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Synthesis of new indirubin derivatives and their in vitro anticancer activity

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Abstract
The opening of epoxy rings from (*2′Z*)-*N*-1-(oxiran-2-ylmethyl)indirubin (**2**) and (*2′Z-3′E*)-indirubin-3′-[*O*-oxiran-2-ylmethyl) oxime] (**6**) with thiols gave 17 new derivatives of indirubin in good yields. Their structures were elucidated by 1D-, 2D-NMR and HRMS spectra. Screening for anticancer activity was performed with four human cancer cell lines: SW480, LU-1, HepG2 and HL-60 in comparison with indirubin, indirubin-3′-oxime and 6-mercaptopurine. The results showed that cytotoxic and anti-proliferative activities of five derivatives were found in the range of 1.35–19.24 μM. Among synthesized derivatives, **4f** showed the strongest activity against all four tested cancer cell lines with IC_{50} values of 1.65, 2.21, 1.90 and 1.35 μM, respectively.

Keywords Indirubin · Indirubin-3′-oxime · 6-mercaptopurine · Cytotoxic

Introduction

The well-known indole skeleton dyes including indigo, isoindigo and (*2′Z*)-indirubin can be early found in a number of plants (Cuong et al. 2010a, b, c; Bektas et al. 2016), and indigo, indirubin and their derivatives are the constituents of a preparation called *indigo naturalis* (Stasiak et al. 2014). In addition, indirubin was determined to be a main active ingredient of a traditional Chinese medicinal recipe including *Indigofera tinctoria* L. and *Isatis tinctoria* L, which were used for treatment of chronic myelocytic leukemia (CML) for decades (Xiao et al. 2002). Indirubin (**1**) could be easily synthesized from istatin and 3-acetoxyindole in alkaline medium (Riepl and Urmann 2012), and it was also reported to inhibit several kinases such as glycogen synthase kinase-3 (GSK-3) and cyclin-dependent kinases (CDKs) by interaction with the ATP-binding site of kinase through van der Waals interaction and hydrogen bonds, and induce apoptosis in human cancer cells (Meijer et al. 2003; Hoessel et al. 1999; Jung et al. 2016; Nam et al. 2005; Lectere et al. 2001). In 2011, Karapetyan reported the synthesis of some indirubin-*N*-glycosides and their activity against several human cancer cell lines such as A-427, Kyse-70 and breast MCF-7. Among these glycosides, the activity of indirubin-α-*N*-rhamnoside and indirubin-*β*-*N*-rhamnoside against the human breast cancer cell line was much higher (10- to 100 fold) than that of the non-glycosylated indirubins tested before (Karapetyan et al. 2011). Indirubin-3′-oxime, an indirubin derivative was considered as potential candidate to inhibit the growth of lymphocytes in leukemia patients. This compound also reduced CDK activity and gene expression (Girard et al. 2007), and indirubin-3′-oxime was found to causes arrest at G0/G1 phase accompanied by a significant decrease in the percentage of cell in S and G2/M phases in cell cycle (Liao and Leung 2013). Lo et al. also recognized that indirubin-3-oxime had anti-proliferative activity and apoptosis in both Ca27 and SHC-3 oral cancer

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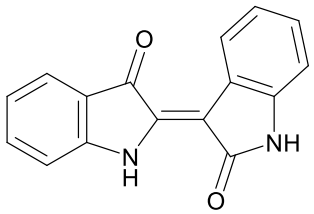
cell lines though the activation of cytochrome C (Lo and Chang 2013). Additionally, indirubin-3′-oxime suppressed migration and invasion in Ca27 by inhibiting the expression of local adhesion kinase, urokinase-type plasminogen inhibitor and matrix metalloproteinase 9 (Lo and Chang 2013). In 2014, the activation of Wnt/b-catenin signaling and the inhibition of adipocyte differentiation and obesity were also reported (Choi et al. 2014). Recently, indirubin-3′-oxime derivatives have also attracted the interest of chemists due to their anticancer activity. Notably, a report of Cuong and colleagues demonstrated that (*2′Z-3′E*)-indirubin-3′-[O-(3-bromoprop-1-yl)-oxime] and (2′Z-3′E)-indirubin-3′-[O-(3-methoxycarrbonylmethyl)-oxime] revealed stimulative effects on MC3T3-E1 osteoblastic cell growth and differentiation (Cuong et al. 2010a, b, c). Another derivative of indirubin, 5-methoxyindirubin 3′-oxime also expressed the induction of cell death in pancreatic ductal adenocarcinoma both in vitro and in vivo (Sano et al. 2017).



(2'Z)-Indirubin (1)

In a search for new agents for cytotoxic effects, we suggest that exploring new indirubin conjugates containing nitrogen and sulfur heterocycles could achieve potential compounds for pharmacological purpose. Therefore, in present study, two series of conjugates from indirubin were designed, synthesized and evaluated for in vitro anticancer activity.

Experimental

All chemicals were purchased from Sigma-Aldrich (USA), Santa cruz (USA) except for **3a**, **3b**, **3c**, **3d** and **3f** were prepared according to the references (Raju et al. 2015; Gilani et al. 2011; Vaughan 1957). Indirubin with (***2′Z***) conformation was isolated from *Strobilanthes cusia* in Vietnam as described by Cuong et al. (2007). Dry solvents were prepared according to procedures of Perrin and Armarego (1988). Melting points were measured in open capillary tubes on a Buchi 530 (Switzerland) melting point apparatus and were uncorrected. NMR spectra were recorded on a Bruker Advance 500 MHz operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Chemical shifts are reported in parts per million (ppm) using tetramethylsilane (TMS) as internal standard. Mass spectra were recorded on an Agilent 6530 Accurate-Mas Q-TOG LC/MS. Progress of the reaction was monitored by thin-layer chromatography (TLC) using precoated TLC sheets (Merck 60F254), and spots were visualized by UV lamp at 254 nm. Multiplicities are shown as follows: s (singlet), d (doublet), t (triplet), m (multiplet). Column chromatography was carried out using silica gel 60 (0.04–0.06 mm) from Scharlau, Spain. Solvents were commercially available materials of reagent grade.

Synthesis of (*2′Z*)-indirubin derivatives 4a-h

Synthesis of (*Z*)-1′-(oxiran-2-ylmethyl)-[2,3′-biindolinylidene]-2′,3-dione (2). A solution of compound **1** (786 mg, 3 mmol), epichlorohydrin (0.784 mL, 10 mmol), K_2CO_3 (1242 mg, 9 mmol), KI (50 mg, 0.3 mmol) and (1-butyl)triethylammonium bromide (71.5 mg, 0.3 mmol) in dried DMF (50 mL) was stirred at 45 °C for 4 days. The mixture was poured in ice water (200 ml) and the resulting precipitate was filtered and washed with water. Compound **2** was purified by chromatography on silica gel eluting with CH_2Cl_2/EtOAc 10:1

(*Z*)-1′-(Oxiran-2-ylmethyl)-[2,3′-biindolinylidene]-2′,3-dione (2). Yield 40%, dark violet solid, m.p. 178–179 °C. **^{1}H NMR (DMSO-*d*$_6$, 500 MHz), *δ*(ppm)**: 11.11 (s, 1H, H-1′), 8.83 (d, $J = 7.50$ Hz, 1H, H-4), 7.68 (d, $J = 7.50$ Hz, 1H, H-4′), 7.61 (m, 1H, H-6′), 7.43 (d, $J = 8.0$ Hz, 1H, H-7′), 7.35 (m, 1H, H-6), 7.21 (d, $J = 8.0$ Hz, 1H, H-7), 7.10 (m, 1H, H-5), 7.06 (m, 1H, H-5′), 4.20 (dd, $J_1 = 15.0$ Hz, $J_2 = 3.75$ Hz, 1H, –N_1–C$\underline{H}_2$–), 3.87 (dd, $J_1 = 15.0$ Hz, $J_2 = 5.25$ Hz, 1H, –N_1–C$\underline{H}_2$–), 3.24 (m, 1H, C$\underline{H}_{epoxy}$), 2.79 (t, $J = 4.50$ Hz, 1H, –C$\underline{H}_{2epoxy}$), 2.66 (dd, $J_1 = 5.0$ Hz, $J_2 = 3.0$ Hz, 1H, –C$\underline{H}_{2\ epoxy}$). **^{13}C NMR (DMSO-*d*$_6$, 125 MHz), *δ*(ppm)**: 189.0 (C-3′), 169.6 (C-2), 153.0 (C-7′a), 141.9 (C-7a), 139.3 (C-2′), 137.7 (C-6′), 129.6 (C-6), 125.0 (C-4), 124.9 (C-4′), 122.4 (C-3a), 121.9 (C-5), 121.1 (C-5′), 119.5 (C-3′a), 114.0 (C-7′), 109.6 (C-7), 105.7 (C-3), 49.9 (–CH–$_{epoxy}$), 45.2 (–CH_2–$_{epoxy}$), 41.6 (–N_1–CH_2–). **HR–ESI–MS**: calculated for $C_{19}H_{14}N_2O_3$ $[M]^+$: 318.1004, found: 318.0998.

