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Design and synthesis of a biotinylated probe of COX-2 inhibitor nimesulide analog JCC76

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ABSTRACT

JCC76 is a derivative of cyclooxygenase-2 (COX-2) selective inhibitor nimesulide and exhibits potent anti-breast cancer activity. It selectively induces apoptosis of Her2 positive breast cancer cells. However, the specific molecular targets of JCC76 still remain unclear, which significantly withdraw the further drug development of JCC76. To identify the molecular targets of JCC76, a six carbon linker and biotin conjugated JCC76 probe was designed and synthesized. The anti-proliferation activity of the probe and its analogs was evaluated.

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Numerous studies have demonstrated overexpression of cyclooxygenase-2 (COX-2) in solid malignancies including breast, prostate, colon, pancreas, nonsmall-cell lung, bladder, and endometrium.^{1–5} Prostaglandin-2 (PGE₂), the product of COX-2, promotes tumor invasiveness, angiogenesis, and progression in various cancers.^{1,4,5} It is therefore reasonable to conclude that inhibition of COX-2 would arrest carcinogenesis and thus, (1) prevent cancer development and (2) regress cancer once developed. Corresponding to these observations and theory, epidemiological, clinical, and pre-clinical studies provide compelling evidence that the use of COX-2 selective inhibitors may reduce the incidence of mammary cancer and colorectal cancer.^{6–9} Theoretically, COX-2 inhibitors achieve all the anti-cancer or cancer preventive activity by targeting COX-2. However, these small molecules may also block other cellular machineries to affect the cancer cell function, which may lead to cell growth inhibition, apoptosis or necrosis. With the extensive studies of COX-2 inhibitors about their anti-cancer or cancer prevention activity, molecular targets of the drugs beyond COX-2 seems play a very important role for their antineoplastic activity. Many researchers even suggested that COX-independent effects might be fully responsible for the anti-cancer properties of some COX-2 inhibitors.^{10,11} The phenomenon is more like an off-target effect, which also underscores the needs to explore targets beyond COX-2, especially in view of emerging toxicity of the COX-2 inhibitors. Developing nonCOX-2 inhibitory analogs as new anti-cancer

drug based on COX-2 inhibitors as lead compound is feasible and practicable. In fact, this principle has been successfully applied in the discovery of some new anti-cancer drugs. For instance, COX-2 inhibitor Celecoxib weakly inhibits PDK-1 kinase in prostate cancer cells. It has been used as a molecular scaffold to develop more potent PDK-1 inhibitors (no COX-2 inhibitory activity) which block PI3K/Akt pathway. The generated new analog, such as OSU-03012 is 20-fold more active than Celecoxib to inhibit tumor cell proliferation and to induce cell apoptosis, which is currently in a phase I clinical trial.^{12,13} Nimesulide (4-nitro-2-phenoxy methane-sulfoanilide) is a nonsteroidal anti-inflammatory drug with a preferential COX-2 inhibitory activity and is available in some Asian and European countries since 1985. Studies suggest that nimesulide could induce apoptosis in liver and lung cancer cells; it also suppressed the development of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced mammary gland carcinogenesis in rats.^{14–17} NonCOX-2 active nimesulide derivatives have been synthesized. Some analogs were much more active than nimesulide in suppressing breast cancer cell growth.¹⁸ Interestingly, the most potent analog JCC76 was selectively potent in Her2 overexpressed breast cancer cells.^{19,20} Both in vitro and in vivo anticancer properties of JCC76 make it a potential new drug candidate or drug lead for Her2 overexpressed breast cancer.

To rational design more potent analogs of JCC76 based on its binding pocket and optimize the structure–activity relationship (SAR), it is a prerequisite to identify the specific molecular target(s) of JCC76. Although JCC76 exhibited good selectivity to Her2 overexpressed breast cancer cells, it did not show any significant block-

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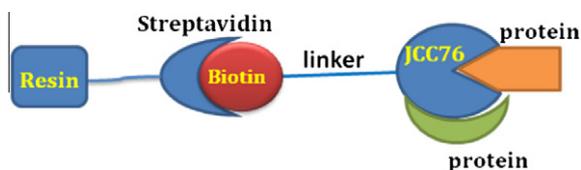


Figure 1. JCC76 biotinylated probe design.

