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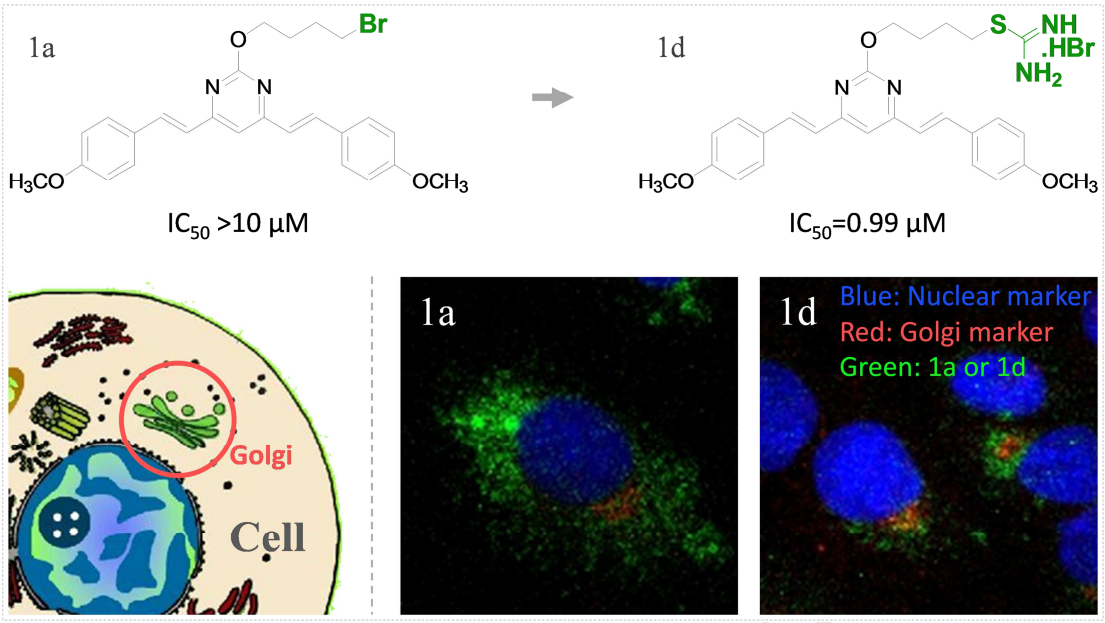

Isothiouronium modification empowers pyrimidine-substituted curcumin analogs potent cytotoxicity and Golgi localization

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ABSTRACT

Most of protein post-translational modifications occur in the Golgi and many human diseases are associated with abnormal Golgi function or improper post translational modifications of proteins in the Golgi. In this study, we designed and synthesized 4×6 series of novel isothiouronium-modified (*E*,*E*)-4,6-bis(styryl)-pyrimidine analogs and found that they localized at the Golgi as visualized by the intrinsic fluorescence of the analogs. The isothiouronium-modified analogs had potent cytotoxicity in both normal (Chinese Hamster Ovary or CHO) and cancer cells. Furthermore, permethylated isothiouronium-modified analogs showed cancer cell-selective cytotoxicity. The molecular mechanisms underlying Golgi localization of

isothiouronium-modified compounds were investigated using 7 CHO and 4 human cancer cell lines and the results indicated that the compounds had binding partners in the Golgi. Thus, isothiouronium-modified analogs might be promising anticancer agents, novel Golgi staining reagents, and useful research tools for studying Golgi functions in normal or cancer cells and in Golgi-related human diseases.

Key words: Isothiouronium; pyrimidine-substituted curcumin; Golgi; cytotoxicity; (*E*,*E*)-4,6-bis(styryl)-pyrimidine; immuno-staining; cancer

1. Introduction

Curcumin is responsible for the therapeutic properties of turmeric in traditional medicine. Its IC_{50s} in cancer cells range from 3 to > 30 μM that are quite high compared to most of anticancer drugs[1,2]. Dietary curcumin exhibits poor bioavailability, rapid metabolism, low solubility, and extensive rapid excretion in clinical trials [1-3]. We have previously reported a family of (*E*,*E*)-4,6-bis(styryl)-pyrimidine analogs that contain the pyrimidine ring instead of the β-dicarbonyl moiety of curcumin. These analogs have significantly improved anticancer activity, chemical stability, and solubility when compared to curcumin [4,5]. Furthermore, the V-shaped D-π-A-π-D structure of the pyrimidine-substituted curcumin analogs generates intensive fluorescence [6]. However, the fluorescent property has not been explored for biological applications.

With only 20,000 to 30,000 human genes, the dynamic complexity of living systems is achieved, in part, through over 400 known post-translational modifications of proteins [7]. Most of protein post-translational modifications, especially N-linked and O-linked glycosylations [8-10], occur in the Golgi and many human diseases are associated with abnormal Golgi function or improper protein post translational modifications in the Golgi [11]. At present, development of small molecule probes that target the morphology and reveal the functions of Golgi is still in its infancy [12]. In fact, only limited numbers of small molecules with different backbone structures

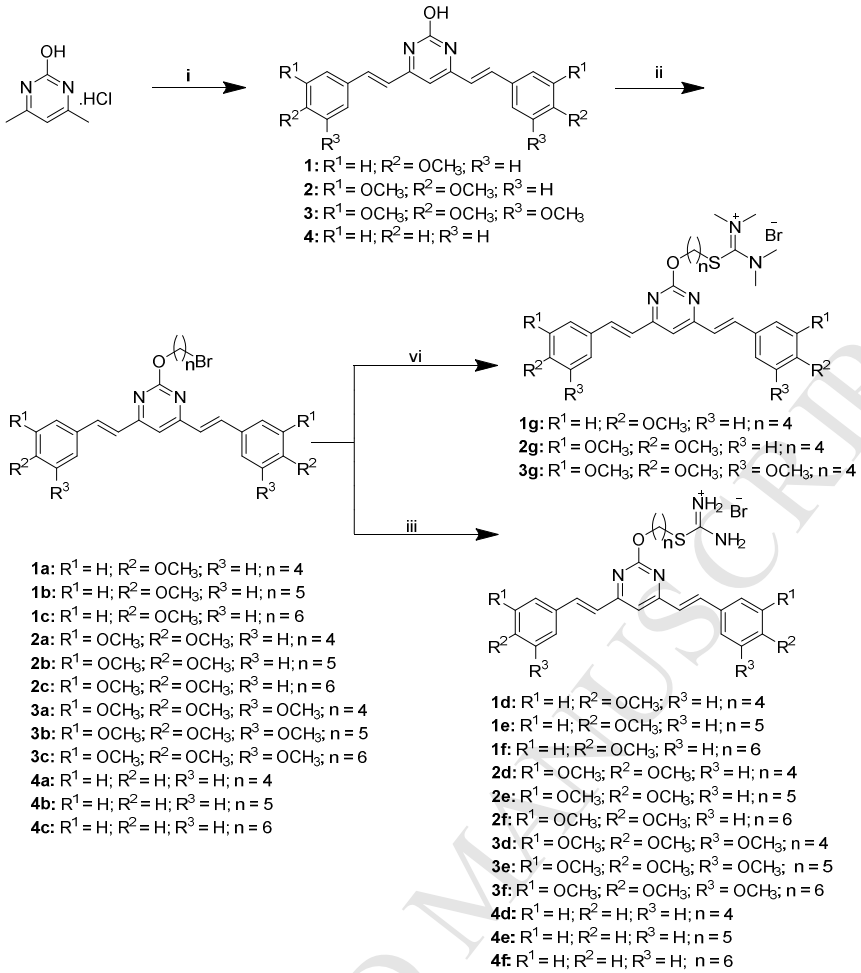
that perturb the function of Golgi have been reported [13-15]. We noticed that positive charges in those small molecules seem to be associated with the Golgi localization [13-15], which is consistent with the fact that Golgi is an acidic compartment with a pH gradient ranging from 5 to 6. Remarkably, a series of isothiouronium-modified compounds exhibit significant pharmacological activities, such as anesthetic [16], antimicrobial [17], and antitumoral [18-21] with unknown mechanisms.

