Note

First Synthesis of Novel Aminophenyl Pyridinium-5-(hydroxybenzoyl)-hydrazonomethyl-2-oxothiazol-3-ide Derivatives and Evaluation of Their Anticancer Activities

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The first total synthesis for large-scale production and anticancer activity of novel aminophenylpyridinium-5-(hydroxybenzoyl)hydrazonomethyl-2-oxothiazol-3-ide (PBHT) (1) and its derivatives are reported. The chemical structure of PBHT was unambiguously determined by utilization of the two-dimensional nuclear Overhauser effect (NOE) technique. The anticancer activity against human colon adenocarcinoma (HCT15) cells of all synthesized compounds was approximately four-fold greater than that of 5-fluorouracil, with IC_{50} values ranging from 10.1 to 14.2 μM. The three structural determinants of hydroxybenzoyl, hydrazinylidene, and pyridinium oxothiazole in the synthesized compounds could be indispensable for exhibiting anticancer activity.

Key words convergent synthesis; oxothiazolide; anticancer activity; nuclear Overhauser effect

Anthracycline antibiotics, for example, daunomycin exhibited anticancer activity *via* intercalation into the DNA double helix. The planar aromatic chromophore portion of the molecule intercalates between two base pairs of the DNA,[1] while the six-membered daunosamine sugar sits in the minor groove.[1] This process inhibits the progression of the enzyme topoisomerase II, which relaxes supercoils in DNA for transcription.[2] Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication.[3] It may also increase free radical production, hence contributing to its cytotoxicity.[4]

We found a novel aminophenylpyridinium-5-(hydroxybenzoyl)hydrazonomethyl-2-oxothiazol-3-ide (PBHT) (**1**) and its preliminary anti-cancer activity by using high-throughput screening of a library of aromatic molecules. Based on its efficient binding to cyclin-dependent kinase (Cdk) as the mode of action, along with its preliminary anticancer activity identified by this laboratory, we propose PBHT (**1**) as a potential anticancer drug candidate. Synthesis and anti-cancer activity of PBHT (**1**) are unknown in the literatures. This paper reports the first total synthesis for large-scale production and also the first anti-cancer activity of PBHT and its derivatives. PBHT (**1**) highly inhibited cancer cell growth in a dose-dependent manner without any toxicity in the screening cells.

Results and Discussion

Chemistry A retrosynthetic analysis was performed to obtain a useful synthetic route as outlined in Chart 1. Target molecule **1** was divided into two compound parts (**2** and **3**) and compound part **3** was further divided into compound parts **4** and **5**.

Based on retrosynthetic analysis, PBHT (**1**) was successfully synthesized in four steps from pyridine in 23% overall yields as outlined in Chart 2. Thus, oxidative coupling of dimethylaniline with pyridine in the presence of cyanuric chloride and aluminium chloride ($AlCl_3$) (r.t., 1.5 h and 100°C, 3h) afforded 4-(*p*-dimethyl aminophenyl)pyridine **5** in 85% yield by a known procedure.[5] Heterocyclic chloro-substituted aldehyde **4** was prepared by phosphorus oxychloride ($POCl_3$) in *N*,*N*-dimethylformamide (DMF) (95°C, 2 h, and 112°C, 20 min) from readily available 2,4-thiazolidine-dione in 47% yield by a known procedure.[6,7]

Convergent and regiospecific coupling of compounds **4** and **5** in DMF (80°C) *via* Michael addition afforded compound **3** in 64% yield as shown in Chart 2. In compound **5**, the pyridine N site was more basic than the aniline N site and the lone pair electrons of the pyridine nitrogen attacked the electrophilic C-4 site of the Michael acceptor **4**, keeping the formyl group intact. Final hydrazonation of compound **3** with (2-hydroxybenzoyl)hydrazine **2** in ethanol (EtOH) (reflux) gave the target hydrazinylidene PBHT (**1**) in 89% yield. These procedures proved to be suitable for the large-scale production.