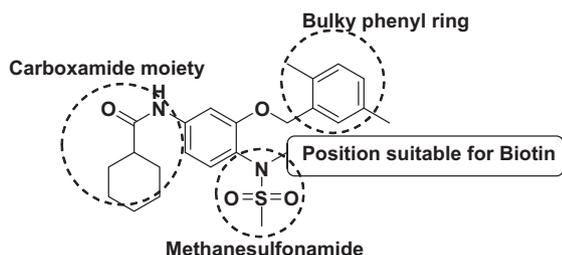


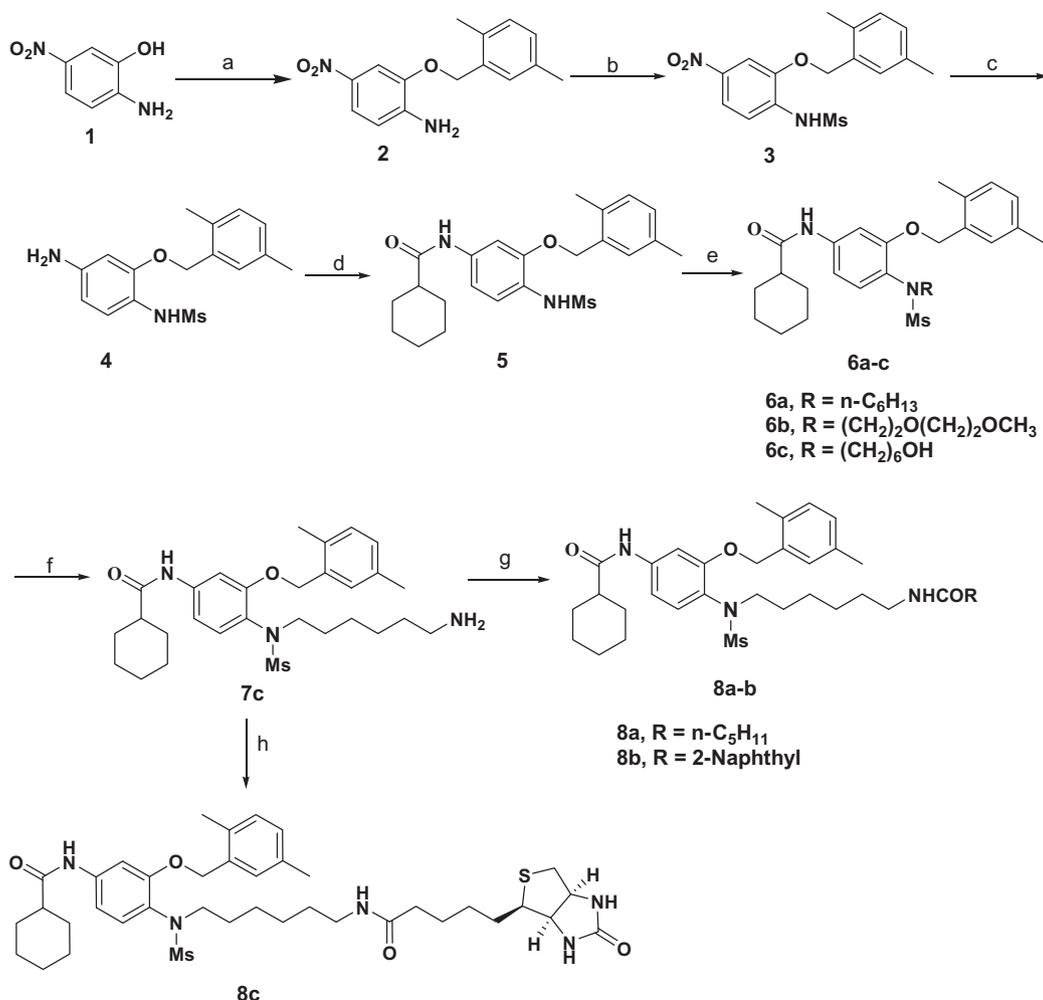
Figure 2. JCC76 structure analysis.