General procedure for the synthesis of (*2′Z*)-indirubin derivatives 4a-h

A solution of compound **2** (47.7 mg, 0.15 mmol), triethylamine (0.45 mmol, 63 µl) and corresponding thiols (**3a-h**) (0.2 mmol) in dried DMF (3 ml) was stirred at room temperature for 40 h. The mixture was then diluted with EtOAc (50 ml) and washed with NaCl 3% solution (3 × 100 ml). The combined EtOAc extract was dried on anhydrous sodium sulfate, and solvent was removed under reduced pressure to afford crudes **4a-h** that was chromatographed

using chloroform: EtOAc 2:1 as solvent to afford **4a-h** in 63.1–77.0.3% yields.

(*Z*)-1′-(2-Hydroxy-3-((5-phenyl-1,3,4-oxadiazol-2-yl) thio)propyl)-[2,3′-biindolinylidene]-2′,3-dione (4a). Yield 73.2%, dark violet solid, m.p. 222–223 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm)**: 11.1 (s, 1H, H-1′), 8.82 (d, J = 8.0 Hz, 1H, H-4), 7.93 (d, J = 7.0 Hz, 2H, H-2″, H-6″), 7.67 (d, J = 7.0 Hz, 1H, H-4′), 7.58 (m, 4H, H-3″, H-5″, H-6′, H-4″), 7.41 (d, J = 8.0 Hz, 1H, H-7′), 7.33 (t, J = 7.50 Hz, 1H, H-6), 7.2 (d, J = 7.50 Hz, 1H, H-7), 7.09 (t, J = 7.50 Hz, 1H, H-5), 7.04 (t, J = 7.25 Hz, 1H, H-5′), 5.71 (d, J = 5.0 Hz, 1H, –CHOH–), 4.24 (m, 1H, –CHOH–), 3.98 (m, 2H, –N–CH$_2$), 3.58 (dd, J_1 = 13.50 Hz, J_2 = 3.50 Hz, 1H, –CH$_2$–S–), 3.4 (dd, J_1 = 13.50 Hz, J_2 = 7.50 Hz, 1H, –CH$_2$–S). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm)**: 189.1 (C-3′), 169.9 (C-2), 165.5 (phenyl–C$_{oxadiazole}$), 164.5 (–S–C$_{oxadiazole}$), 152.9 (C-7′a), 142.3 (C-7a), 139.1 (C-2′), 137.7 (C-6′), 132.4 (C-4″), 129.9 (C-3″, C-5″), 129.5 (C-6), 126.8 (C-2″, C-6″), 124.9 (C-4), 124.8 (C-4′), 123.5 (C-1″), 122.2 (C-3a), 121.9 (C-5), 121.3 (C-5′), 119.5 (C-3′a), 113.9 (C-7′), 109.6 (C-7), 106.1 (C-3), 67.5(–CHOH–), 45.2 (–N–CH$_2$–), 37.6(–CH$_2$–S–). **HR–ESI–MS:** Caculated for $C_{27}H_{20}N_4O_4S$: $[M]^+$: 496.1205, found: 496.1195.

(*Z*)-1′-(2-Hydroxy-3-((5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl)thio)propyl)-[2,3′-biindolinylidene]-2′,3-dione (4b). Yield 71.8%, dark violet solid, mp: 225–226 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 11.09 (s, 1H, H-1′), 8.83 (d, J = 7.5 Hz, 1H, H-4), 7.85 (d, J = 8.5 Hz, 2H, H-2″, H-6″), 7.67 (d, J = 7.5 Hz, 1H, H-4′), 7.59 (t, J = 7.5 Hz, 1H, H-6′), 7.41 (d, J = 8.0 Hz, 1H, H-7′), 7.33 (t, J = 7.75 Hz, 1H, H-6), 7.2 (d, J = 8.0 Hz, 1H, H-7), 7.10 (m, 3H, H-5, H-3″, H-5″), 7.04 (t, J = 7.5 Hz, 1H, H-5′), 5.71 (d, J = 5.0 Hz, 1H, –CHOH–), 4.22 (m, 1H, –CHOH–), 3.97 (m, 2H, –N–CH$_2$–), 3.83 (s, 3H, CH$_3$O–), 3.56 (dd, J_1 = 13.5 Hz, J_2 = 4.0 Hz, 1H, –CH$_2$–S–), 3.38 (m, 1H,–CH$_2$–S–). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm)**: 189.1 (C-3′), 169.9 (C-2), 165.4 (phenyl–C$_{oxadiazole}$), 163.6 (–S–C$_{oxadiazole}$), 162.4 (C-4″), 152.9 (C-7′a), 142.3 (C-7a), 139.1 (C-2′), 137.7 (C-6′), 129.5 (C-6), 128.6 (C-2″, C-6″), 124.9 (C-4), 124.8 (C-4′), 122.2 (C-3a), 121.9 (C-5), 121.3 (C-5′), 119.5 (C-3′a), 115.9 (C-1″), 115.3 (C-3″, C-5″), 113.9 (C-7′), 109.6 (C-7), 106.1 (C-3), 67.6 (–CHOH–), 55.9 (CH$_3$O–), 45.2 (–N–CH$_2$–), 37.6 (–CH$_2$–S–). **HR–ESI–MS:** Caculated for $C_{28}H_{22}N_4O_5S$: $[M]^+$: 526.1311, found: 526.1303.

(*Z*)-1′-(2-Hydroxy-3-((5-(4-nitrophenyl)-1,3,4-oxadiazol-2-yl)thio)propyl)-[2,3′-biindolinylidene]-2′,3-dione (4c). Yield 66.1%, dark violet solid, mp: 168–169 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 11.1 (s, 1H, H-1′), 8.82 (d, J = 7.0 Hz, 1H, H-4), 8.36 (dd, J_1 = 7.0 Hz, J_2 = 2.0 Hz, 2H, H-3″, H-5″); 8.16 (dd, J_1 = 7.0 Hz, J_2 = 2.0 Hz, 2H, H-2″, H-6″), 7.65 (d, J = 7.50 Hz, 1H, H-4′), 7.58 (m, 1H, H-6′), 7.39 (d, J = 8.0 Hz, 1H, H-7′), 7.33 (m, 1H, H-6), 7.2 (d, J = 7.5 Hz, 1H, H-7), 7.09 (m, 1H, H-5), 7.03 (m, 1H, H-5′), 5.74 (d, J = 5.50 Hz, 1H, –CHOH-), 4.26 (m, 1H, –CHOH–), 4.01 (dd, J_1 = 14.50 Hz, J_2 = 5.50 Hz, 1H, –N–CH$_2$-), 3.95 (dd, J_1 = 14.50 Hz, J_2 = 6.50 Hz, 1H, –N–CH$_2$-), 3.61 (dd, J_1 = 13.50 Hz, J_2 = 4.50 Hz, 1H, –CH$_2$–S–), 3.43 (dd, J_1 = 13.50 Hz, J_2 = 7.50 Hz, 1H, –CH$_2$–S–). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm)**: 189 (C-3′), 169.9(C-2), 165.9 (phenyl–C$_{oxadiazole}$), 164.1 (–S–C$_{oxadiazole}$), 152.9 (C-7′a), 149.5 (C-4″), 142.3 (C-7a), 139.1 (C-2′), 137.7 (C-6′), 129.5 (C-6), 129 (C-1″), 128.1 (C-2″, C-6″), 125 (C-3″, C-5″), 124.9 (C-4), 124.8 (C-4′), 122.3 (C-3a), 121.9 (C-5), 121.3 (C-5′), 119.5 (C-3′a), 113.9 (C-7′), 109.6 (C-7), 106 (C-3), 67.4(–CHOH–), 45.1(–N–CH$_2$-), 37.6(–CH$_2$–S–). **HR-ESI–MS:** Caculated for $C_{27}H_{19}N_5O_6S$: $[M]^+$: 541.1056, found: 541.1047.