The *E*-configuration at the imine group and the *s-cis* form of the single bond between imine C-1′ and C-5 of the oxothiazolide part of compound **1** were unambiguously determined as shown in Chart 2 by utilization of the two dimensional nuclear Overhauser effect (NOE) spectroscopy (NOESY)[8,9] technique (Fig. 1). No NOE enhancement was observed between 2-H (δ 8.94, d, J=6.8 Hz) of the pyridine part and 1′-H (δ 8.21, s) of the imine part, establishing that the configuration of the imine was *E*. However, the NOE spectrum showed interaction between 1′-H (δ 8.21, s) of the imine and N 3′-H (δ 12.14, s), demonstrating that the N-2′ and N-3′ single bond was *s-trans*.

Similarly, analogs **6** and **7** were successfully prepared from compound **4** by the same procedures *via* intermediates **3a**, **b** in 61 and 56% yields, respectively (Chart 3).

Anticancer Activity *In vitro* anticancer activities of benzoylhydrazinylidene phenylpyridinium oxothiazolides **1**, **3, 6** and **7** were tested against the human cancer cell lines colon adenocarcinoma (HCT15) and gastric adenocarcinoma (MKN74) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method. The results

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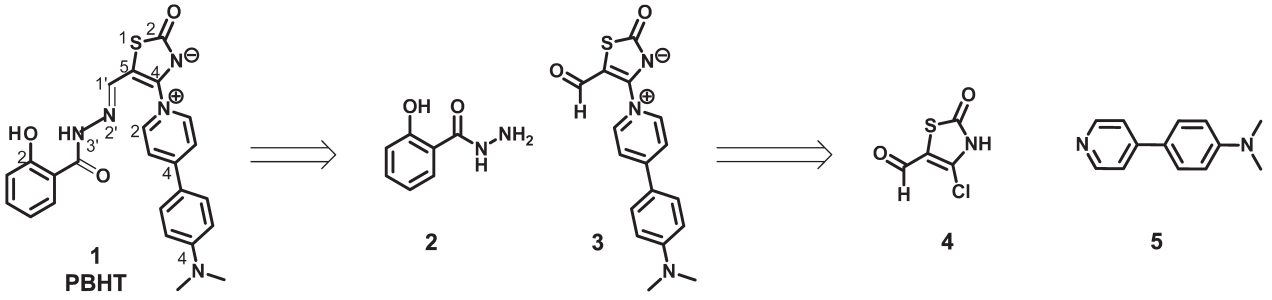


Chart 1. Retrosynthesis of PBHT (**1**)