Based on these facts, we decided to introduce positively charged isothiouronium group to pyrimidine-substituted curcumin analogs and to screen for Golgi-localized compounds by taking advantage of the unique fluorescent properties of the (*E*,*E*)-4,6-bis(styryl)-pyrimidine analogs. Indeed, our data showed that the isothiouronium-modified analogs had greatly enhanced anticancer activities in addition to their unique Golgi localization compared to the unmodified compounds.

2. Chemistry

We first designed and synthesized 4×6 series of isothiouronium-modified (*E*,*E*)-4,6-bis-(styryl)-pyrimidine analogs in three steps as outlined in Scheme 1. The compounds **1**-**4** were prepared from the appropriate aromatic aldehydes according to the procedure we reported previously [4]. Subsequently, the compounds were allowed to react individually with excessive 1,ω-dibromoalkane in the presence of K_2CO_3 to yield the ω-bromoalkyl derivatives **1a**~**c**-**4a**~**c**, which were separated by chromatography in 30%~50% yield. Then **1a**~**c**-**4a**~**c** reacted with thiourea in boiling acetone to generate the target compounds **1d**~**f**-**4d**~**f** in 50%~70% yield.

We also synthesized three 1,1,3,3-tetramethylated isothiouronium derivatives by mixing compounds **1a**-**3a** with 1,1,3,3-tetramethylthiourea in boiling acetone in 50%~60% yield.



Scheme 1. Synthesis of the isothiouronium derivatives of curcumin-pyrimidine analogs.

Reagents and conditions: (i) appropriate aromatic aldehydes, HCl, EtOH, rf, 50%~75%; (ii) $Br(CH_2)_nBr$, K_2CO_3, DMF, 80°C, 30%~50%;(iii)Thiourea, acetone, rf, 50%~70%; (vi) 1,1,3,3-Tetramethylthiourea, acetone, rf, 50%~60%.

3. Results and discussion

3.1 Cytotoxicity and IC_{50}

Four human cancer cell lines A549 (lung), H1299 (lung), HCT116 (colon), and HT29 (colon) were treated with 10 μM of compounds **1a, 1d, 1g, 2a, 2d, 2g, 3a, 3d,** and **3g** or compound-free control for 48 h and the cell viability was measured as an indicator for cytotoxicity of the compounds (Figure 1).

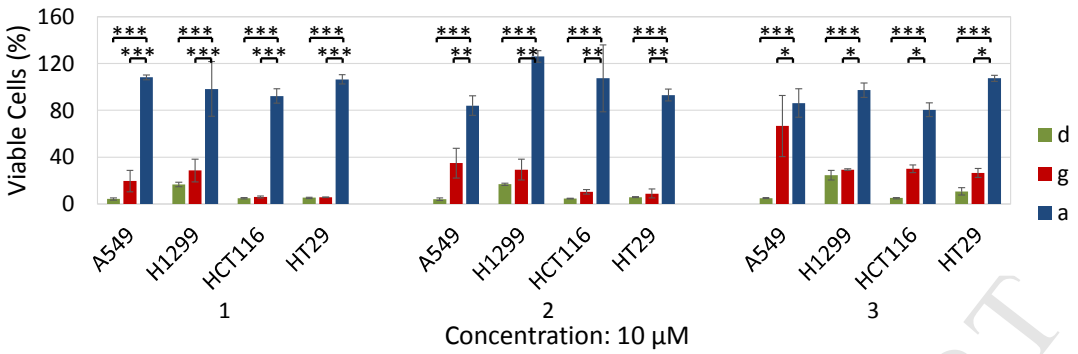


Figure 1. Effects of the tested compounds on cell viability of four human cancer cell lines. Percentage of viable cells (A549, H1299, HCT116, HT29) after 48 h exposure to the curcumin-pyrimidine analogues (**1a, 1d, 1g, 2a, 2d, 2g, 3a, 3d,** and **3g**) at a concentration of 10 µM compared to the compound-free control received an equal volume of DMSO (100% viability) (***P<0.001, **P<0.01, *P < 0.05; Student's t-test). The analogues were formulated initially in DMSO and then diluted in complete growth media. Each value was calculated from 2 independent experiments performed in triplicate. Data are shown as mean ± SD.

To obtain IC_{50} values for each compound, all four cancer cells were treated with series of concentrations of each compound and the data were summarized in Table 1. All isothiouronium-modified compounds (**1d, 1e, 1f, 2d, 2e, 2f, 3d, 3e, 3f, 4d, 4e,** and **4f**) had IC_{50} values at low µM ranges. The 1,1,3,3-tetramethylated isothiouronium-modified compounds (**1g, 2g,** and **3g**) had higher IC_{50} values compared to their unmethylated counterparts (**1d, 2d,** and **3d**). In contrast, all Br-modified compounds (**1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 3c, 4a, 4b,** and **4c**) had IC_{50} values over 20 µM.

The IC_{50} values indicated that the compounds with one methoxyl group were most toxic in all four cancer cell lines tested. In general, when the compounds were modified by methoxyl groups, the fewer the number of methoxyl groups, the stronger the cytotoxicity (1>2>3). In contrast, the toxicities of compounds with no methoxyl modification were between the compounds with two and three methoxyl groups. When the compounds had 4, 5, or 6 methylene spacer (-(CH_2)-) separating isothiouronium from the (*E*,*E*)-4,6-bis(styryl)-pyrimidine, the spacer effect on IC_{50}

values was minimal in all four cell lines tested. Furthermore, the cytotoxicity declined when the isothiouronium was fully methylated (**1g, 2g,** and **3g**).

Table 1. IC_{50} of the compounds in four human cancer cell lines

Comp	IC_{50} (μM)[a]			
	HT29	HCT116	H1299	A549
1a	＞20	＞20	＞20	＞20
1b	＞20	＞20	＞20	＞20
1c	＞20	＞20	＞20	＞20
1d	0.79±0.16	1.00±0.13	0.92±0.14	0.99±0.19
1e	2.03±0.04	1.93±0.03	2.96±0.74	1.97±0.03
1f	2.14±0.13	1.98±0.04	3.69±0.42	1.97±0.13
1g	5.97±1.04	3.00±0.39	7.49±0.58	2.26±0.18
2a	＞20	＞20	＞20	＞20
2b	＞20	＞20	＞20	＞20
2c	＞20	＞20	＞20	＞20
2d	0.61±0.17	1.86±0.05	1.53±0.62	2.36±0.70
2e	1.91±0.13	1.81±0.05	4.04±0.44	1.99±0.05
2f	1.72±0.26	2.02±0.19	3.75±0.25	2.09±0.14
2g	4.10±1.35	3.89±1.4	5.81±0.91	9.71±0.94
3a	＞20	＞20	＞20	＞20
3b	＞20	＞20	＞20	＞20
3c	＞20	＞20	＞20	＞20
3d	2.19±0.04	3.77±0.16	5.1±0.72	4.24±0.91
3e	1.86±0.23	2.08±0.09	4.13±0.08	2.88±0.89
3f	1.17±0.49	1.87±0.03	3.09±1.22	2.00±0.03
3g	10.49±2.28	5.41±0.35	14.97±3.6	14.36±4.97
4a	＞20	＞20	＞20	＞20
4b	＞20	＞20	＞20	＞20
4c	＞20	＞20	＞20	＞20
4d	2.19±0.04	3.77±0.16	5.1±0.72	4.24±0.91
4e	1.86±0.23	2.08±0.09	4.13±0.08	2.88±0.89
4f	1.17±0.49	1.87±0.03	3.09±1.22	2.00±0.03

a. Each IC_{50} value was calculated from 3 independent experiments performed in triplicate. Data are shown as mean ± SD.