(*Z*)-1′-(2-Hydroxy-3-((5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl)thio)propyl)-[2,3′-biindolinylidene]-2′,3-dione (4d). Yield 65.0%, dark violet solid, mp: 183–184 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), $\delta\delta$(ppm):** 11.1 (s, 1H, H-1′), 8.82 (d, J = 7.0 Hz, 1H, H-4), 8.78 (dd, J_1 = 4.50 Hz, J_2 = 1.50 Hz, 2H, H-2″, H-6″), 7.84 (dd, J_1 = 4.50 Hz, J_2 = 1.50 Hz, 2H, H-3″, H-5″), 7.66 (d, J = 7.50 Hz, 1H, H-4′), 7.59 (m, 1H, H-6′), 7.4 (d, J = 8.0 Hz, 1H, H-7′), 7.32 (m, 1H, H-6), 7.19 (d, J = 8.0 Hz, 1H, H-7), 7.09 (m, 1H, H-5), 7.03 (m, 1H, H-5′), 5.73 (d, J = 5.50 Hz, 1H, –CHOH), 4.25 (m, 1H, –CHOH–), 3.99 (m, 2H, –N–CH$_2$-), 3.61 (dd, J_1 = 13.50 Hz, J_2 = 4.0 Hz, 1H, –CH$_2$–S–), 3.42 (dd, J_1 = 13.50 Hz, J_2 = 7.50 Hz, 1H, –CH$_2$–S–). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 189 (C-3′), 169.9 (C-2), 166 (pyridine–C$_{oxadiazole}$), 163.9 (–S–C$_{oxadiazole}$), 152.9 (C-7′a), 151.4 (C-2″, C-6″), 142.3 (C-7a), 139.1 (C-2′), 137.7 (C-6′), 130.5 (C-4″), 129.5 (C-6), 124.9 (C-4), 124.8 (C-4′), 122.2 (C-3a), 121.9 (C-5), 121.3 (C-5′), 120.4 (C-3″, C-5″), 119.5 (C-3′a), 113.9 (C-7″), 109.6 (C-7), 106.1 (C-3), 67.5(–CHOH–), 45,1(–N–CH$_2$-), 37,6(–CH$_2$–S–). **HR-ESI–MS:** Caculated for $C_{26}H_{19}N_5O_4S$ $[M]^+$: 497.1158, found: 497.1157.

(*Z*)-1′-(3((7*H*-Purin-6-yl)thio)-2-hydroxypropyl)-[2,3′-biindolinylidene]-2′,3-dione (4e). Yield 63.1%, dark violet solid, mp: 172–173 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 13.49 (s, 1H, NH$_{purine}$), 11.11 (s, 1H, H-1′), 8.83 (d, J = 7.50 Hz, 1H, H-4), 8.49 (s, 1H, H-8″), 8.41 (s, 1H, H-2″), 7.67 (d, J = 7.50 Hz, 1H, H-4′), 7.59 (t, J = 7.75 Hz, 1H, H-6′), 7.41 (d, J = 8.0 Hz, 1H, H-7′), 7.31 (t, J = 7.75 Hz, 1H, H-6), 7.16 (d, J = 8.0 Hz, 1H, H-7), 7.08 (t, J = 7.75 Hz, 1H, H-5), 7.04 (t, J = 7.50 Hz, 1H, H-5′), 5.56 (d, J = 5.0 Hz, 1H, –CHOH-), 4.16 (m, 1H, –CHOH–), 4.0 (dd, J_1 = 14.0 Hz, J_2 = 5.0 Hz, 1H, –N–CH$_2$-), 3.92 (dd, J_1 = 14.0 Hz, J_2 = 7.0 Hz, 1H, –N–CH$_2$-), 3.7 (m, 1H, –CH$_2$–S–), 3.33 (m, 1H, –CH$_2$–S–). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 189.1 (C-3′), 169.9 (C-2), 159.1 (C-6″), 153 (C-7′a), 151.7 (C-4″, C-8″), 149.6 (C-5″), 143.4 (C-2″), 142.5 (C-7a), 138.9 (C-2′), 137.7 (C-6′), 129.5 (C-6), 124.9 (C-4), 124.8 (C-4′), 122.1 (C-3a), 121.9 (C-5), 121.3 (C-5′), 119.5 (C-3′a), 113.9 (C-7′), 109.7 (C-7), 106.2

(C-3), 68.2 (–CHOH–), 45.5 (–N–CH_2-), 33.2 (–CH_2–S–). **HR-ESI–MS:** Caculated for $C_{24}H_{18}N_6O_3S$ $[M]^+$: 470.1161, found: 470.1159.

(*Z*)-1′-(2-Hydroxy-3-((5-(phenylamino)-1,3,4-thiadiazol-2-yl)thio)propyl)-[2,3′-biindolinylidene]-2′,3-dione (4f). Yield 77.0%, dark violet solid, mp: 232–233 °C. **IR (KBr, ν_{max} (cm^{-1}))**: 3416 (O–H), 3316 (N–H), 2923 (C–H), 1710 (C=O), 1643 (C=N), 1607 and 1499 (C=C), 1319 (C–N), 1093 (N–N), 660 (C–S–C). **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 11.1 (s, 1H, H-1′), 10.36 (s, 1H, NH-phenyl), 8.82 (d, J = 8.0 Hz, 1H, H-4), 7.67 (d, J = 7.50 Hz, 1H, H-4′), 7.6 (m, 1H, H-6′), 7.55 (d, J = 7.50 Hz, 2H, H-2″, H-6″), 7.42 (d, J = 8.0 Hz, 1H, H-7′), 7.33–7.31 (m, 3H, H-3″, H-5″, H-6), 7.18 (d, J = 8.0 Hz, 1H, H-7), 7.08 (t, J = 7.50 Hz, 1H, H-5), 7.04 (m, 1H, H-5′), 7.0 (m, 1H, H-4″), 5.61 (d, J = 5.50 Hz, 1H, –CHOH-), 4.16 (m, 1H, –CHOH–), 3.94 (m, 2H, –N–CH_2-), 3.43 (dd, J_1 = 13.25 Hz, J_2 = 4.50 Hz, 1H, –CH_2–S–), 3.26 (dd, J_1 = 13.25 Hz, J_2 = 7.25 Hz, 1H, –CH_2–S–). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 189.1 (C-3′), 169.9 (C-2), 165.1 (–NH–$C_{thiadiazole}$-), 153.7 (–CH_2–S–$C_{thiadiazole}$), 152.9 (C-7′a), 142.4 (C-7a), 140.9 (C-1″), 139 (C-2′), 137.7 (C-6′), 129.6 (C-3″, C-5″), 129.4 (C-6), 124.9 (C-4), 124.8 (C-4′), 122.4 (C-4″), 122.2 (C-3a), 121.9 (C-5), 121.3 (C-5′), 119.5(C-3′a), 117.8 (C-2″, C-6″), 113.9 (C-7′), 109.7 (C-7), 106.2 (C-3), 67.9 (–CHOH–), 45.4 (–N–CH_2-), 39.4 (–CH_2–S–). **HR-ESI–MS:** Caculated for $C_{27}H_{21}N_5O_3S_2$ $[M]^+$: 527.1086, found: 527.1082.

(*Z*)-1′-(3-((5-((2,3-Dimethylphenyl)amino)-1,3,4-thiadiazol-2-yl)thio)propyl)-[2,3′-biindolinylidene]-2′,3-dione (4 g). Yield 69.5%, dark violet solid, mp: 247–248 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 11.09 (s, 1H, H-1′), 9.54 (s, 1H, NH-phenyl), 8.81 (d, J = 8.0 Hz, 1H, H-4), 7.67 (d, J = 7.50 Hz, 1H, H-4′), 7.59 (m, 1H, H-6′), 7.44 (d, J = 8.0 Hz, 1H, H-4″), 7.41 (d, J = 8.0 Hz, 1H, H-7′), 7.31 (m, 1H, H-6), 7.16 (d, J = 8.0 Hz, 1H, H-7), 7.08 (m, 2H, H-5, H-5″), 7.04 (t, J = 7.50 Hz, 1H, H-5′), 6.99 (d, J = 7.50 Hz, 1H, H-6″), 5.58 (d, J = 5.50 Hz, 1H, –CHOH-), 4.12 (m, 1H, –CHOH–), 3.92 (m, 2H, –N–CH_2-), 3.37 (m, 1H, –CH_2–S–), 3.21 (dd, J_1 = 13.50 Hz, J_2 = 7.0 Hz, 1H, –CH_2–S–), 2.26 (s, 3H, CH_3–C-3″), 2.13 (s, 3H,CH_3–C-2″). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 189 (C-3′), 169.9 (C-2), 168.6 (–NH–$C_{thiadiazole}$-), 152.9 (C-7′a), 152.7 (–CH_2–S–$C_{thiadiazole}$), 142.4(C-7a), 139.5 (C-1″), 139(C-2′), 137.9 (C-3″), 137.7 (C-6′), 129.7 (C-2″), 129.5 (C-6), 126.9 (C-4″), 126.4 (C-5″), 124.9 (C-4), 124.8 (C-4′), 122.2 (C-3a), 121.9 (C-5), 121.3 (C-5′), 121 (C-6″), 119.5 (C-3′a), 113.9 (C-7′), 109.6 (C-7), 106.1 (C-3), 67.9 (–CHOH), 45.3(–N–CH_2-), 40(–CH_2–S–), 20.7 (CH_3–C-3″), 14.3 (CH_3–C-2″). **HR-ESI–MS:** Caculated for $C_{29}H_{25}N_5O_3S_2$ $[M]^+$: 555.1399, found: 555.1396.