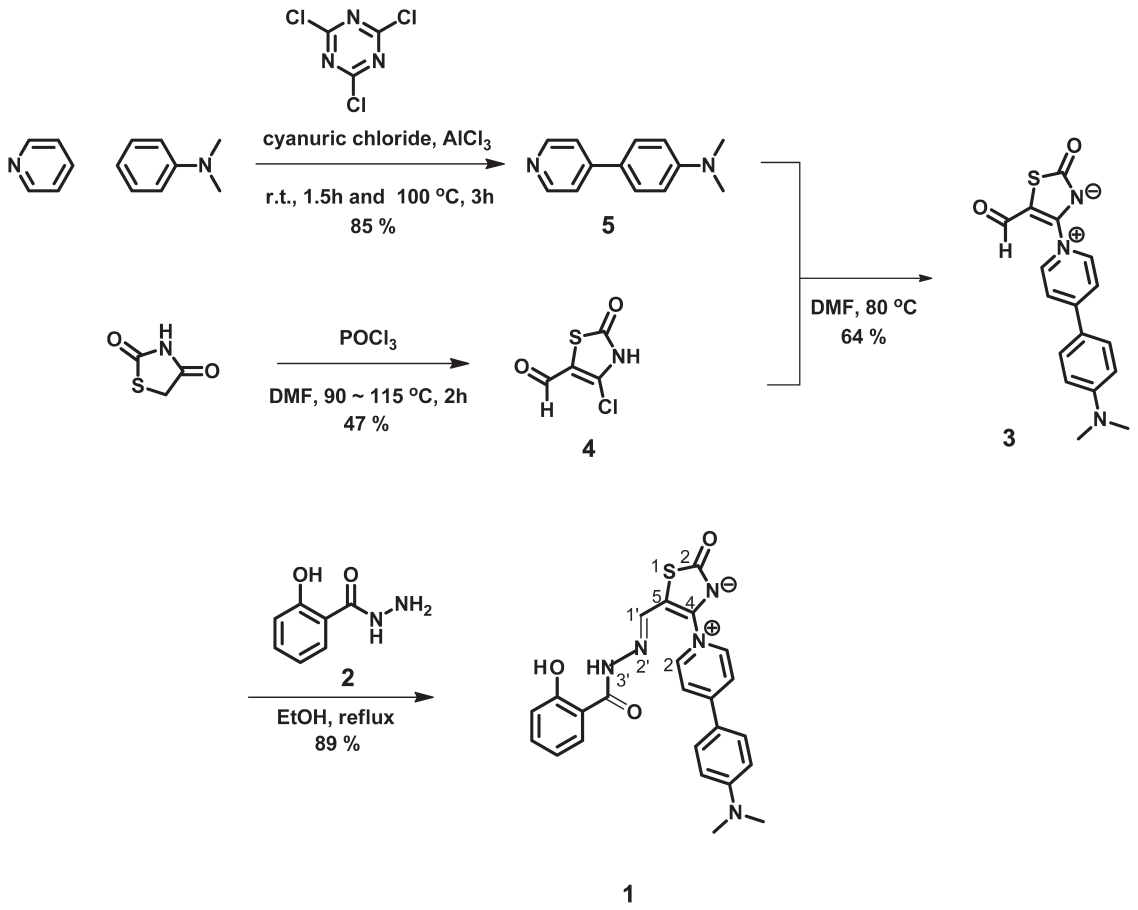


Chart 2. Total Synthesis of PBHT (**1**)

of these assays are summarized in Table 1. The drug standards used for comparison were gefitinib[10–14] (Iressa®) and 5-Fu.

Under our conditions, all synthesized compounds (PBHT **1**, **6** and **7**) exhibited good anticancer activity, even better than 5-Fu in both cell lines. The anticancer activities against HCT15 of compounds **1**, **6** and **7** was approximately four times greater than 5-Fu with IC_{50} values of 10.1, 13.1 and 14.2 μM, respectively. However, all compounds showed activity that was comparable activity to that of Iressa®.

However, PBHT (**1**) failed to show notable activity at concentrations below 50 μM against MKN74. The *N*,*N*-dimethylaniline function seems to interfere with the anticancer activity in this cancer cell line (MKN74). For the MKN74 cancer cell line, compounds **6** and **7** showed better activity than the reference 5-Fu with superior IC_{50} values of 11.1 and 33.1 μM, respectively, but displayed again a comparable activity with that of Iressa®. The three structural determinants of hydroxybenzoyl, hydrazinylidene and pyridinium oxothiazole in the synthesized compounds could be indispensable for exhibiting anticancer activity. The drastic decrease of anticancer activity of the compound **3** seemed to be due to the absence of hydroxybenzoyl determinant.

Conclusion

In conclusion, PBHT and its derivatives were synthesized *via* a convergent and regiospecific synthesis for the first time from readily available *N*,*N*-dimethylaniline. The chemical structure of PBHT was unambiguously determined by utilization of the two dimensional NOE technique. They showed potent anti-colon and anti-gastric cancer activity. It is also noteworthy that the absence of the *N*,*N*-dimethylaniline functionality seems to enhance dramatic increases in anticancer