ing effects on the well-documented Her2 downstream kinases.^{19,20} It is difficult to elucidate the molecular targets of JCC76 by checking the signal transduction net in Her2 overexpressed breast can-

cer cells. In an effort to identify the bio-molecule(s) that bind to JCC76, we planned to use biotin–streptavidin affinity purification. For this purpose, biotinylated derivative of JCC76 with considerable bioactivity should be designed and synthesized. Generally, a biotinylated linker can be incorporated at the suitable position of JCC76, so that the biological activity of the small molecule can be conserved. Moreover, a linker between small molecule and biotin should have appropriate length in order to provide enough space for biotin to interact with streptavidin (Fig. 1).

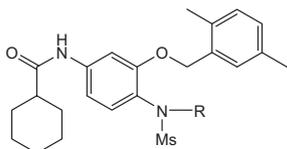
The previous structure–activity analysis indicated that the inhibition of breast cancer cell growth by nimesulide derivatives requires a hydrophobic group substituted bulky phenyl ring, a methanesulfonamide and a hydrophobic carboxamide moiety.¹⁸ Those three moieties might not be suitable for incorporation of biotinylated linker. *N*-methyl group on the sulfonamide group is the only position feasible for the introduction of biotin (Fig. 2).

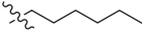
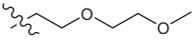
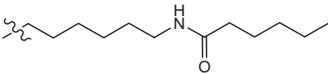
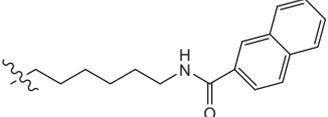
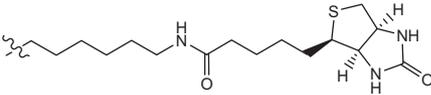
Replacement of *N*-methyl group with hydrogen or alkyl chains of different length generated a series of novel derivatives. The synthetic approach used is shown in Scheme 1. The key intermediate **5** was prepared according to the procedure previously used for synthesis of JCC76.¹⁸ The starting material **1** was treated with 2,5-dimethylbenzylbromide in the presence of potassium carbonate to afford compound **2**, followed by mesylation and hydrolysis to generate compound **3**. Instead of *N*-methylation of sulfonamide group, compound **3** was directly reduced in the presence of Zn/FeCl₃ to generate substituted aniline **4**. Coupling with cyclohexanecarbonyl



Scheme 1. Regents and conditions: (a) 2,5-dimethylbenzylbromide, K₂CO₃, DMF; (b) (1) MsCl, NaH, DMF; (2) NaOH, MeOH; (c) FeCl₃, Zn, DMF/H₂O; (d) cyclohexanecarbonyl chloride, K₂CO₃, 1,4-dioxane; (e) RX (X = Br or I), K₂CO₃, DMF; (f) (1) MsCl, Et₃N, DCM; (2) NH₃·H₂O, EtOH; (g) RCOCl, K₂CO₃, 1,4-dioxane; (h), (+)-D-biotin, PyBOP, Et₃N, DMF.