(*Z*)-1′-(3-((5-((2,4-Dimethylphenyl)amino)-1,3,4-thiadiazol-2-yl)thio)propyl)-[2,3′-biindolinylidene]-2′,3-dione (4 h). Yield 67.4%, dark violet solid, mp: 241–242 °C (decomposition). **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 11.09 (s, 1H, H-1′), 9.42 (s, 1H, NH-phenyl), 8.81 (d, J = 7.50 Hz, 1H, H-4), 7.67 (d, J = 7.50 Hz, 1H, H-4′), 7.60 (m, 2H, H-6′, H-5″), 7.41 (d, J = 8.0 Hz, 1H, H-7′), 7.31 (1H, m, H-6), 7.16 (d, J = 8.0 Hz, 1H, H-7), 7.09 (d, J = 7.50 Hz, 1H, H-5), 7.04 (m, 2H, H-5′, H-3″), 6.98 (d, J = 8.0 Hz, 1H, H-6″), 5.58 (d, J = 5.50 Hz, 1H, –CHOH-), 4.13 (m, 1H, –CHOH–), 3.92 (m, 2H, –N–CH_2-), 3.37 (m, 1H, –CH_2–S–), 3.21 (dd, J_1 = 13.50 Hz, J_2 = 7.0 Hz, 1H, –CH_2–S–), 2.24 (s, 3H, CH_3–C-4″), 2.19 (s, 3H, CH_3–C-2″). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 189 (C-3′), 169.9 (C-2), 168.1 (–NH–$C_{thiadiazole}$-), 152.9 (C-7′a), 152.8 (–CH_2–S–$C_{thiadiazole}$), 142.4 (C-7a), 139 (C-2′), 137.7 (C-6′), 137.1 (C-1″), 133.9 (C-4″), 131.8 (C-5″), 130.1 (C-2″), 129.5 (C-6), 127.6 (C-3″), 124.9 (C-4), 124.8 (C-4′), 122.4 (C-6″), 122.2 (C-3a), 121.9 (C-5), 121.3 (C-5′), 119.5 (C-3′a), 113.9 (C-7′), 109.6 (C-7), 106.1 (C-3), 67.9 (–CHOH–), 45.3 (–N–CH_2-), 40 (–CH_2–S–), 20.9 (CH_3–C-4″), 18.3 (CH_3–C-2″). **HR-ESI–MS:** Caculated for $C_{29}H_{25}N_5O_3S_2$ $[M]^+$: 555.1399, found: 555.1393.

Synthesis of *(2′Z-3′E)*-indirubin-3′-oxime derivatives 7a-i

Synthesis of (2*Z*,3*E*)-3-((Oxiran-2-ylmethoxy)imino)-[2,3′-biindolinylidene]-2′-one (6)

Indirubin-3′-oxime **5** with *(2′Z-3′E)* conformation was first obtained by the reaction of indirubin (**1**) with hydroxylamine in pyridine according to the procedure as described by Cuong and et al. (2010a, b, c), indirubin-3′-oxime (**5**) was then *O*-alkylated with epibromohydrin using a procedure described by Ichimaru (Ichimaru et al. 2015) to give *((2′Z-3′E)*-Indirubin-3′-(*O*-oxiran-2-ylmethyl)oxime**) 6** in 70% yield.

General procedure for the synthesis of indirubin-3′-oxime derivatives 7a-i

A solution of compound **6** (50 mg, 0.15 mmol), triethylamine (0.45 mmol, 63 µl) and the corresponding thiols **3a-3i** (0.2 mmol) in dried DMF (3 ml) was stirred at room temperature for 40 h. The mixture was then diluted with EtOAc (50 ml) and washed with NaCl 3% solution (3 × 100 ml). The combined EtOAc extract was dried on anhydrous sodium sulfate and solvent was removed under reduced pressure. Crude **7a-i** was chromatographed using chloroform: EtOAc as solvent to afford **7a-i** in 59.2–75.0% yields.

(*2′Z-3′E*)-Indirubin-3′-[*O*-2-hydroxy-3-(5-phenyl-1,3,4-oxadiazole-2-thio)prop-1-yl]oxime (7a). Yield 67.1%, red solid, m.p. 196–197 °C. **^{1}H NMR (DMSO-d_6,**

500 MHz), δ(ppm): 11.68 (s, 1H, H-1′), 10.75 (s, 1H, H-1), 8.58 (d, J = 7.50 Hz, 1H, H-4), 8.22 (d, J = 7.50 Hz, 1H, H-4′), 7.89 (m, 2H, H-2″, H-6″), 7.6 (m, 1H, H-4″), 7.54 (t, J = 7.50 Hz, 2H, H-3″, H-5″), 7.43 (m, 2H, H-6′, H-7′), 7.11 (m, 1H, H-6), 7.03 (m, 1H, H-5′), 6.96 (t, J = 7.75 Hz, 1H, H-5), 6.87 (d, J = 7.50 Hz, 1H, H-7), 5.86 (d, J = 5.50 Hz, 1H, –CHOH-), 4.7 (dd, J_1 = 11.0 Hz, J_2 = 5.0 Hz, 1H, –O–CH$_2$-), 4.65 (dd, J_1 = 11.0 Hz, J_2 = 6.0 Hz, 1H, –O–CH$_2$-), 4.42 (m, 1H, –CHOH–), 3.69 (dd, J_1 = 13.50 Hz, J_2 = 4.5 Hz, 1H, –CH$_2$–S–), 3.53 (dd, J_1 = 13.5 Hz, J_2 = 7.0 Hz, 1H, –CH$_2$–S–). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 171.3 (C-2), 165.5(phenyl–C$_{oxadiazole-}$), 164.4(–S–C$_{oxadiazole}$), 152.2 (C-3′), 146 (C-2′), 144.3 (C-7′a), 139.2 (C-7a), 133.4 (C-6′), 132.4 (C-4″), 129.8 (C-3″, C-5″), 129.2 (C-4′), 126.9 (C-6), 126.7 (C-2″, C-6″), 123.8 (C-4), 123.5 (C-1″), 122.7 (C-3a), 121.9 (C-5′), 121.1 (C-5), 116.7 (C-3′a), 112.2 (C-7′), 109.4 (C-7), 100.8 (C-3), 79.5 (–O–CH$_2$-), 67.9 (–CHOH–), 36.6(–CH$_2$–S–). **HR-ESI–MS:** calculated for $C_{27}H_{21}O_4N_5S$ $[M]^+$: 511.1314, found: 511.1305.

(*2′Z-3′E*)-Indirubin-3′-[*O*-2-hydroxy-3-(5-(4-methoxyphenyl)-1,3,4-oxadiazole-2-thio)prop-1-yl] oxime (7b). Yield 69.4%, red solid, m.p. 207–208 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm)**: 11.67 (s, 1H, H-1′), 10.75 (s, 1H, H-1), 8.58 (d, J = 8.0 Hz, 1H, H-4), 8.21 (d, J = 8.0 Hz, 1H, H-4′), 7.8 (dd, J_1 = 6.75 Hz, J_2 = 2.25 Hz, 2H, H-2″, H-6″), 7.43 (m, 2H, H-6′, H-7′), 7.12 (m, 1H, H-6), 7.04 (m, 3H, H-3″, H-5″, H-5′), 6.96 (m, 1H, H-5), 6.88 (d, J = 7.50 Hz, 1H, H-7), 5.86 (d, J = 5.50 Hz, 1H, –CHOH-), 4.67 (m, 2H, –O–CH$_2$-), 4.42 (m, 1H, –CHOH–), 3.84 (s, 3H, CH$_3$O-), 3.66 (dd, J_1 = 13.25 Hz, J_2 = 4.75 Hz, 1H, –CH$_2$–S–), 3.5 (dd, J_1 = 13.75 Hz, J_2 = 7.25 Hz,1H, –CH$_2$–S–). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 171.3(C-2), 165.5(phenyl–C$_{oxadiazole-}$), 163.5 (–S–C$_{oxadiazole}$), 162.4 (C-4″), 152.2 (C-3′), 146 (C-2′), 144.2 (C-7′a), 139.2 (C-7a), 133.4 (C-6′), 129.1(C-4′), 128.6 (C-2″, C-6″), 126.9 (C-6), 123.8 (C-4), 122.7 (C-3a), 121.9 (C-5′), 121.1 (C-5), 116.7 (C-3′a), 115.8 (C-1″), 115.2 (C-3″, C-5″), 112.2 (C-7′), 109.4 (C-7), 100.8 (C-3), 79.4 (–O–CH$_2$-), 67.9 (–CHOH–), 55.9 (CH$_3$O-), 36.7 (–CH$_2$–S–). **HR-ESI–MS:** calculated for $C_{28}H_{23}N_5O_5S$ $[M]^+$: 541.1420, found: 541.1414.