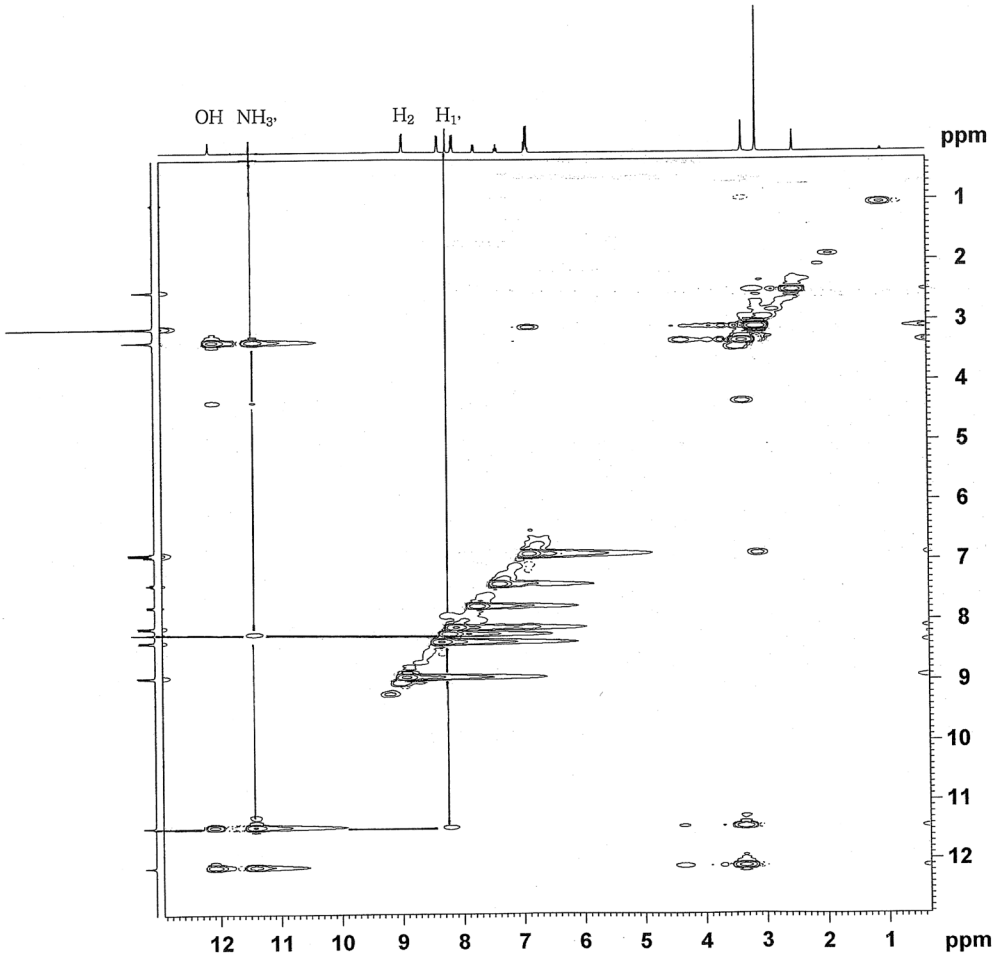


Fig. 1. NOESY Spectra of PBHT (**1**)