Table 1
IC₅₀ of inhibition of SKBR-3 breast cancer cells growth by JCC76 probes



Compound	R	Inhibition of SKBR-3 cell growth (IC ₅₀ μM)
JCC76	Me	3.43 ± 1.57
5	H	83.68 ± 42.77
6a		81.68 ± 31.18
6b		12.95 ± 4.92
8a		6.00 ± 2.52
8b		45.28 ± 12.47
8c		2.21 ± 0.78

SKBR-3 cells were treated with indicated compounds at various concentrations by triplicates for 48 h and cell viability was measured by MTT assay.²²

chloride afforded **5**. *N*-alkylation of sulfonamide group with alkyl bromide/iodide in the presence of potassium carbonate yielded **6a–c**. Conversion of hydroxyl group in **6c** to amino group followed by coupling with alkyl or aromatic carbonyl chloride generated **8a–b**.²¹

The anti-proliferation effect of compounds **5**, **6a–b**, and **8a–b** was evaluated against breast cancer cell line SKBR-3. The results are summarized in Table 1. It can be seen that alkyl group on the nitrogen of sulfonamide appreciably affects the biological activity of JCC76 derivatives. Replacement of methyl group with hydrogen or hydrophobic alkyl chain *n*-C₆H₁₃ almost abolishes the inhibitory activity. Replacement of methyl group with hydrophilic (CH₂)₂O (CH₂)₂OCH₃ to afford **6b** and results in an about fourfold loss in inhibitory potency. It seems that hydrophilic linker is better than hydrophobic linker. However, it is difficult to further modify the end structure of the hydrophilic linker **6b**. Extension of the linear C₆ chain with another alkyl chain or an aromatic ring through formation of an amide bond restores the biological activity to some extent. Compared to **6a**, compound **8b** is about twofold more potent and compound **8a** even exhibits 14-fold more potency. Noticeably, the inhibitory potency of compound **8a** is comparable to that of JCC76, which indicates that the long chain in **8a** is well tolerated. A survey of several JCC76 derivatives with different *N*-alkyl chains shows that an appropriate alkyl group can be introduced in lead compound JCC76 without jeopardizing the biological activity.

Based on the information obtained from this preliminary SAR study, we designed and synthesized compound **8c**, a novel biotinylated derivative of JCC76. PyBOP-mediated coupling of intermediate **7c** with biotin efficiently yielded compound **8c**.²¹ This compound has a very similar long chain as in compound **8a**. Hypothetically, with this long chain the biotin moiety should protrude for the interaction with streptavidin. The anti-proliferation effect of **8c** on SKBR-3 was evaluated and its IC₅₀ value was determined to be 2.21 ± 0.78 μM. This biotinylated derivative has comparable cell growth inhibition potency with lead compound JCC76. Biotin did not show any inhibitive activity even at 100 μM, which further

prove that JCC76 was the moiety that cause the anti-proliferation activity of the biotinylated probe.

In conclusion, we synthesized a biotin-tagged probe of anti-proliferative agent JCC76 in this work. The biotin moiety was successfully introduced in the molecule without loss of the biological activity of the parental compound. JCC76 is nonCOX-2 active agent developed based on COX-2 inhibitor nimesulide as a lead compound. It selectively inhibits Her2 over expressed breast cancer cell proliferation,^{19,20} but its specific anti-cancer molecular target(s) still remain unclear. There are numerous studies demonstrate that COX inhibitors exhibit anti-cancer activity via COX independent mechanism(s).^{1–5} However, it is difficult to elucidate the specific nonCOX targets of these small molecules. Our work can provide a feasible approach for the identification of the new molecular targets of the COX inhibitors. We currently focus on the purification and identification of JCC76 targeted protein(s) by protein pull down and proteomic approach based on the biotin-tagged JCC76 probe.