(*2′Z-3′E*)-Indirubin-3′-[*O*-2-hydroxy-3-(5-(4-nitrophenyl)-1,3,4-oxadiazole-2-thio)prop-1-yl] oxime (7c). Yield 75.0%, red solid, m.p. 213–214 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 11.64 (s, 1H, H-1′), 10.71 (s, 1H, H-1), 8.53 (d, J = 8.0 Hz, 1H, H-4), 8.3 (m, 2H, H-3″, H-5″), 8.21 (d, J = 7.50 Hz, 1H, H-4′), 8.06 (m, 2H, H-2″, H-6″), 7.45 (m, 1H, H-6′), 7.4 (d, J = 7.50 Hz, 1H, H-7′), 7.1 (m, 1H, H-6), 7.03 (m, 1H, H-5′), 6.94 (m, 1H, H-5), 6.83 (d, J = 7.50 Hz, 1H, H-7), 5.89 (d, J = 5.50 Hz, 1H, –CHOH-), 4.7 (dd, J_1 = 11.50 Hz, J_2 = 5.50 Hz, 1H, –O–CH$_2$-), 4.65 (dd, J_1 = 11.25 Hz, J_2 = 5.75 Hz, 1H, –O–CH$_2$-), 4.44 (m, 1H, –CHOH–), 3.72 (dd, J_1 = 13.50 Hz, J_2 = 5.0 Hz, 1H, –CH$_2$–S–), 3.57 (dd, J_1 = 13.50 Hz, J_2 = 6.50 Hz, 1H, –CH$_2$–S–). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 171.3 (C-2), 165.7 (phenyl–C$_{oxadiazole-}$), 164.1 (–S–C$_{oxadiazole}$), 152.2 (C-3′), 149.4 (C-4″), 146 (C-2′), 144.1 (C-7′a), 139.1 (C-7a), 133.4 (C-6′), 129.2 (C-4′), 128.9 (C-1″), 127.9 (C-2″, C-6″), 126.9 (C-6), 124.8 (C-3″, C-5″), 123.7 (C-4), 122.7 (C-3a), 121.9 (C-5′), 121 (C-5), 116.6 (C-3′a), 112.2 (C-7′), 109.4 (C-7), 100.8 (C-3), 79.1 (–O–CH$_2$-), 67.8 (–CHOH), 36.7 (–CH$_2$–S–). **HR-ESI–MS:** calculated for $C_{27}H_{20}O_6N_6S$ $[M]^+$: 556.1165, found: 556.1155.

(*2′Z-3′E*)-Indirubin-3′-[*O*-2-hydroxy-3-(5-(pyridin-4-yl)-1,3,4-oxadiazole-2-thio)prop-1-yl]oxime (7d). Yield 61.7%, red solid, m.p. 216–217 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 11.67 (s, 1H, H-1′), 10.74 (s, 1H, H-1), 8.75 (dd, J_1 = 4.50 Hz, J_2 = 1.50 Hz, 2H, H-2″, H-6″), 8.56 (d, J = 8.0 Hz, 1H, H-4), 8.22 (d, J = 7.50 Hz, 1H, H-4′), 7.78 (dd, J_1 = 4.25 Hz, J_2 = 1.75 Hz, 2H, H-3″, H-5″), 7.43 (m, 2H, H-6′, H-7′), 7.1 (t, J = 7.50 Hz, 1H, H-6), 7.03 (m, 1H, H-5′), 6.96 (t, J = 7.75 Hz, 1H, H-5), 6.85 (d, J = 7.50 Hz, 1H, H-7), 5.88 (d, J = 5.50 Hz, 1H, –CHOH-), 4.7 (dd, J_1 = 11.25 Hz, J_2 = 5.25 Hz, 1H, –O–CH$_2$-), 4.65 (dd, J_1 = 11.25 Hz, J_2 = 5.75 Hz, 1H, –O–CH$_2$-), 4.43 (m, 1H, –CHOH–), 3.72 (dd, J_1 = 13.50 Hz, J_2 = 5.0 Hz, 1H, –CH$_2$–S–), 3.56 (dd, J_1 = 13.50 Hz, J_2 = 7.0 Hz, 1H, –CH$_2$–S–). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm)**: 171.3 (C-2), 165.8 (pyridine–C$_{oxadiazole-}$), 164 (–S–C$_{oxadiazole}$), 152.2 (C-3′), 151.3 (C-2″, C-6″), 146 (C-2′), 144.2 (C-7′a), 139.1 (C-7a), 133.4 (C-6′), 130.5 (C-4″), 129.2 (C-4′), 126.9 (C-6), 123.8 (C-4), 122,7 (C-3a), 121.9 (C-5′), 121.1 (C-5), 120.3 (C-3″, C-5″), 116.7 (C-3′a), 112.2 (C-7′), 109.4 (C-7), 100.8 (C-3), 79,3(–O–CH$_2$-), 67.8 (–CHOH–), 36.7(–CH$_2$–S–). **HR-ESI–MS:** calculated for $C_{26}H_{20}O_4N_6S$ $[M]^+$: 512.1267, found: 512.1259.

(*2′Z-3′E*)-Indirubin-3′-[*O*-2-hydroxy-3-(purine-6-thio)prop-1-yl]oxime (7e). Yield 59.2%, red solid, m.p. 255–256 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 13.52 (s, 1H, NH$_{purine}$), 11.69 (s, 1H, H-1′), 10.76 (s, 1H, H-1), 8.56 (s, 1H, H-8″), 8.55 (d, J = 8.0 Hz, 1H, H-4), 8.44 (s, 1H, H-2″), 8.24 (d, J = 7.50 Hz, 1H, H-4′), 7.44 (m, 2H, H-6′, H-7′), 7.08 (t, J = 7.50 Hz, 1H, H-6), 7.03 (m, 1H, H-5′), 6.86 (m, 2H, H-5, H-7), 5.74 (d, J = 5.0 Hz, 1H, –CHOH-), 4.69 (dd, J_1 = 11.0 Hz, J_2 = 5.0 Hz, 1H, –O–CH$_2$-), 4.64 (dd, J_1 = 11.0 Hz, J_2 = 6.0 Hz, 1H, –O–CH$_2$-), 4.35 (m, 1H,–CHOH), 3.79 (dd, J_1 = 13.50 Hz, J_2 = 5.50 Hz, 1H, –CH$_2$–S–), 3.56 (dd, J_1 = 13.75 Hz, J_2 = 6.75 Hz, 1H, –CH$_2$–S–). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 171.3 (C-2), 159.1 (C-6″), 152 (C-3′), 151.7 (C-4″, C-8″), 149.7 (C-5″), 145.9 (C-2′), 144.3 (C-7′a), 143.5 (C-2″), 139.1 (C-7a), 133.4 (C-6′), 129.1 (C-4′), 126.8 (C-6), 123.8 (C-4), 122.7 (C-3a), 121.9 (C-5′), 121 (C-5), 116.7 (C-3′a), 112.2 (C-7′), 109.4 (C-7), 100.7 (C-3), 79.9 (–O–CH$_2$-), 68.5 (–CHOH–), 31.9 (–CH$_2$–S–). **HR-ESI–MS:** calculated for $C_{24}H_{19}N_7O_3S$ $[M]^+$: 485.1270, found: 485.1268.