3. 2 Golgi localization of isothiouronium-modified pyrimidine-substituted curcumin analogs

The potent cytotoxicity of the isothiouronium-modified (*E*,*E*)-4,6-bis(styryl)-pyrimidine analogs (Figure 1 and Table 1) prompted us to consider that the negatively charged DNAs in nucleus might be the molecular targets of the positively charged analogs. To verify this hypothesis, we performed live cell imaging to evaluate the uptake and subcellular localization of the pyrimidine-substituted curcumin analogs. A549 or HCT116 cells were seeded on glass coverslips in complete cell culture media

before being treated with or without the compounds **1a** and **1d** at 10 μM for 4 h at 37 ºC. The cells were imaged for the unique green fluorescence in living cells under laser scanning confocal microscope. Surprisingly, in contrast to the even distribution of compound **1a** in cytoplasm, the isothiouronium-modified compound **1d** had an apparent Golgi localization.

To confirm the Golgi localization of isothiouronium-modified compound, we used Golgi marker Golgin-97 and the nuclear marker DAPI to co-stain A549 and HCT116 cells. The cells were seeded on glass coverslips in complete cell culture media before being treated with 10 μM of **1a, 1d, 1e, 1f,** and **1g** for 4 h at 37 ºC. Subsequently, the media were removed and the cells were fixed with 4% formaldehyde followed by permeabilizing with 0.2% Triton-100 in PBS. After being blocked with 5% bovine serum albumin (BSA), the cells were incubated with the anti-Glogin-97 primary antibody followed by incubation with fluorescent secondary antibody (red fluorescence). The nucleus was stained with DAPI (blue fluorescence). The cells stained by the compounds (green fluorescence), Golgin-97 (red fluorescence), and DAPI (blue fluorescence) were imagined by laser scanning confocal microscope and the results were shown in Figure 2.

Merging the cell images in green (compounds), red (Golgin-97), and blue (nucleus) channels showed the absence of green fluorescence and the presence of the red fluorescence (Golgin-97) in the Golgi next to the nucleus (blue fluorescence) in the blank control (**Control,** Figure 2). Br-modified compound **1a** was evenly distributed in the cytoplasm (**1a,** Figure 2). In contrast, isothiouronium-modified compound **1d** was mostly co-localization with Golgi marker Golgin-97 (**Insert 1** and **2**, Figure 2). Although compounds **1d, 1e**, and **1f** with four, five, and six methylene spacer groups did not show significant difference in their IC_{50} values (Table 1), their Golgi localization showed different patterns: the fluorescence generated by **1d** was mostly associated with the Golgi while the green fluorescence from **1e** and **1f** was associated with vesicles radiated away from the Golgi in the order of 6 > 5 > 4 of methylene spacer groups (**Inserts 3, 4, 5,** and **6,** Figure 2), suggesting the methylene

spacer groups in the compounds contributed to the subtle differences in their Golgi localization.

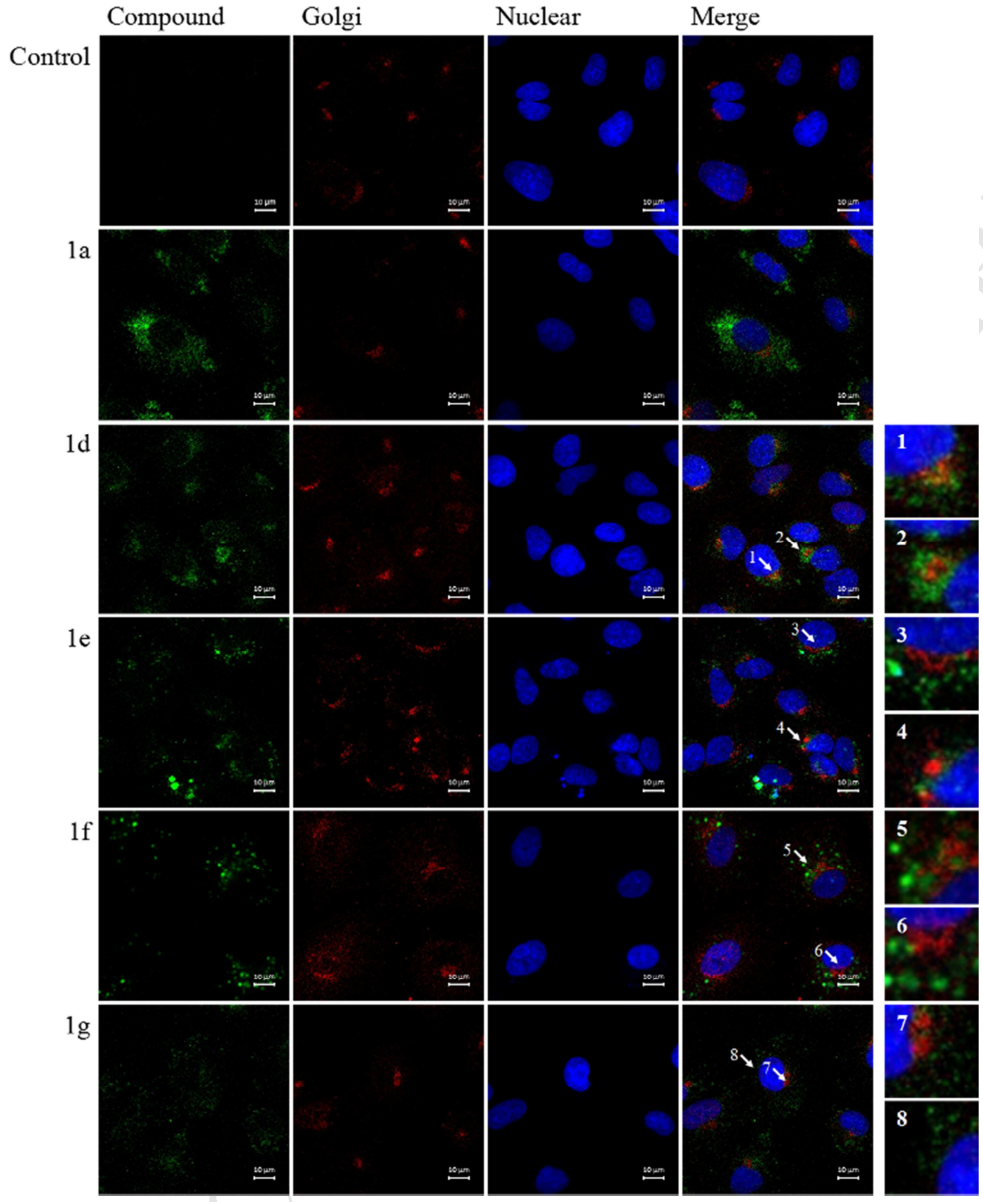


Figure 2. Fluorescence images of A549 showing the location of Compounds. Laser scanning confocal microscope images of A549 cells incubated with or without compounds (green) (**1a, 1d, 1e, 1f,** and **1g**) followed by Golgi (red) and nucleus (blue) staining with Golgin-97 and DAPI. Scale bars: 10 μm. Control: non-derivative treated cells recevied an equal volum of DMSO. Sub-Figure 1-8: zoom in of the arrows in **1d-1g**. The results have been repeated.

The IC_{50} values (Table 1) was obtained 48 h after the cells were treated with the compounds. Within this time frame, the isothiouronium-modified compounds were highly toxic. It raised the question if the compounds had binding partners in the Golgi

or they were accumulated in the Golgi as a result of their Golgi-targeted cytotoxicity. To address this issue, we conducted three sets of experiments.

First, we performed the live cell imaging using A549 cells treated with compounds **1a** and **1d** for 1, 2, 4, and 12 h. We observed that compound **1a** was evenly distributed in cytoplasm whereas the isothiouronium-modified compound **1d** had the Golgi localization at all-time points tested, indicating that compound **1d** may have binding partners in the Golgi.