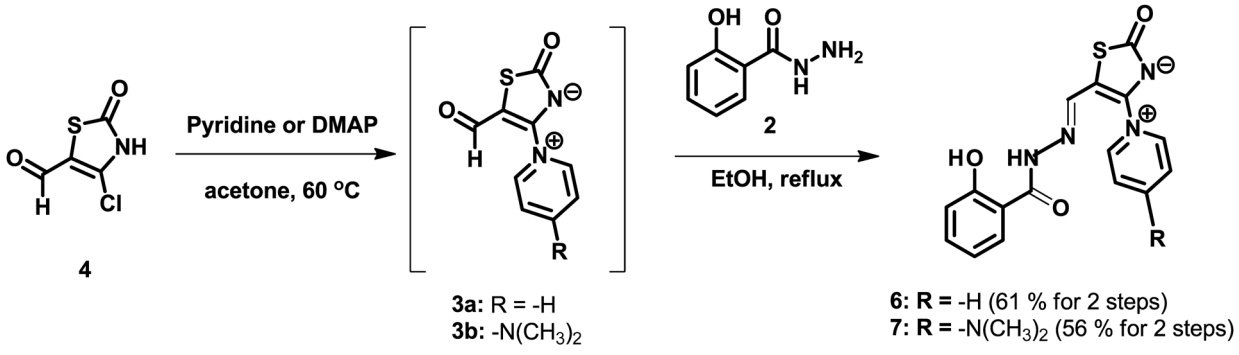


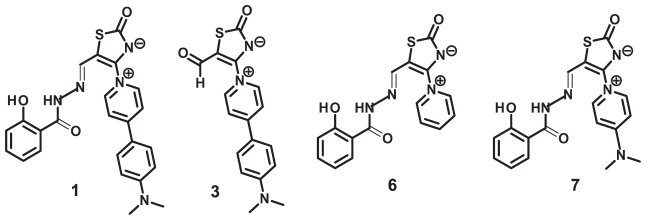
Chart 3. Synthesis of PBHT Analogs

activity against the MKN74 cancer cell line. The compounds including PBHT deserve further evaluation as possible anti-colon and anti-gastric cancer drug candidates.

Experimental

Chemistry General Procedures Reaction solvents were distilled from calcium hydride for dichloromethane and from sodium metal and benzophenone for tetrahydrofuran. All other commercial reagents and solvents were used as received without further purification. The reactions were monitored and the *Rf* values determined using analytical thin layer chromatography (TLC) with Merck silica gel 60, F-254 precoated plates (0.25 mm thickness). Spots on the TLC plates were visualized using ultraviolet light (254 nm), a cerium sulfate/ammonium dimolybdate/sulfuric acid solution followed by heating on a hot plate. Flash column chromatography was performed with Merck silica gel 60 (230–400 mesh) or purification of reaction mixture was recrystallized by diethyl ether in dichloromethane. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DRX 250 at 250 MHz and 63 MHz or 400 at 400 MHz and 100 MHz, respectively. Proton chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS, δ 0.00) or with the solvent reference relative to TMS employed as the internal standard ($CDCl_3$, δ 7.26 ppm; dimethyl sulfoxide

Table 1. Cytotoxic Activities of PBHT Analogs (**1**, **3**, **6** and **7**) against Two Human Cancer Cells (μM)



Compounds	IC_{50} (μM) in cell line[a]	
	HCT15[b]	MKN74[c]
1	10.1	>50
3	>50	>50
6	14.2	11.1
7	13.1	33.3
Iressa®	13.5	14.7
5-Fu	45.5	>50

a) Human cancer cell lines. *b*) HCT15 (human colon adenocarcinoma). *c*) MKN74 (human gastric adenocarcinoma).

(DMSO), δ 2.5 ppm). Data are reported as follows: chemical shift {multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m)], coupling constants [Hz], integration}. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the respective solvent resonance as the internal standard ($CDCl_3$, δ 77.16 ppm; DMSO, δ 39.52 ppm). Infrared (IR) spectra were recorded on a JASCO FT/IR-430 spectrometer. Data are reported in wave numbers (cm^{-1}). Electrospray ionization (ESI)-LC-MS was recorded on a Waters ZQ 4000 LC-MS spectrometer. Melting points (mp) were determined on a BIBBY Stuart Scientific melting point apparatus SMP3.