(*2′Z-3′E*)-Indirubin-3′-[*O*-2-hydroxy-3-(5-phenylamino)-1,3,4-thiadiazole-2-thio)prop-1-yl]oxime (7f). Yield 69.6%, red solid, m.p. 236–237 °C. **IR (KBr, ν_{max} (cm^{-1})):** 3416 (O–H overlap N–H), 1735 (C=O), 1616 (C=N), 1600 and 1524 (C=C), 1338 (C–N), 1086 (N–N), 642 (C–S–C). **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 11.67 (s, 1H, H-1′), 10.72 (s, 1H, H-1), 10.32 (s, 1H, NH-phenyl), 8.58 (d, J=7.50 Hz, 1H, H-4), 8.19 (d, J=7.50 Hz, 1H, H-4′), 7.53 (d, J=8.0 Hz, 2H, H-2″, H-6″), 7.41 (m, 2H, H-6′, H-7′), 7.32 (t, J=7.75 Hz, 2H, H-3″, H-5″), 7.1 (t, J=7.50 Hz, 1H, H-6), 7.01 (m, 3H, H-5′, H-4″, H-5), 6.89 (d, J=7.50 Hz, 1H, H-7), 5.76 (d, J=5.50 Hz, 1H, –CHOH-), 4.66 (dd, J_1=11.0 Hz, J_2=4.50 Hz, 1H, –O–CH$_2$-), 4.59 (dd, J_1=11.0 Hz, J_2=6.0 Hz, 1H, –O–CH$_2$-), 4.34 (m, 1H, –CHOH), 3.53 (dd, J_1=13.50 Hz, J_2=5.0 Hz, 1H, –CH$_2$–S–), 3.37 (1H, –CH$_2$–S–). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 171.4 (C-2), 165.2 (–NH–C$_{thiadiazole-}$), 153.5 (–CH$_2$–S–C$_{thiadiazole}$), 152.1 (C-3′), 145.9 (C-2′), 144.3 (C-7′a), 140.9 (C-1″), 139.1 (C-7a), 133.3 (C-6′), 129.6 (C-3″, C-5″), 129.2 (C-4′), 126.9 (C-6), 123.9 (C-4), 122.8 (C-3a), 122.5 (C-4″), 121.9 (C-5′), 121.2 (C-5), 117.9 (C-2″, C-6″), 116.7 (C-3′a), 112.1 (C-7′), 109.4 (C-7), 100.8 (C-3), 79.6 (–O–CH$_2$-), 68.3 (–CHOH–), 38.3 (–CH$_2$–S–). **HR-ESI–MS:** calculated for $C_{27}H_{22}O_3N_6S_2$ $[M]^+$: 542.1195, found: 542.1190.

(*2′Z-3′E*)-Indirubin-3′-[*O*-2-hydroxy-3-(5-((2,3-dimethylphenyl)amino)-1,3,4-thiadiazole-2-thio)prop-1-yl]oxime (7 g). Yield 72.3%, red solid, m.p. 231–232 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 11.7 (s, 1H, H-1′), 10.77 (s, 1H, H-1), 9.55 (s, 1H, NH-phenyl), 8.58 (d, J=8.0 Hz, 1H, H-4), 8.18 (d, J=7.50 Hz, 1H, H-4′), 7.43 (m, 3H, H-6′, H-7′, H-4″), 7.13 (m, 1H, H-6), 7.05 (m, 1H, H-5″), 7.00 (m, 3H, H-5′, H-6″, H-5), 6.9 (d, J=7.50 Hz, 1H, H-7), 5.75 (d, J=5.50 Hz, 1H, –CHOH-), 4.66 (dd, J_1=11.0 Hz, J_2=4.50 Hz, 1H, –O–CH$_2$-), 4.59 (dd, J_1=11.0 Hz, J_2=6.50 Hz, 1H, –O–CH$_2$-), 4.31 (m, 1H, –CHOH–), 3.47 (dd, J_1=13.50 Hz, J_2=5.0 Hz, 1H, –CH$_2$–S–), 3.34 (1H, –CH$_2$–S–), 2.26 (s, 3H, CH$_3$ –C-3″), 2.11 (s, 3H, CH$_3$–C-2″). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 171.3 (C-2), 168.8 (–NH–C$_{thiadiazole-}$), 152.6 (–CH$_2$–S–C$_{thiadiazole}$), 152.1 (C-3′), 146 (C-2′), 144.3 (C-7′a), 139.5 (C-7a), 139.1 (C-1″), 138 (C-3″), 133.3 (C-6′), 129.7 (C-2″), 129.1 (C-4′), 127 (C-4″), 126,9 (C-6), 126.4 (C-5″), 123.9 (C-4), 122.8 (C-3a), 121.9 (C-5′), 121.2 (C-5), 121.1 (C-6″), 116.6 (C-3′a), 112.2 (C-7′), 109.4 (C-7), 100.7 (C-3), 79.6 (–O–CH$_2$), 68.3 (–CHOH–), 38.5 (–CH$_2$–S–), 20.7 (CH$_3$–C-3″), 14.2 (CH$_3$–C-2″). **HR-ESI–MS:** calculated for $C_{29}H_{26}O_3N_6S_2$ $[M]^+$: 570.1508, found: 570.1501.

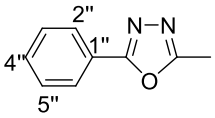
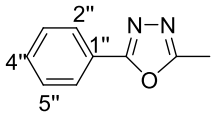
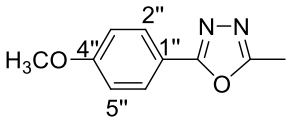
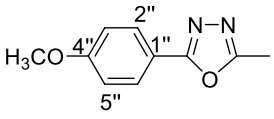
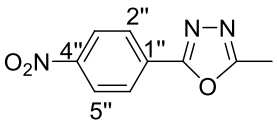
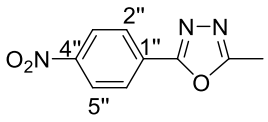
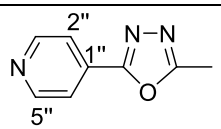
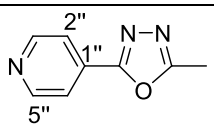
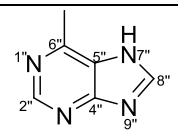
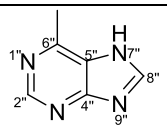
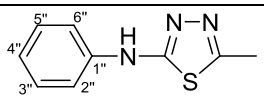
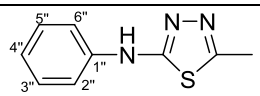
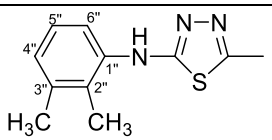
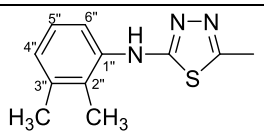
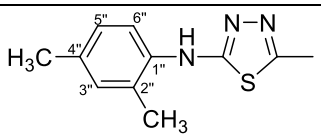
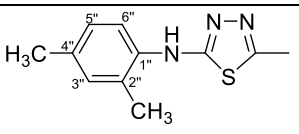
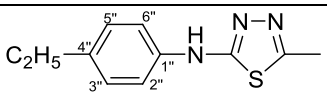
(*2′Z-3′E*)-Indirubin-3′-[*O*-2-hydroxy-3-(5-((2,4-dimethylphenyl)amino)-1,3,4-thiadiazole-2-thio)prop-1-yl]oxime (7 h). Yield 66.8%, red solid, m.p. 233–234 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 11.78 (s, 1H, H-1′), 10.87 (s, 1H, H-1), 9.53 (s, 1H, NH-phenyl), 8.69 (d, J=7.50 Hz, 1H, H-4), 8.28 (d, J=8.0 Hz, 1H, H-4′), 7.65 (d, J=8.0 Hz, 1H, H-5″), 7.53 (m, 2H, H-6′, H-7′), 7.23 (m, 1H, H-6), 7.10 (m, 4H, H-5′, H-3″, H-6″, H-5), 7.01 (d, J=8.0 Hz, 1H, H-7), 5.85 (d, J=5.50 Hz, 1H, –CHOH-), 4.76 (dd, J_1=11.0 Hz, J_2=4.50 Hz, 1H, –O–CH$_2$-), 4.69 (dd, J_1=11.0 Hz, J_2=6.0 Hz, 1H, –O–CH$_2$-), 4.42 (m, 1H, –CHOH–), 3.58 (dd, J_1=13.50 Hz, J_2=5.50 Hz, 1H, –CH$_2$–S–), 3.44 (1H, –CH$_2$–S–), 2.35 (s, 3H, CH$_3$–C-4″), 2.12 (s, 3H, CH$_3$–C-2″). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 171.3 (C-2), 168.2 (–NH–C$_{thiadiazole-}$), 152.6 (–CH$_2$–S–C$_{thiadiazole}$), 152.1 (C-3′), 146 (C-2′), 144.3 (C-7′a), 139.1 (C-7a), 137 (C-1″), 133.9 (C-4″), 133.3 (C-6′), 131.8 (C-5″), 130.1 (C-2″), 129.1 (C-4′), 127.6 (C-3″), 126.9 (C-6), 123.7 (C-4), 122.8 (C-3a), 122.4 (C-6″), 121.9 (C-5′), 121.2 (C-5), 116.7 (C-3′a), 112.2 (C-7′), 109.4 (C-7), 100.8 (C-3), 79.6 (–O–CH$_2$-), 68.3 (–CHOH–), 38.4 (–CH$_2$–S–), 20.9 (CH$_3$–C-4″), 18.2 (CH$_3$–C-2″). **HR-ESI–MS:** calculated for $C_{29}H_{26}O_3N_6S_2$ $[M]^+$: 570.1508, found: 570.1502.

