 4.0) as implemented through the graphical user interface AutoDockTools (ADT 1.4.6).

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