It was reported that the positively charged guanidinium-rich scaffolds bind to negatively charged phosphate, sulfate, and carboxyl groups on cell surface through hydrogen binding [22]. We reasoned that if the isothiouronium-modified compound binds to its binding partners in the Golgi through hydrogen bond via isothiouronium, such binding will be weakened if the isothiouronium is fully methylated. Therefore, in the next experiment, we synthesized permethylated compounds **1g**, **2g**, and **3g**, and measured their cytotoxicity, followed by imaging the cellular localization of these compounds. Indeed, not only the cytotoxicity of compounds **1g, 2g**, and **3g** in all four cancer cell lines were decreased (Table 1), but also compound **1g** almost lost the Golgi localization (**Inserts 1, 2, 3, 4, 5, 6, 7,** and **8,** Figure 2). These results suggested that compounds **1d, 1e**, and **1f** bind to their binding partners in the Golgi through hydrogen bonds.

The same Golgi localization and cytotoxicity (data not shown) were observed when HCT116 cells were used in the same experiment, suggesting that the analogs have the same molecular actions in different types of cancer cells.

Finally, we investigated if the binding partners of the isothiouronium-modified (*E*,*E*)-4,6-bis(styryl)-pyrimidine analogs were glycosaminoglycans (GAGs), the most negatively charged biopolymers assembled on the core protein of proteoglycans in the Golgi since it was reported that the binding of positively charged guanidinoneomycin to cells is exclusively dependent on the cell surface glycosaminoglycans [23]. The normal fibroblast CHO cells used in current study include wild-type K1, GAG-deficient 745, heparan sulfate-deficient 677, 3-O-sulfotransferase 1-overexpressing 3.1, 3-O-sulfotransferase 2-overexpressing 3.2, 3-O-sulfotransferase 3-overexpressing 3.3,

3-O-sulfotransferase 4-overexpressing 3.4 that we have used and described in details previously [24]. We evaluated the cytotoxicity of compounds **1a, 1d, 1g, 2a, 2d**, and **2g** in all the CHO cells and the lung cancer cells A549 (Figure 3). The isothiouronium modified compounds **1d** and **2d** were highly toxic to all 8 cell lines including the GAG-deficient 745 cells, indicating that the binding partners in the Golgi might not be the GAGs (Figure 3). The Br-modified compound **1a** was not toxic whereas **1d**, **2a**, and **2d** were toxic to all cell lines tested. This evidence further supported the hypothesis that isothiouronium-modified compounds **1d** and **2d** targeted the common partners in the Golgi both in normal and cancer cells. Even though permethylated compounds **1g** and **2g** were toxic to all four cancer cells tested (Table 1, Figure 1, and Figure 3), they stimulated CHO cell growth instead, suggesting that **1g** and **2g** had methylation-related toxicity towards cancer cells only (Figure 3).

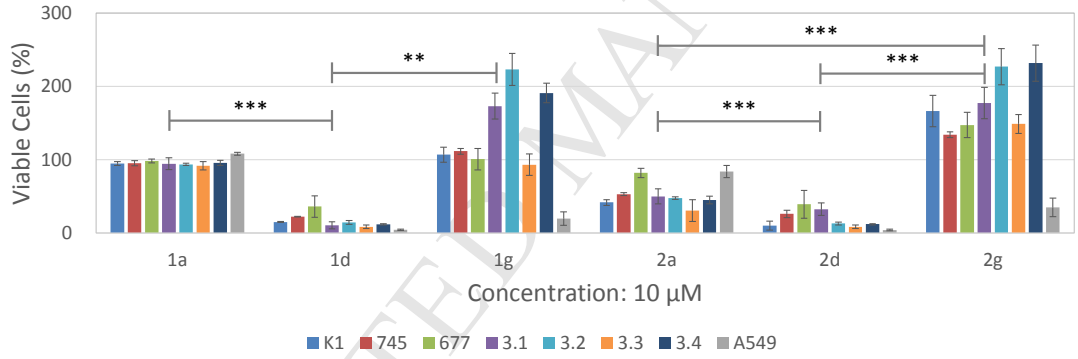


Figure 3. Effects of the tested compounds on cytotoxicity to all the CHO cells and the lung cancer cells A549. Percentage of viable cells (CHOK1, 745, 677, 3.1, 3.2, 3.3, 3.4, and human cancer cells A549) after 48 h exposure to the curcumin-pyrimidine analogues (**1a, 1d, 1g, 2a, 2d,** and **2g**) at a concentration of 10 µM compared to the compound-free control received an equal volume of DMSO (100% viability) (***P<0.001, **P<0.01, *P < 0.05; Student's t-test). The analogues were formulated initially in DMSO and then diluted in complete growth media. Each value was calculated from 2 independent experiments performed in triplicate. Data are shown as mean ± SD.

4. Conclusions

In current study, we designed and synthesized a series of novel isothiouronium-modified pyrimidine-substituted curcumin analogs. Their unexpected Golgi

localization, potent cytotoxicity, and the molecular mechanisms underlying Golgi localization were revealed. These compounds provide several advantages over the current tools to image and study the function of the Golgi. First, they could be used in live cells and do not require fixation and permeation as common for the application of conventional immunofluorescence methods. Second, the probes were localized in the Golgi over a 12 h period that provided sufficient time frame to perform detailed functional studies of the Golgi-associated post translational modification-defects associated with various human diseases. Third, isothiouronium-modified (*E*,*E*)-4,6-bis(styryl)-pyrimidine analogs were toxic to both CHO and cancer cells whereas permethylated analogs were only toxic to cancer cells (Figure 3). Such property might provide a novel way for selecting and developing permethylated compounds as selective anti-cancer drugs.

In conclusion, identifying the precise binding partner(s) of the isothiouronium-modified pyrimidine-substituted curcumin analogs in the Golgi will be our next goal. This information is critical for understanding the isothiouronium-modified (*E*,*E*)-4,6-bis(styryl)-pyrimidine analog-induced cell death. In addition, the chemical and biological approach used in current study in developing Golgi targeting fluorescent probes can be useful tools for the booming field of protein post translational modification research in the near future.

5. EXPERIMENTAL SECTION

5.1. Chemical Synthesis

5.1.1. Materials and Methods

All chemicals were purchased from commercial vendors and used without further purification unless otherwise noted. Thin-layer chromatography (TLC) was performed on precoated E. Merck silica-gel 60 F254 plates. Column chromatography was performed on silica gel (200-300 mesh). Melting points were determined on a Mitamura-Riken micro-hot stage and were not corrected. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Jeol JNM-ECP 600 spectrometer with tetramethylsilane (Me_4Si) as the internal standard and chemical shifts were recorded as δ values in ppm.

Following abbreviations are used: s = singlet, d = doublet, t = triplet, q= quartet, m = multiplet, dd = double-doublet. Mass spectra were recorded on a Q-TOF Global mass spectrometer.

4,6-Dimethyl-2-hydroxyl-pyrimidine hydrochloride was prepared according to literature procedures [25]. The syntheses of (*E,E*)-4,6-bis(4-methoxystyryl)-2-hydroxyl-pyrimidine (**1**), (*E,E*)-4,6-bis(3,4-dimethoxystyryl)-2-hydroxyl-pyrimidine (**2**), (*E,E*)-4,6-bis(3,4,5-trimethoxystyryl)-2-hydroxylpyrimidine (**3**) have been described previously [4].

5.1.2. Synthesis of Compound (*E,E*)-4,6-bisstyryl-2-hydroxyl-pyrimidine (4).

A mixture of 2-hydroxy-4,6-dimethylpyrimidine hydrochloride (0.16 g, 1 mmol), benzaldehyde (306 μL, 3 mmol) in anhydrous ethyl alcohol (100 mL) was stirred under an N_2 atmosphere at room temperature for 1 h. Then hydrochloric acid (600 μL, 1.2 mmol) was added to the reaction mixture which was refluxed for 36 h. After completion of the reaction, the solvent was evaporated under reduce pressure, and the saturated $NaHCO_3$ solution (250 mL) was added into the brown oil residue for desalting. After stirring at room temperature for 5 h, the resulting precipitate was filtered, washed with water followed with ether, and then purified by chromatography on silica gel to give **4** as a yellow powder (0.15g , 50%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.73 (s, 1H), 7.85 (d, J = 16.3 Hz, 2H), 7.67 (d, J = 6.7 Hz, 4H), 7.52-7.35 (m, 6H), 7.05 (d, J = 16.2 Hz, 2H), 6.96 (s, 1H); MS (ESI) m/z ESI$^+$ 301.1 $[M+H]^+$.