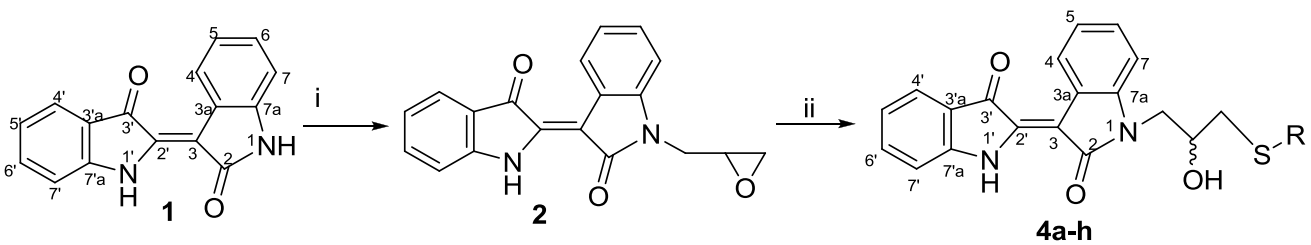
(*2′Z-3′E*)-**Indirubin-3′-[*O*-2-hydroxy-3-(5-((4-ethylphenyl)amino)-1,3,4-thiadiazole-2-thio)prop-1-yl]oxime (7i).** Yield 65.6%, red solid, m.p. 221–222 °C. **^{1}H NMR (DMSO-d_6, 500 MHz), δ(ppm):** 11.68 (s, 1H, H-1′), 10.74 (s, 1H, H-1), 10.24 (s, 1H, NH-phenyl), 8.58 (d, J=7.50 Hz, 1H, H-4), 8.19 (d, J=8.0 Hz, 1H, H-4′), 7.43 (d, J=8.50 Hz, 2H, H-2″, H-6″), 7.40 (m, 2H, H-6′, H-7′), 7.15 (d, J=8.50 Hz, 2H, H-3″, H-5″), 7.1 (m, 1H, H-6), 7.03 (m, 1H, H-5′), 6.98 (m, 1H, H-5), 6.88 (d, J=8.0 Hz, 1H, H-7), 5.75 (d, J=5.50 Hz, 1H, –CHOH-), 4.66 (dd, J_1=11.0 Hz, J_2=4.50 Hz, 1H, –O–CH$_2$-), 4.59 (dd, J_1=11.0 Hz, J_2=6.50 Hz, 1H, –O–CH$_2$-), 4.34 (m, 1H, –CHOH–), 3.52 (dd, J_1=13.50 Hz, J_2=5.0 Hz, 1H, –CH$_2$–S–), 3.34 (1H, –CH$_2$–S–), 2.55 (q, J=7.50 Hz, 2H, CH$_3$–CH$_2$-), 1.16 (t, J=7.50 Hz, 3H, CH$_3$–CH$_2$-). **^{13}C NMR (DMSO-d_6, 125 MHz), δ(ppm):** 170.9 (C-2), 165.0 (–NH–C$_{thiadiazole-}$), 152.5(–CH$_2$–S–C$_{thiadiazole}$), 151.6 (C-3′), 145.5 (C-2′), 143.8 (C-7′a), 138.7 (C-7a), 138.2 (C-1″), 137.5 (C-4″), 132.9 (C-6′), 128.7 (C-4′), 128.3 (C-3″, C-5″), 126.4 (C-6), 123.4 (C-4), 122.3 (C-3a), 121.5 (C-5′), 120.8 (C-5), 117.6 (C-2″, C-6″), 116.2 (C-3′a), 111.7 (C-7′), 108.9 (C-7), 100.3 (C-3), 79.2 (–O–CH$_2$-), 67.8 (–CHOH–), 37.9 (–CH$_2$–S–), 27.5 (CH$_3$–CH$_2$-), 15.7 (CH$_3$–CH$_2$-). **HR-ESI–MS:** calculated for $C_{29}H_{26}O_3N_6S_2$ $[M]^+$: 570.1508, found: 570.1502.

Results and discussion

(*2′Z*)-Indirubin derivatives **4a-h** (Table 1) were synthesized via a two-step procedure (Scheme 1). Indirubin was first converted into the key intermediate **2** by the nucleophilic substitution at *N*1 position with epichlorohydrin using K_2CO_3 as a catalyst in the presence of a transfer calalyst (1-butyl) triethylammonium bromide) to give **2** in 40% yield. The *N*1-alkylation of indirubin was confirmed by the interactions

Table 1 Structures and numbering of **4a-h** and **7a-i**

No	Compounds	R	No	Compounds	R
1	4a		1	7a	
2	4b		2	7b	
3	4c		3	7c	
4	4d		4	7d	
5	4e		5	7e	
6	4f		6	7f	
7	4g		7	7g	
8	4h		8	7h	
9			9	7i	



Scheme 1 Preparation of new derivatives 4a-h: Reagents and conditions: (i) epichlorohydrin, DMF, K2CO3, KI, (1-butyl)triethylammonium bromide; (ii) thiols 3a-h, DMF, rt, 40 h

of protons in $-N_1-CH_2-$ at 4.20 ppm (dd, $J_1 = 15.0$ Hz, $J_2 = 3.75$ Hz) and 3.88 ppm (dd, $J_1 = 15.0$ Hz, $J_2 = 5.25$ Hz) with C-2 (169.6 ppm) and C-7a (141.9 ppm) of indirubin in HMBC spectrum. Epoxy **2** was then opened with thiols **3a-h** to obtain target compounds **4a-h** in 63.1–77.0% yields (Scheme 1).

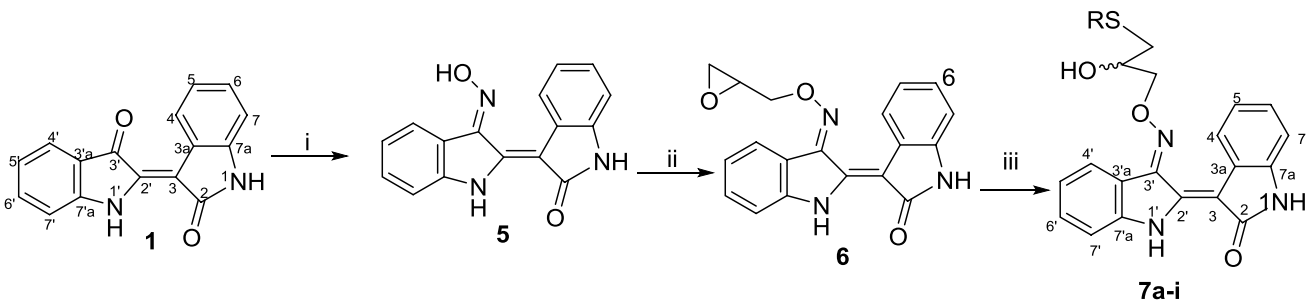
The structure of **4a-h** was determined by 1D, 2D and HRMS spectra. The spectral signal distinction among derivatives **4a-h** was found due to the resonance of protons and carbons in thiol moieties. Compound **4f** was selected to assign structure supported extensively by HSQC and HMBC correlations. The data given from ^{1}H- and ^{13}C-NMR spectra of **4f** as well as the support from HSQC and HMBC spectra agreed well with the structure **4f**. The structure of the remaining was in good agreement with NMR and HRMS data.

Series of new (*2′Z-3′E*)-indirubin-3′-oxime derivatives (**7a-i**) were produced by opening epoxy ring at oxime position of the key intermediate **6** that was synthesized from indirubin (**1**) in two steps. Compound **5** with (2*E*,3*Z*) conformation was first obtained in 93% yield by a condensation reaction of indirubin with hydroxylamine hydrochloride in pyridine (Cuong et al. 2010a, b, c). Compound **5** was then *O*-alkylated using epibromohydrin catalyzed by triethylamine according to a known procedure (Ichimaru et al. 2015) to afford **6** in 70% yield (Scheme 2). In the last step, **6** was opened with the corresponding thiols **3a-i** in DMF in the presence of triethylamine as a catalyst to give **7a-i** in 59.2–75.0% yields (Table 1).