4-*p*-(*N*,*N*-Dimethylaminophenyl)pyridine (5)[5] A solution of cyanuric chloride (22.8 g, 0.124 mol, 0.3 eq) in pyridine (100 mL, 1.24 mol, 3 eq) was heated at 70°C for 15 min, then cooled. To this solution was added $PhNMe_2$ (52 mL 0.413 mol, 1 eq) followed by $AlCl_3$ (55 g, 0.413 mol, 1 eq) at less than 30°C and the mixture was stirred for 1.5 h at room temp., heated at 100°C for 3 h and cooled to room temp. The crude solids were added to 10% HCl (100 mL) and dissolved at above 80°C. The temperature of the acidic solution was decreased to warm and the solution was filtered through Celite. The acidic filtrates were extracted with methylene chloride (1 L). The aqueous layer was neutralized with sodium hydroxide (NaOH) and extracted with methylene chloride (1 L×2). The organic layer was dried, filtered and evaporated under reduced pressure. The crude solids were dissolved with methylene chloride (500 mL). The suspended solution was evaporated at 0°C under reduced pressure. The precipitated solids were collected and washed with ether to give pure product **5** as pale pink solid (69.5 g, 85% yield); mp 236°C (lit.[5] 233–234°C); IR (KBr) ν_{max} cm^{-1} 3436, 2923, 1608, 1592, 1562, 1535, 1493, 1446, 1365, 1293, 1229, 1176; ^{1}H-NMR (250 MHz $CDCl_3$) δ: 8.56 (2H, d, J 5.6 Hz), 7.60 (2H, d, J=8.8 Hz), 7.51 (2H, d, J=6.0 Hz), 6.80 (2H, d, J=8.9 Hz), 3.04 (6H, s); ^{13}C-NMR (63 MHz, $CDCl_3$) δ: 140.1, 129.2, 120.7, 112.5, 40.2, 31.1; LC-MS (ESI+) m/z 199.18 $[M+H]^+$ (Calcd for $C_{13}H_{14}N_2$. Found: 198.12). *Anal.* Calcd for $C_{13}H_{14}N_2$: C, 78.79; H, 7.07; N, 14.14%. Found: C, 78.83; H, 7.15; N, 14.07%.

2,4-Chloro-5-formyl-2-thiazolinone (4)[6,7] A suspension of 2,4-thiazolidinedione (25 g, 0.213 mol, 1 eq) in $POCl_3$ (60 mL, 0.640 mol, 3 eq) was cooled to 0°C with an ice bath. To this suspension was added dropwise cool DMF (25 mL, 0.320 mol, 1.5 eq) over 15 min. The reaction mixture was heated to 90°C for 2 h then at 115°C for 20 min. After 20 min, the reaction was cooled to 90°C and maintained for an additional hour. After 1 h the mixture was heated to 115°C for 15 min. The hot reaction mixture was poured into 1 L of water with vigorously stirring. After 10 min the mixture is filtered. The aqueous phase was extracted 5 times with ethyl ether (600 mL) and the organic layer was separated, dried, filtered and evaporated under reduced pressure. The solid residue was dissolved in a minimum volume of aq. sat. $NaHCO_3$. The mixture was carefully acidified with 6 M HCl to pH=2, whereupon a precipitate formed after about 30 min. Filtration yielded 16.3 g of pure product **4** as a yellow solid in 47% yield; mp 197°C (lit.[6,7] 219°C); IR (KBr) ν_{max} cm^{-1} 3450, 2980, 2860, 2672, 1684, 1646, 1563, 1457, 1384, 1233, 1173; ^{1}H-NMR (250 MHz $CDCl_3$) δ: 9.79 (1H, s, CHO), 8.95 (1H, s, NH); ^{13}C-NMR (63 MHz, DMSO) δ: 180.2, 180.1, 168.5, 132.5, 113.7; LC-MS (ESI+) m/z 164.00 $[M+H]^+$ (Calcd for $C_4H_2ClNO_2S$. Found: 162.95). *Anal.* Calcd for $C_4H_2ClNO_2S$: C, 29.45; H, 1.23; N, 8.59%. Found: C, 29.49; H, 1.29; N, 8.44%.

4-[4-(4-Dimethylamino)phenyl)pyridinium-1-yl]-5-formyl-2-oxothiazol-3-ide (3) A mixture of 4-chloro-5-formyl-2-thiazolinone **4** (5.0 g, 30.6 mmol, 1 eq) and *p*-(*N*,*N*-dimethylphenyl)pyridine **5** (6.1 g, 30.6 mmol, 1 eq) in 50 mL of DMF was heated at 80°C until a precipitate formed. The precipitated solids were collected, washed with ether to give pure product **3** as a brown solid (6.4 g, 64% yield); mp 230°C; IR (KBr) ν_{max} cm^{-1} 3437, 2928, 1585, 1540, 1495, 1384, 1315, 1220, 1146; ^{1}H-NMR (250 MHz DMSO) δ: 9.20 (1H, s, CHO), 9.04 (2H, d, J=6.7 Hz), 8.34 (2H, d, J=7.0 Hz), 8.13 (2H, d, J=9.0 Hz), 6.89 (2H, d, J 8.9 Hz), 3.11 (6H, s, NCH_3); ^{13}C-NMR (100.6 MHz, DMSO) δ: 176.8, 155.6, 153.6, 142.7, 130.4, 125.8, 119.8, 118.3, 112.4, 39.7; LC-MS (ESI+) m/z 326.37 $[M+H]^+$ (Calcd for $C_{17}H_{15}N_3O_2S$. Found: 325.09). *Anal.* Calcd for $C_{17}H_{15}N_3O_2S$: C, 62.77; H, 4.62; N, 12.92%. Found: C, 62.89; H, 4.69; N, 12.94%.