5.1.3. General procedure for Compounds 1a~c-4a~c.

To a solution of **1-4** (1.0 mmol) in anhydrous N,N-dimethylformamide (DMF, 30 mL) at 80 °C was added anhydrous potassium carbonate (3.0 mmol), 1,ω-dibromoalkan (15.0 mmol) and the mixture was stirred for 12 h. After the reaction was judged complete by TLC, the solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 and washed with water. The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated. The resulting residue was purified by chromatography on silica gel to give compounds **1a~c-4a~c.**

5.1.4 (*E,E*)-2-(4-bromobutoxy)-4,6-bis(4-methoxystyryl)pyrimidine (1a)

Yellow powder (193 mg, yield 39%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.87 (s, 1H), 7.84 (s, 1H), 7.54 (d, J = 8.4 Hz, 4H), 6.92 (d, J = 8.4 Hz, 4H), 6.90 (s, 1H), 6.86 (s, 2H), 4.51 (t, J = 6.1 Hz, 2H), 3.84 (s, 6H), 3.52 (t, J = 6.5 Hz, 2H), 2.18-2.10 (m, 2H), 2.08-2.00 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.22, 165.17, 160.60, 136.40, 129.12, 128.59, 123.58, 114.29, 110.71, 66.06, 55.35, 33.61, 29.41, 27.67; MS (ESI) m/z ESI$^+$ 495.1 $[M+H]^+$.

5.1.5. (*E*,*E*)-2-(5-bromopentyloxy)-4,6-bis(4-methoxystyryl)pyrimidine (1b)

Yellow powder (178 mg, yield 35%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.87 (s, 1H), 7.84 (s, 1H), 7.54 (d, J = 8.7 Hz, 4H), 6.92 (d, J = 8.7 Hz, 4H), 6.90 (s, 1H), 6.87 (s, 2H), 4.48 (t, J = 6.5 Hz, 2H), 3.85 (s, 6H), 3.45 (t, J = 6.8 Hz, 2H), 2.02-1.95 (m, 2H), 1.94-1.88 (m, 2H), 1.73-1.66 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.29, 165.15, 160.57, 136.33, 129.10, 128.61, 123.63, 114.28, 110.62, 66.83, 55.35, 33.65, 32.60, 28.22, 24.88; MS (ESI) m/z ESI$^+$ 509.1 $[M+H]^+$.

5.1.6. (*E*,*E*)-2-(6-bromohexyloxy)-4,6-bis(4-methoxystyryl)pyrimidine (1c)

Yellow powder (161 mg, yield 31%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.87 (s, 1H), 7.84 (s, 1H), 7.54 (d, J = 8.7 Hz, 4H), 6.92 (d, J = 8.7 Hz, 4H), 6.90 (s, 1H), 6.87 (s, 2H), 4.48 (t, J = 6.5 Hz, 2H), 3.85 (s, 6H), 3.45 (t, J = 6.8 Hz, 2H), 2.02-1.95 (m, 2H), 1.94-1.88 (m, 2H), 1.73-1.66 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.11, 160.59, 136.40, 129.12, 128.61, 123.60, 114.28, 110.51, 67.08, 55.35, 33.89, 32.75, 28.87, 28.00, 25.36; MS (ESI) m/z ESI$^+$ 523.1 $[M+H]^+$.

5.1.7. (*E*,*E*)-2-(4-bromobutoxy)-4,6-bis(3,4-dimethoxystyryl)pyrimidine (2a)

Yellow powder (266 mg, yield 48%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.84 (d, J = 15.8 Hz, 2H), 7.18-7.12 (m, 4H), 6.93-6.85 (m, 5H), 4.52 (t, J = 6.1 Hz, 2H), 3.94 (d, J = 11.8 Hz, 12H), 3.53 (t, J = 6.5 Hz, 2H), 2.14 (dt, J = 13.4, 6.6 Hz, 2H), 2.04 (dt, J = 12.7, 6.4 Hz, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.23, 165.09, 150.30, 149.15, 136.70, 128.80, 123.78, 121.72, 111.09, 110.53, 109.42, 66.09, 55.96, 33.67, 29.40, 27.64; MS (ESI) m/z ESI$^+$ 555.1 $[M+H]^+$.

5.1.8. (*E*,*E*)-2-(5-bromopentyloxy)-4,6-bis(3,4-dimethoxystyryl)pyrimidine (2b)

Yellow powder (210 mg, yield 37%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.85 (s, 1H), 7.82 (s, 1H), 7.18-7.12 (m, 4H), 6.92-6.89 (m, 3H), 6.87 (d, J = 3.7 Hz, 2H), 4.49 (t, J

= 6.5 Hz, 2H), 3.93 (d, J = 13.3 Hz, 12H), 3.45 (t, J = 6.8 Hz, 2H), 2.01-1.95 (m, 2H), 1.94-1.87 (m, 2H), 1.70 (dd, J = 15.3, 8.0 Hz, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.33, 165.07, 150.31, 149.18, 136.61, 128.86, 123.88, 121.70, 111.14, 110.40, 109.49, 77.26, 77.01, 76.76, 66.85, 55.95, 33.66, 32.58, 28.22, 24.87; MS (ESI) m/z ESI$^+$ 569.1 $[M+H]^+$.

5.1.9 (*E*,*E*)-2-(6-bromohexyloxy)-4,6-bis(3,4-dimethoxystyryl)pyrimidine (2c)

Yellow powder (257 mg, yield 44%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.85 (s, 1H), 7.82 (s, 1H), 7.19-7.11 (m, 4H), 6.92-6.85 (m, 5H), 4.48 (t, J = 6.4 Hz, 2H), 3.93 (d, J = 13.4 Hz, 12H), 3.43 (t, J = 6.7 Hz, 2H), 1.95-1.85 (m, 4H), 1.56 (s, 4H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.39, 165.05, 150.27, 149.15, 136.58, 128.85, 123.90, 121.72, 111.09, 110.38, 109.39, 67.07, 55.95, 33.94, 32.74, 28.90, 27.99, 25.38; MS (ESI) m/z ESI$^+$ 583.1 $[M+H]^+$.

5.1.10. (*E*,*E*)-2-(4-bromobutoxy)-4,6-bis(3,4,5-trimethoxystyryl)pyrimidine (3a)

Red powder (221 mg, yield 36%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.80 (s, 1H), 6.95 (s, 1H), 6.93 (s, 1H), 6.90 (s, 1H), 6.82 (s, 4H), 4.53 (t, J = 6.1 Hz, 2H), 3.90 (d, J = 13.8 Hz, 18H), 3.53 (t, J = 6.5 Hz, 2H), 2.14 (dd, J = 13.7, 6.2 Hz, 2H), 2.05 (dd, J = 14.0, 6.1 Hz, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.27, 164.94, 153.44, 139.40, 136.91, 131.32, 125.13, 110.79, 104.79, 66.17, 60.98, 56.15, 33.58, 29.39, 27.62; MS (ESI) m/z ESI$^+$ 615.1 $[M+H]^+$.

5.1.12. (*E*,*E*)-2-(5-bromopentyloxy)-4,6-bis(3,4,5-trimethoxystyryl)pyrimidine (3b)

Red powder (270 mg, yield 43%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.81 (d, J = 15.8 Hz, 2H), 6.94 (d, J = 6.1 Hz, 2H), 6.90 (s, 1H), 6.82 (s, 4H), 4.49 (t, J = 6.2 Hz, 2H), 3.90 (d, J = 13.9 Hz, 18H), 3.45 (t, J = 6.6 Hz, 2H), 2.02-1.94 (m, 2H), 1.94-1.87 (m, 2H), 1.74-1.66 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.35, 164.90, 153.42, 139.31, 136.83, 131.35, 125.20, 110.74, 104.72, 66.96, 60.99, 56.14, 33.71, 32.55, 28.21, 24.87; MS (ESI) m/z ESI$^+$ 629.1 $[M+H]^+$.