The structure of **7a-i** was confirmed by 1D, 2D and HRMS spectra. The similarity of the multiplicity and signal position of indirubin moiety was easily observed in ^{1}H- and ^{13}C-NMR spectra of compounds **7a-i** and the distinction among those was also found due to the different structure of thiol components. Compound **7f** was selected to assign structure supported extensively by HSQC and HMBC correlations. In ^{1}H-NMR spectra, protons H-1, H-1′ of indirubin and NH of thiol moiety appeared as singlet signals at 10.72, 11.67 and 10.32 ppm respectively. Two doublets at 8.58 ppm ($J = 7.50$ Hz) and 8.19 ppm ($J = 7.50$ Hz) attributed to H-4 and H-4′ protons, and the overlapped doublet signal (δ: 7.53 ppm, $J = 8.0$ Hz) was assigned to protons H-2′′ and H-6′′. Protons H-3′′ and H-5′′ were recognized by a triplet at 7.32 ppm ($J = 7.75$ Hz) and other triplet at 7.10 ppm ($J = 7.50$ Hz) arose from H-6. Besides a multiplet at 7.41 ppm arising from H-6′ and H-7′ of indirubin moiety, signal of the remaining thiol proton H-4′′ overlapped by protons H-5 and H-5′ of indirubin component was found due to a multiplet at (7.01 ppm). The final proton H-7 in indirubin resonates as a doublet ($J_1 = 7.50$ Hz) at 6.89 ppm. In 2-ol-1,3-propylen linker, a doublet ($J = 5.50$ Hz) at 5.76 ppm was assigned to proton –OH and methine proton attached to this group appeared as a multiplet at 4.34 ppm. In addition, doublet–doublet signals at 4.66 ppm ($J_1 = 11.0$ Hz, $J_2 = 4.50$ Hz) and 4.59 ppm ($J_1 = 11.0$ Hz, $J_2 = 6.0$ Hz) were determined to be resonance signals of protons $O-CH_2-$, and the signals at 3.53 ppm and 3.37 ppm were characteristic of two protons, which were neighbor to sulfur ($S-CH_2$). Based on HSQC spectrum, the chemical shifts of the corresponding carbons were elucidated as: C-4: 123.9, C-4′: 129.2, (C-2′′, C-6′′): 117.9, (C-3′′, C-5′′): 129.6; $C_{\underline{C}HOH}$: 68.3, $C-O_{\underline{C}H2-}$: 79.6, $C_{S-\underline{C}H2-CHOH-CH2-}$: 38.3 ppm. In addition, the key cross-picks of $C_{thiadiazole}$ (153.5 ppm) with $H_{S-\underline{C}H2-CHOH-}$ in HMBC approved the correct structure of **7f** (Scheme 2).

Bioactivity

In the present study, indirubin (**1**), indirubin-3′-oxime (**5**) and 6-mercaptopurine (**6-MP**), a medicine for treatment of blood cancer were also evaluated for cytotoxicity against SW480, LU-1, HepG2 and HL-60 cell lines along with indirubin and indirubin-3′-oxime derivatives (**4a-h** and **7a-i**), and ellipticine was used as positive control for SAR evaluation (Table 1). Cytotoxic activity against SW480, LU-1, HepG2 cell lines and anti-proliferative activity of HL-60 cell line were undertaken using the method as described by Monks (Monks et al. 1991). The derivatives **4a-h**, **7a-i** were judged to have no activity when their IC_{50} values > 20 μM.



Scheme 2 Preparation of new derivatives 7a-i: Reagents and conditions: (i) hydroxylamine, pyridine, 120 °C; (ii) epibromohydrin, TEA, DMF; (iii) thiols 3a-i, TEA, DMF, rt, 40 h

Table 2 *In vitro* cytotoxic activity of indirubin **4a-h** and indirubin-3-oxime derivatives **7a-i**

No	Compounds	IC_{50} (μM)			
		SW480	LU-1	HepG2	HL60
1	**4a**	>20	>20	>20	>20
2	**4b**	>20	>20	>20	>20
3	**4c**	>20	>20	>20	>20
4	**4d**	>20	>20	>20	>20
5	**4e**	>20	>20	>20	>20
6	**4f**	**1.65**	**2.21**	**1.90**	**1.35**
7	**4 g**	>20	>20	>20	>20
8	**4 h**	**4.19**	**4.53**	**4.47**	**4.28**
9	**7a**	**19.24**	**19**	>20	>20
10	**7b**	>20	>20	>20	>20
11	**7c**	>20	>20	>20	>20
12	**7d**	>20	>20	>20	>20
13	**7e**	>20	>20	>20	>20
14	**7f**	**16.38**	**16.90**	**17.67**	**9.52**
15	**7 g**	**15.96**	**19.11**	**15.34**	**12.03**
16	**7 h**	>20	**19.20**	>20	**16.40**
17	**7i**	>20	>20	>20	>20
18	**6-MP**	**19.10**	**18.75**	**19.72**	**16.04**
19	**indirubin**	>20	>20	>20	>20
20	**Indirubin-3′-oxim**	**14.26**	**13.11**	**15.87**	**14.90**
21	**Ellipticine**	**2.19**	**1.91**	**1.79**	**2.40**

The reference substance, ellipticine, exhibited cytotoxic activity against SW 480 (ATCC-CCL-228), LU-1 (ATCC-HTB-57), HepG2 (ATCC-HB-8065) and HL-60 (ATCC-CCL-240) with IC_{50} values of 2.19, 1.91, 1.79 and 2.40 μM, respectively. The values shown for these compounds are the average of three determinations

In screening for cytotoxic and anti-proliferative activities against four cancer cell lines: SW480, LU-1, HepG2 and HL 60, indirubin did not show direct activity. This was also observed in some its derivative **4a-h** excluding **4f** and **4 h** (Table 2). Obviously, the derivatives derived from *N1* position did not give the clear advantages to parent compound, indirubin, even derivative **4e** formed by the opening epoxy with 6-mercaptopurine (**6-MP**). However, in some cases, the structure of thiol component has a significant effect on cytotoxic and anti-proliferative activities. Compounds **4f**, **4 h** derived from indirubin and 5-(phenylamino)-1,3,4-thiadiazole-2-thiol or 5-((2,4-dimethylphenyl)amino)-1,3,4-thiadiazole-2-thiol showed strong activity against SW480, LU-1, HepG2 and HL-60 cell lines in which compound **4f** exhibited the strongest activity with IC_{50} values of 1.65, 2.21, 1.90 and 1.35 μM, respectively, much stronger than those of 6-mercaptopurin, indirubin-3′-oxime and nearly equivalent to those of ellipticine.

In series **7a-i**, data on cytotoxic and anti-proliferative activities indicated that 3′-oxime group could be an important anticancer activity center of indirubin-3′-oxime. The chemical intervention in this group resulted in the decreased activity, and the synthesized derivatives showed no activity against SW480, LU-1, HepG2, and HL-60 cell lines except derivatives **7a**, **7f**, **7 g** and **7 h** containing phenylaminothiadiazole, phenylthiazole and 6-mecarptopurin exhibited weak activity with IC_{50} values in range of 15.34–19.24 μM and not so prominent. An expected result was also found with derivative **7e**, a conjugate of indirubin-3′-oxime and a medicine for treatment of blood cancer, 6-mecarptopurine was negative with tested cell lines. Evidently, the evaluation of cytotoxic and anti-proliferative activities of indirubin and indirubin-3′-oxime derivatives resulted in conclusion that the particular structure of parent compound, (*2′Z*)-indirubin and 3′-oxime group were main activity centers of (*2′Z-3′E*)-indirubin-3′-oxime. We suggest this be an important orientation for the design, synthesis of indirubin-3-oxime derivatives in a search for new cytotoxic agents in our future research.

Conclusions

The design, synthesis and screening for cytotoxic and anti-proliferative activities of indirubin derivatives containing thiols were successfully performed. The cytotoxic and anti-proliferative activities of five in 17 new derivatives were also carried out. Data on cytotoxic and anti-proliferative activities of two series **4a-h** and **7a-i** could lead to conclusion that the combination of specific configuration of indirubin and 3′-oxime group in its derivative was necessary for anticancer activity. Therefore, chemical transformation at oxime group did not improve anticancer activity while chemical intervention at *N*1 position could produce new derivatives that showed strong cytotoxic and anti-proliferative activities in some cases. This may be an important orientation in finding candidates from indirubin that can be used in cancer treatment in the future.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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