(*E*)-4-(4-(4-(Dimethylamino)phenyl)pyridin-1-ium-1-yl)-5-((2-(2-hydroxybenzoyl)hydrazono)methyl)-2-oxothiazol-3-ide (PBHT) (1) A solution of 4-(4-(4-(dimethylamino)-phenyl)pyridinium-1-yl)-5-formyl-2-oxothiazol-3-ide **3** (6.0 g, 18.4 mmol) and 2-hydroxybenzohydrazide **2** (2.8 g, 18.4 mmol) in EtOH (200 mL) was refluxed for overnight. The solids were collected and washed with acetone to give product **1** as a brown-yellow solid (7.5 g, 89% yield); mp 239°C; IR (KBr) ν_{max} cm^{-1} 3442, 2923, 1638, 1586, 1492, 1444, 1382, 1353, 1315, 1218, 1172, 1139; ^{1}H-NMR (250 MHz DMSO) δ: 12.14 (1H, s, NH), 11.46 (1H, s, OH), 8.94 (2H, d, J=6.8 Hz), 8.36 (2H, d, J=7.0 Hz), 8.21 (1H, s), 8.12 (2H, d, J=9.0 Hz), 7.76 (1H, d, J=7.8 Hz), 7.39 (1H, t, J=7.5 Hz), 6.89 (4H, m), 3.10 (6H, s); ^{13}C-NMR (100.6 MHz, DMSO) δ: 174.8, 164.4, 159.6, 154.6, 153.5, 148.0, 142.7, 141.2, 133.7, 130.1, 127.6, 119.9, 118.7, 118.3, 117.4, 115.2, 112.4, 105.4, 39.6, 18.6; LC-MS (ESI+) m/z 460.29 $[M+H]^+$ (Calcd for $C_{24}H_{21}N_5O_3S$. Found: 459.14). *Anal.* Calcd for $C_{24}H_{21}N_5O_3S$: C, 62.75; H, 4.58; N, 15.25%. Found: C, 62.89; H, 4.59; N, 15.28%.

(*E*)-5-((2-(2-Hydroxybenzoyl)hydrazono)methyl)-2-oxo-4-(pyridin-1-ium-1-yl)thiazol-3-ide (6) A mixture of 4-chloro-5-formyl-2-thiazolinone **4** (0.15 g, 0.92 mmol, 1 eq)

and pyridine (0.22 mL, 2.75 mmol, 3 eq) in 2 mL of acetone was heated at 60°C until a precipitate formed. The solvents were evaporated under reduced pressure. The residue was added to 2-hydroxybenzohydrazide **2** (0.14 g, 0.92 mmol) in EtOH (3 mL). And the mixture was refluxed for overnight. The solids were collected and washed with acetone to give product **6** as a brown-yellow solid (190 mg, 61% yield); mp 236°C; IR (KBr) ν_{max} cm^{-1} 3435, 3050, 1684, 1546, 1491, 1467, 1351, 1308, 1221, 1171, 1107; ^{1}H-NMR (250 MHz DMSO) δ: 12.02 (1H, s, NH), 11.83 (1H, brs, OH), 9.46 (2H, d, J=7.5 Hz), 8.91 (1H, t, J=7.5 Hz), 8.41 (3H, m), 7.90 (1H, d, J=10.0 Hz), 7.41 (1H, t, J=7.5 Hz), 7.01 (1H, d, J=7.5 Hz), 6.90 (1H, t, J=7.5 Hz); ^{13}C-NMR (100.6 MHz, DMSO) δ: 168.5, 164.3, 158.8, 149.1, 146.0, 138.5, 133.9, 128.8, 128.3, 119.0, 117.3, 115.7, 111.2; LC-MS (ESI+) *m/z* 341.27 $[M+H]^+$ (Calcd for $C_{16}H_{12}N_4O_3S$. Found: 340.06). *Anal.* Calcd for $C_{16}H_{12}N_4O_3S$: C, 56.47; H, 3.53; N, 16.37%. Found: C, 56.59; H, 3.59; N, 16.44%.