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21. Compound **5**: White powder, ^1H NMR (400 MHz, CDCl_3) δ 7.970 (1H, d, $J = 2$ Hz), 7.453 (1H, d, $J = 8.4$ Hz), 7.232 (1H, s), 7.126 (3H, m), 6.696 (1H, dd, $J = 2, 8.4$ Hz), 6.555 (1H, s), 5.047 (2H, s), 2.834 (3H, s), 2.336 (3H, s), 2.313 (3H, s), 2.242 (1H, tt, $J = 3.6, 12$ Hz), 1.245–1.985 (10H, m); **Compound 6a**: White powder, ^1H NMR (400 MHz, CDCl_3) δ 7.995 (1H, d, $J = 2.4$ Hz), 7.410 (1H, s), 7.210 (1H, d, $J = 8.4$ Hz), 7.094 (3H, m), 6.643 (1H, dd, $J = 2.4, 8.4$ Hz), 5.036 (2H, s), 3.482 (2H, b), 2.693 (3H, s), 2.317 (6H, s), 2.258 (1H, tt, $J = 3.6, 12$ Hz), 1.970–1.174 (18H, m), 0.842 (3H, t, $J = 6.8$ Hz); **Compound 6b**: White powder, ^1H NMR (400 MHz, CDCl_3) δ 7.902 (1H, d, $J = 2.4$ Hz), 7.509 (1H, s), 7.230 (1H, d, $J = 8.4$ Hz), 7.178 (1H, s), 7.091 (2H, m), 6.696 (1H, dd, $J = 2.4, 8.8$ Hz), 5.001 (2H, s), 3.471 (8H, m), 3.338 (3H, s), 2.792 (3H, s), 2.317 (3H, s), 2.311 (3H, s), 2.253 (1H, tt, $J = 3.6, 12$ Hz), 1.956–1.254 (10H, m); **Compound 8a**: White powder, ^1H NMR (400 MHz, CDCl_3) δ 8.027 (1H, s), 7.883 (1H, s), 7.134 (4H, m), 6.686 (1H, d, $J = 8.4$ Hz), 5.677 (1H, b), 5.024 (2H, s), 3.487 (2H, b), 3.184 (2H, d, $J = 6.4$ Hz), 2.691 (3H, s), 2.312 (7H, m), 2.178–1.290 (26H, m), 0.878 (3H, t, $J = 6.8$ Hz); **Compound 8b**: White powder, ^1H NMR (400 MHz, CDCl_3) δ 8.294 (1H, s), 8.006 (1H, s), 7.854 (5H, m), 7.528 (2H, m), 7.185 (1H, d, $J = 8.4$ Hz), 7.077 (3H, m), 6.696 (1H, dd, $J = 2, 8.4$ Hz), 6.589 (1H, s), 4.997 (2H, s), 3.455 (4H, m), 2.680 (3H, s), 2.289 (7H, m), 1.931–1.192 (18H, m); **Compound 8c**: White powder, ^1H NMR (400 MHz, CDCl_3) δ 8.201 (1H, s), 8.085 (1H, s), 7.203 (1H, d, $J = 8.4$ Hz), 7.101 (3H, m), 6.763 (1H, dd, $J = 2, 8.4$ Hz), 6.186 (1H, s), 6.115 (1H, t, $J = 6$ Hz), 5.223 (1H, s), 5.040 (2H, s), 4.498 (1H, m), 4.314 (1H, m), 3.488 (2H, m), 3.149 (3H, m), 2.900 (1H, dd, $J = 4.8, 128$ Hz), 2.717 (1H, d, $J = 124$ Hz), 2.684 (3H, s), 2.314 (7H, m), 2.195 (2H, t, $J = 7.2$ Hz), 1.951–1.283 (24H, m); HRMS calculated for $\text{C}_{39}\text{H}_{58}\text{N}_5\text{O}_6\text{S}_2$ ($\text{M}+\text{H}$) $^+$ 756.3828, found 756.3847.
22. The effect of JCC76 probes on SKBR-3 breast cancer cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay (MTT) in triplicates. Cells were grown in custom medium in 96-well, flat-bottomed plates for 24 h, and were exposed to various concentrations of nimesulide derivatives dissolved in DMSO (final concentration $\leq 0.1\%$) in media for 72 h. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. The medium was removed, replaced by 200 μl of 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide in fresh media, and cells were incubated in the CO_2 incubator at 37 $^\circ\text{C}$ for 2 h. Supernatants were removed from the wells, and the reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide dye was solubilized in 200 μl /well DMSO. Absorbance at 570 nm was determined on a plate reader.