5.1.12. (*E*,*E*)-2-(6-bromohexyloxy)-4,6-bis(3,4,5-trimethoxystyryl)pyrimidine (3c)

Red powder (264 mg, yield 41%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 (d, J = 15.6 Hz, 2H), 6.94 (d, J = 16.6 Hz, 3H), 6.82 (s, 4H), 4.48 (t, J = 6.3 Hz, 2H), 3.89 (d,

J = 13.4 Hz, 18H), 3.43 (t, J = 6.7 Hz, 2H), 1.90 (dt, J = 13.1, 6.7 Hz, 4H), 1.55 (s, 4H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.81,153.41, 139.32, 138.7.11, 131.30, 125.02, 110.57, 104.72, 67.28, 61.00, 56.14, 33.97, 32.69, 28.85, 27.95, 25.35; MS (ESI) m/z ESI$^+$ 643.1 $[M+H]^+$.

5.1.13. (*E*,*E*)-2-(4-bromobutoxy)-4,6-distyrylpyrimidine (4a)

White powder (148 mg, yield 34%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.93 (s, 1H), 7.89 (s, 1H), 7.60 (d, J = 7.3 Hz, 4H), 7.40 (t, J = 7.4 Hz, 4H), 7.35 (t, J = 7.0 Hz, 2H), 7.03 (d, J = 15.9 Hz, 2H), 6.95 (s, 1H), 4.53 (t, J = 6.2 Hz, 2H), 3.53 (t, J = 6.6 Hz, 2H), 2.15 (dt, J = 14.5, 6.5 Hz, 2H), 2.05 (dt, J = 12.7, 6.3 Hz, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.28, 165.06, 136.95, 135.76, 129.30, 128.84, 127.64, 125.76, 111.20, 66.22, 33.56, 29.40, 27.65; MS (ESI) m/z ESI$^+$ 435.1 $[M+H]^+$.

5.1.14. (*E*,*E*)-2-(5-bromopentyloxy)-4,6-distyrylpyrimidine (4b)

White powder (207 mg, yield 46%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.93 (s, 1H), 7.90 (s, 1H), 7.61 (d, J = 7.4 Hz, 4H), 7.40 (t, J = 7.3 Hz, 4H), 7.37-7.32 (m, 2H), 7.05 (s, 1H), 7.01 (s, 1H), 6.94 (s, 1H), 4.50 (t, J = 6.5 Hz, 2H), 3.46 (t, J = 6.8 Hz, 2H), 1.98 (dt, J = 15.3, 7.6 Hz, 2H), 1.92 (dd, J = 14.4, 7.0 Hz, 2H), 1.74-1.66 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.35, 165.02, 136.88, 135.78, 129.29, 128.84, 127.65, 125.80, 111.17, 67.00, 33.68, 32.60, 28.21, 24.88; MS (ESI) m/z ESI$^+$ 449.1 $[M+H]^+$.

5.1.15. (*E*,*E*)-2-(6-bromohexyloxy)-4,6-distyrylpyrimidine (4c)

White powder (143 mg, yield 31%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.93 (s, 1H), 7.90 (s, 1H), 7.60 (d, J = 7.5 Hz, 4H), 7.40 (t, J = 7.3 Hz, 4H), 7.37-7.33 (m, 2H), 7.05 (s, 1H), 7.01 (s, 1H), 6.94 (s, 1H), 4.49 (t, J = 6.4 Hz, 2H), 3.48-3.40 (m, 2H), 1.91 (dd, J = 13.0, 6.5 Hz, 4H), 1.59 (d, J = 19.6 Hz, 4H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.41, 165.00, 136.84, 135.79, 129.27, 128.84, 127.64, 125.83, 111.12, 67.21, 33.92, 32.75, 28.87, 28.01, 25.38; MS (ESI) m/z ESI$^+$ 463.1 $[M+H]^+$.

5.1.16 General procedure for 1d~f-4d~f

A mixture of compound **1a~c-4a~c** (0.1 mmol) and thiourea (0.5 mmol) in anhydrous acetone (1 mL) was heated to reflux under nitrogen for 12 h and monitored with TLC. When the reaction was finished, the solvent was evaporated under reduce pressure, and the resulting residue was purified by chromatography on silica gel to

give compound **1d~f-4d~f**.

5.1.17 (*E,E*)-4-(4,6-bis(4-methoxystyryl)pyrimidin-2-yloxy)butyl carbamimidothioate hydrobromide (1d)

Yellow powder (388 mg, yield 68%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.77 (s, 1H), 7.74 (s, 1H), 7.60 (t, J = 12.1 Hz, 4H), 7.17 (s, 1H), 6.98 (s, 1H), 6.96-6.92 (m, 5H), 4.36 (t, J = 5.7 Hz, 2H), 3.74 (s, 6H), 3.17 (t, J = 7.0 Hz, 2H), 1.87-1.81 (m, 2H), 1.77 (d, J = 6.9 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 170.49, 165.29, 164.92, 160.73, 136.78, 129.77, 128.36, 123.68, 114.82, 110.92, 66.27, 55.65, 30.32, 27.57, 25.56; MS (ESI) m/z ESI$^+$ 491.2 $[M+H]^+$.

5.1.18. (*E,E*)-5-(4,6-bis(4-methoxystyryl)pyrimidin-2-yloxy) pentyl carbamimidothioate hydrobromide (1e)

Yellow powder (292 mg, yield 50%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.83 (s, 1H), 7.80 (s, 1H), 7.65 (d, J = 8.5 Hz, 4H), 7.24 (s, 1H), 7.05 (d, J = 16.0 Hz, 2H), 7.00 (d, J = 8.5 Hz, 4H), 4.39 (t, J = 5.8 Hz, 2H), 3.79 (s, 6H), 3.18 (t, J = 7.2 Hz, 2H), 1.83-1.75 (m, 2H), 1.73-1.65 (m, 2H), 1.56 (d, J = 6.5 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 170.22, 165.33, 165.23, 160.78, 136.53, 129.76, 128.52, 124.06, 114.88, 110.95, 66.65, 55.74, 30.48, 28.61, 28.34, 25.04; MS (ESI) m/z ESI$^+$ 505.2 $[M+H]^+$.

5.1.19. (*E,E*)-6-(4,6-bis(4-methoxystyryl)pyrimidin-2-yloxy)hexyl carbamimidothioate hydrobromide (1f)

Yellow powder (316 mg, yield 53%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.80 (s, 1H), 7.77 (s, 1H), 7.62 (d, J = 8.1 Hz, 4H), 7.21 (s, 1H), 7.02 (s, 1H), 7.00-6.92 (m, 5H), 4.34 (s, 2H), 3.76 (s, 6H), 3.13-3.07 (m, 2H), 1.72 (s, 2H), 1.62 (s, 2H), 1.43 (s, 4H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 170.42, 165.30, 165.17, 160.74, 136.57, 129.76, 128.44, 123.92, 114.86, 110.90, 66.81, 55.70, 30.50, 28.65, 28.58, 27.89, 25.36; MS (ESI) m/z ESI$^+$ 519.4 $[M+H]^+$.

5.1.20. (*E,E*)-4-(4,6-bis(3,4-dimethoxystyryl)pyrimidin-2-yloxy)butyl carbamimidothioate hydrobromide (2d)

Yellow powder (321 mg, yield 51%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.79 (d, J = 15.8 Hz, 2H), 7.29 (s, 2H), 7.22 (d, J = 9.3 Hz, 3H), 7.07 (d, J = 15.8 Hz,

2H), 6.98 (d, J = 7.9 Hz, 2H), 4.40 (s, 2H), 3.79 (d, J = 18.7 Hz, 12H), 3.21 (s, 2H), 1.83 (d, J = 30.3 Hz, 4H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 170.32, 165.35, 165.09, 150.55, 149.34, 137.03, 128.70, 124.07, 122.36, 112.11, 111.07,110.26, 66.20, 55.97, 30.31, 27.67, 25.71; MS (ESI) m/z ESI^+ 551.1 $[M+H]^+$.