(*E*)-4-(4-(Dimethylamino)pyridin-1-ium-1-yl)-5-((2-(2-hydroxybenzoyl)hydrazono)methyl)-2-oxothiazol-3-ide (7) A mixture of 4-chloro-5-formyl-2-thiazolinone **4** (0.15 g, 0.92 mmol, 1 eq) and 4-(dimethylamino)pyridine (DMAP) (0.34 g, 2.75 mmol, 3 eq) in 2 mL of acetone was heated at 60°C until a precipitate formed. The solvents were evaporated under reduced pressure. The residue was added to 2-hydroxybenzohydrazide **2** (0.14 g, 0.92 mmol) in EtOH (3 mL). And the mixture was refluxed for overnight. The solids were collected and washed with acetone to give product **7** as a brown-yellow solid (200 mg, 56% yield); mp 231°C; IR (KBr) ν_{max} cm^{-1} 3435, 2928, 1644, 1577, 1490, 1358, 1217, 1146; ^{1}H-NMR (250 MHz DMSO) δ: 12.23 (1H, brs, OH), 11.42 (1H, s, NH), 8.49 (2H, d, J=7.5 Hz), 8.14 (1H, s), 7.78 (1H, d, J=7.5 Hz), 7.40 (1H, t, J=7.5 Hz), 7.11 (2H, d, J=7.5 Hz), 6.89 (2H, m), 3.28 (6H, s); ^{13}C-NMR (100.6 MHz, DMSO) δ: 175.1, 164.4, 159.8, 156.2, 148.6, 141.5, 141.4, 133.6, 127.5, 118.6, 117.4, 115.1, 107.2, 103.9, 40.1; LC-MS (ESI+) *m/z* 384.18 $[M+H]^+$ (Calcd for $C_{18}H_{17}N_5O_3S$. Found: 383.11). *Anal.* Calcd for $C_{18}H_{17}N_5O_3S$: C, 56.40; H, 4.44; N, 18.28%. Found: C, 56.49; H, 4.57; N, 18.34%.

Biology Materials Dulbecco's modified Eagle's medium (DMEM), DMEM/F12, fetal bovine serum (FBS), RPMI1640 were purchased from Gibco BRL (Rockville, MD, U.S.A.). The MTT, DMSO, cholera toxin, hydrocortisone, insulin, transferrin, and triiodothyronine (T3) were obtained from Sigma-Aldrich Chemical (St. Louis, MO, U.S.A.).

Cell Viability Assay The viability of cancer cells was determined *via* the MTT assay. HCT15 and MKN74 human cancer cells were cultured in DMEM supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 at 37°C. Cancer cells were cultured in DMEM/F12 (3 : 1) supplemented with 10% FBS, 1×10^{-10} M cholera toxin, 0.4 mg/mL hydrocortisone, 5 μg/mL insulin, 5 μg/mL transferrin or 2×10^{-11} M T3. The compounds were dissolved in DMSO and diluted with culture media. Cancer cells (1.0×10^4 cells/mL) were seeded onto each well of a 96-well plate with the respective media and incubated to adhere overnight. The cells were then treated with various concentrations of each newly synthesized compound in serum-free medium for 24 h. The MTT solution (20 μL, 5 mg/mL) was added to each well, and the cells were incubated for 4 h at 37°C. The medium was then removed, and 200 μL of DMSO was added to each well. The absorbance was determined at 570 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

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Conflict of Interest The authors declare no conflict of interest.

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