5.1.21. (*E*,*E*)-5-(4,6-bis(3,4-dimethoxystyryl)pyrimidin-2-yloxy) pentyl carbamimidothioate hydrobromide (2e)

Yellow powder (361 mg, yield 56%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.70 (s, 1H), 7.67 (s, 1H), 7.19 (s, 2H), 7.13 (d, J = 8.1 Hz, 2H), 7.09 (s, 1H), 6.97 (s, 1H), 6.95-6.90 (m, 3H), 4.29 (s, 2H), 3.72 (t, J = 18.8 Hz, 12H), 3.06 (t, J = 7.1 Hz, 2H), 1.69 (d, J = 6.6 Hz, 2H), 1.65-1.59 (m, 2H), 1.47 (d, J = 6.8 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 170.64, 165.23, 165.00, 150.35, 149.12, 136.97, 128.63, 123.89, 122.33, 111.94, 110.03, 66.75, 55.85, 30.50, 28.22, 28.12, 24.78; MS (ESI) m/z ESI^+ 565.3 $[M+H]^+$.

5.1.22. (*E*,*E*)-6-(4,6-bis(3,4-dimethoxystyryl)pyrimidin-2-yloxy)hexyl carbamimidothioate hydrobromide(2f)

Yellow powder (421 mg, yield 64%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.78 (d, J = 15.9 Hz, 2H), 7.29 (s, 2H), 7.20 (d, J = 8.3 Hz, 3H), 7.07 (d, J = 15.8 Hz, 2H), 6.97 (d, J = 7.2 Hz, 2H), 4.35 (s, 2H), 3.78 (d, J = 19.1 Hz, 12H), 3.11 (s, 2H), 1.72 (s, 2H), 1.61 (s, 2H), 1.43 (s, 4H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 170.03, 165.32, 165.24, 150.57, 149.39, 136.91, 128.75, 124.20, 122.30, 112.13, 110.91, 66.75, 55.98, 30.45, 28.71, 27.97, 26.89, 25.44; MS (ESI) m/z ESI^+ 579.3 $[M+H]^+$.

5.1.23. (*E*,*E*)-4-(4,6-bis(3,4,5-trimethoxystyryl)pyrimidin-2-yloxy)butyl carbamimidothioate hydrobromide (3d)

Red powder (380 mg, yield 55%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.83 (s, 1H), 7.79 (s, 1H), 7.28 (s, 1H), 7.20 (s, 1H), 7.17 (s, 1H), 7.02 (s, 4H), 4.42 (t, J = 5.8 Hz, 2H), 3.82 (s, 12H), 3.68 (s, 6H), 3.23 (t, J = 7.1 Hz, 2H), 1.87 (dd, J = 14.0, 7.1 Hz, 2H), 1.81 (d, J = 6.9 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 170.21, 165.24, 165.14, 153.52, 139.04, 137.13, 131.57, 125.73, 111.51, 105.50, 66.26, 60.61, 56.40, 30.26, 27.70, 25.78; MS (ESI) m/z ESI^+ 611.1 $[M+H]^+$.

5.1.24. (*E*,*E*)-5-(4,6-bis(3,4,5-trimethoxystyryl)pyrimidin-2-yloxy)pentyl carbamimidothioate hydrobromide (3e)

Red powder (470 mg, yield 67%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.80 (s, 1H), 7.77 (s, 1H), 7.27 (s, 1H), 7.18 (s, 1H), 7.15 (s, 1H), 7.00 (s, 4H), 4.38 (s, 2H), 3.82 (s, 12H), 3.67 (s, 6H), 3.15 (t, J = 7.1 Hz, 2H), 1.77 (s, 2H), 1.68 (d, J = 7.0 Hz, 2H), 1.55 (d, J = 6.4 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 170.39, 165.23, 153.52, 139.07, 137.09, 131.58, 125.78, 111.42, 105.52, 66.81, 60.64, 56.42, 30.51, 28.46, 28.29, 24.97; MS (ESI) m/z ESI^+ 625.4 $[M+H]^+$.

5.1.25. (*E*,*E*)-6-(4,6-bis(3,4,5-trimethoxystyryl)pyrimidin-2-yloxy)hexyl carbamimidothioate hydrobromide (3f)

Red powder (452 mg, yield 63%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.68 (s, 1H), 7.65 (s, 1H), 7.13 (s, 1H), 7.04 (s, 1H), 7.01 (s, 1H), 6.88 (s, 4H), 4.27 (s, 2H), 3.75 (s, 12H), 3.63 (s, 6H), 3.02 (t, J = 7.1 Hz, 2H), 1.64 (s, 2H), 1.55 (s, 2H), 1.35 (s, 4H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 170.66, 165.07, 165.03, 153.33, 138.84, 136.95, 131.57, 125.55, 111.35, 105.29, 66.98, 60.68, 56.29, 30.49, 28.53, 28.37, 27.79, 25.25; MS (ESI) m/z ESI^+ 639.3 $[M+H]^+$.

5.1.26. (*E*,*E*)-4-(4,6-distyrylpyrimidin-2-yloxy)butyl carbamimidothioate hydrobromide (4d)

Yellow powder (261 mg, yield 51%); ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.07 (s, 4H), 7.91 (d, J = 16.0 Hz, 2H), 7.73 (d, J = 6.7 Hz, 4H), 7.50-7.34 (m, 7H), 7.25 (d, J = 16.0 Hz, 2H), 4.45 (s, 2H), 3.28 (s, 2H), 1.86 (d, J = 40.1 Hz, 4H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.27, 165.23, 136.95, 135.91, 129.88, 129.40, 128.17, 126.47, 111.74, 66.29, 30.30, 27.80, 25.9; MS (ESI) m/z ESI^+ 431.3 $[M+H]^+$.

5.1.27. (*E*,*E*)-5-(4,6-distyrylpyrimidin-2-yloxy)pentyl carbamimidothioate hydrobromide (4e)

Yellow powder (346 mg, yield 66%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.85 (d, J = 16.0 Hz, 2H), 7.67 (d, J = 7.5 Hz, 4H), 7.42 (t, J = 7.3 Hz, 4H), 7.39-7.35 (m, 2H), 7.33 (s, 1H), 7.18 (d, J = 16.0 Hz, 2H), 4.37 (t, J = 6.0 Hz, 2H), 3.14 (t, J = 7.1 Hz, 2H), 1.82-1.72 (m, 2H), 1.67 (dd, J = 14.4, 7.2 Hz, 2H), 1.53 (d, J = 6.8 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 170.38, 165.20, 136.99, 135.73,

129.96, 129.44, 128.53, 128.10, 126.28, 111.59, 66.84, 30.47, 28.47, 28.23, 24.91; MS (ESI) m/z ESI^+ 445.3 $[M+H]^+$.

5.1.28. (*E*,*E*)-6-(4,6-distyrylpyrimidin-2yloxy)hexyl carbamimidothioate hydrobromide (4f)

Yellow powder (350 mg, yield 65%); ^1H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.85 (d, J = 16.0 Hz, 2H), 7.68 (d, J = 7.2 Hz, 4H), 7.42 (t, J = 7.2 Hz, 4H), 7.38 (d, J = 7.1 Hz, 2H), 7.33 (s, 1H), 7.19 (d, J = 16.0 Hz, 2H), 4.37 (t, J = 5.9 Hz, 2H), 3.11 (t, J = 7.1 Hz, 2H), 1.73 (s, 2H), 1.62 (s, 2H), 1.43 (s, 4H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 170.33, 165.23, 165.19, 136.96, 135.75, 129.95, 129.44, 128.52, 128.10, 126.31, 111.35, 66.98, 30.48, 28.64,28.61, 27.91, 25.37; MS (ESI) m/z ESI^+ 459.3 $[M+H]^+$.

5.1.29. General procedure for Compounds 1g-3g

A mixture of compound **1a-3a** (0.1 mmol) and tetramethylthiourea (0.5 mmol) in anhydrous acetone (1 mL) was heated to reflux under nitrogen for 12 h and monitored with TLC. When the reaction was finished, the solvent was evaporated under reduce pressure, and the resulting residue was purified by chromatography on silica gel to give compound **1g-3g**.

5.1.30. (*E*,*E*)-2-(4-(4,6-bis(4-methoxystyryl)pyrimidin-2-yloxy)butyl)-1,1,3,3-tetramethylisothiouronium hydrobromide (1g)

Red powder (330 mg, yield 53%); ^1H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.78 (d, J = 15.8 Hz, 2H), 7.61 (d, J = 7.9 Hz, 4H), 7.21 (s, 1H), 7.01 (s, 1H), 6.96 (d, J = 8.1 Hz, 5H), 4.39 (s, 2H), 3.73 (d, J = 22.5 Hz, 6H), 3.18 (s, 12H), 3.07 (s, 2H), 1.84 (s, 2H), 1.75 (s, 2H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 174.78, 165.35, 165.01, 160.76, 136.72, 129.79, 128.38, 123.78, 114.86, 110.99, 66.30, 55.68, 43.72, 33.98, 27.60, 26.41; MS (ESI) m/z ESI^+ 547.2 $[M+H]^+$.

5.1.31. (*E*,*E*)-2-(4-(4,6-bis(3,4-dimethoxystyryl)pyrimidin-2-yloxy)-butyl)-1,1,3,3-tetramethylisothiouronium hydrobromide (2g)

Red powder (376 mg, yield 55%); ^1H NMR (500 MHz, CD_3OD+ D_2O) δ 7.80 (s, 1H), 7.77 (s, 1H), 7.24 (d, J = 1.9 Hz, 2H), 7.18 (d, J = 1.8 Hz, 1H), 7.16 (d, J = 1.8 Hz, 1H), 7.14 (s, 1H), 6.97 (d, J = 4.5 Hz, 2H), 6.94 (d, J = 3.0 Hz, 2H), 4.50 (t, J =

5.9 Hz, 2H), 3.88 (s, 6H), 3.85 (s, 6H), 3.30 (s, 12H), 3.16 (t, J = 7.2 Hz, 2H), 1.96 (dd, J = 13.1, 6.2 Hz, 2H), 1.91 (dd, J = 14.6, 7.5 Hz, 2H); ^{13}C NMR (126 MHz, CD_3OD+ D_2O) δ 175.77, 165.52, 164.78, 150.69, 149.36, 137.16, 128.86, 123.12, 121.85, 111.36, 110.00, 109.87, 66.07, 55.04, 42.89, 33.95, 27.54, 26.37; MS (ESI) m/z ESI$^+$ 607.1 $[M+H]^+$.

5.1.32. (*E*,*E*)-2-(4-(4,6-bis(3,4,5-trimethoxystyryl)pyrimidin-2-yloxy)-butyl)-1,1,3,3-tetramethylisothiouronium hydrobromide (3g)

Red powder (388 mg, yield 52%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 7.77 (d, J = 15.9 Hz, 2H), 7.25 (s, 1H), 7.14 (d, J = 15.9 Hz, 2H), 6.97 (s, 4H), 4.40 (s, 2H), 3.80 (s, 12H), 3.64 (d, J = 18.9 Hz, 6H), 3.18 (s, 12H), 3.06 (dd, J = 16.0, 9.2 Hz, 2H), 1.84 (d, J = 6.3 Hz, 2H), 1.76 (d, J = 6.8 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6 + D_2O) δ 174.80, 165.22, 165.04, 153.47, 139.02, 137.14, 131.56, 125.64, 105.46, 66.41, 60.67, 56.40, 43.72, 33.99, 27.64, 26.41; MS (ESI) m/z ESI$^+$ 667.2 $[M+H]^+$.

5.2. Bioactivity Study

5.2.1. Cell culture

Both human colon cancer cells (HCT116 and HT29) and lung cancer cells (H1299 and A549) were obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). McCoy's 5A medium was used for colon cancer cell culture and RPMI 1640 medium was used for lung cancer cell culture, respectively. Both media were supplemented with 5% fetal bovine serum (HyClone), penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) (HyClone). Chinese hamster ovary (CHO) cell lines (K1, 745, 677, 3.1, 3.2, 3.3 and 3.4) were described in detail during the past by both Esko [26] and by us [24,27]. The CHO cells were cultured with Ham's F-12 medium (Hyclone) supplemented with 10% FBS and antibiotics. All the cells were cultured under an atmosphere of 5% CO_2 and 100% relative humidity at 37 ºC, and passaged with trypsin every 2-4 days and revived periodically from frozen stocks.

5.2.2 Antibodies

Anti-Golgin-97 (human) antibody was purchased from Life technologies, USA. Golgin-97 is a trans-Golgi network peripheral membrane protein and localizes

exclusively on the cytoplasmic face of the Golgi. Golgin-97 acts as an essential player to the cell during the trafficking from the trans-Golgi network to the recycling endosome and/or early endosome.

5.2.3 Cellular viability assay and IC_{50} calculation

All synthesized curcumin-pyrimidine analogues were dissolved in DMSO (Solarbio, CN) in a final concentration of 0.1% initially and further diluted with cell culture medium to desired mol/L concentrations for cellular viability assay.

For cellular viability assay, a 96-wells plate was seeded with 2000 cells/well in 100 μL complete cell culture medium. After 24 h, 100 μL complete medium containing serial concentrations of each compound was added to each well. Either cancer or CHO cells were cultured 48 h, followed by adding 20 μL of resazurin (2 mg/mL dissolved in water, catalog no. R7017-5G, Sigma) to the media for 16 h. The fluorescent signal was monitored using 544 nm excitation wavelength and 595 nm emission wavelength by Spectramax M5 plate reader (Molecular Devices). The relative fluorescence unit (RFU) generated from the assay was proportional to the number of living cells in each well [28]. The IC_{50} value of each drug was calculated by the Logit approach.

5.2.4. Confocal analysis

The confocal analysis was performed according to Hao et al [29]. For confocal imaging, A549 or HCT116 cells were seeded on glass coverslips in complete cell culture media overnight before treated with or without the compounds at the concentration of 10 μM for 4 h at 37 ºC. After that, the media were removed and the cells were fixed with 4% formaldehyde in complete media for 15 min followed by permeabilized with 0.2% Triton-100 in PBS for 30 min. After blocked with 5% bovine serum albumin (BSA, BovoMax) for 60 min, the cells were incubated with Golgin-97 primary antibody (Invitrogen) overnight followed by incubated with fluorescent secondary antibody (LI-COR IRDye® 680LT) for 45 min. The nucleus was stained with DAPI (Beyotime, CN) for 15 min. Among each steps, the cells were washed with PBS for 3 times. The fluorescence of the compounds and secondary antibody were measured at 488 nm and 680 nm excitation wavelength by Laser

Scanning Confocal Microscope (Zeiss LSM 710, GER), respectively.

5.2.5. Statistical analysis

The statistical significance of differences between the groups was assessed by Student's t-test. P values < 0.05 were considered as statistically significant.

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Title Caption

Table 1. IC_{50} of the tested compounds in four human cancer cell lines.

Figure 1. Effects of the tested compounds on cell viability of four human cancer cell lines.

Figure 2. Fluorescence images of A549 showing the location of Compounds.

Figure 3. Effects of the tested compounds on cytotoxicity to all the CHO cells and the lung cancer cells A549.

Scheme 1. Synthesis of the isothiouronium derivatives of curcumin-pyrimidine analogs.

Highlights:

1. Novel isothiouronium-modified pyrimidine-substituted curcumin analogs were synthesized.
2. Isothiouronium modifications greatly enhanced anticancer activities of the analogs.
3. Only the fluorescence compound with isothiouronium modification showed unique Golgi localization.
4. The isothiouronium analogs were novel Golgi staining compounds.
5. The isothiouronium analogs were useful in studying the biological functions of Golgi.