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Synthesis and Evaluation of Novel Erlotinib−NSAID Conjugates as More Comprehensive Anticancer Agents

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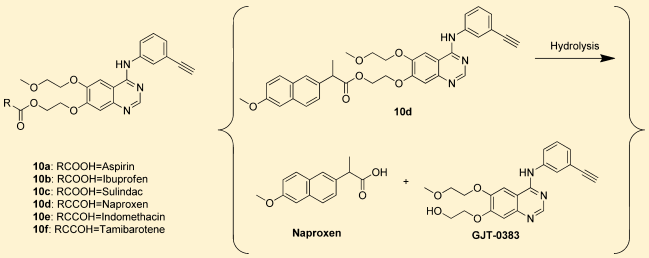
Supporting Information

ABSTRACT: A series of novel anticancer agents were designed and synthesized based on coupling of different nonsteroidal anti-inflammatory drugs (NSAIDs) with the epidermal growth-factor receptor (EGFR) tyrosine kinase inhibitor, erlotinib. Both the antiproliferative and pharmacokinetic activity of the target compounds were evaluated using HCC827 and A431 tumor cell lines. Among the derivatives made, compounds **10a**, **10c**, and **21g** showed superb potency, comparable to that of erlotinib. Furthermore, preliminary SAR analysis showed that when the NSAIDs were conjugated via linkage to C-6 OH versus linkage to C-7 OH of the quinazoline nucleus, superior anticancer activity was achieved. Finally, the *in vitro* pharmacokinetic profile of several conjugates demonstrated the desired dissociation kinetics as the coupled molecules were effectively hydrolyzed, releasing both erlotinib and the specific NSAID in a time-dependent manner. The conjugation strategy represents a unique and simplified approach toward combination therapy, particularly for the treatment of cancers where both EGFR overexpression and inflammation play a direct role in disease progression.



KEYWORDS: *NSAIDs, EGFR, Erlotinib, cancer, COX, conjugate*

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Lung cancer, in particular nonsmall cell lung carcinoma (NSCLC), is a disease associated with a high mortality rate due to its complexity and heterogeneity.[1−3] Although there has been significant progress made in its diagnosis, management, and treatment, the five year survival rate for this disease is still low.[4] Chemotherapy is a primary option for patients with lung cancers like NSCLC and the use of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors has become a frontline approach due to their demonstrable effectiveness in slowing the progression of the disease as well as increasing the overall survival rate of patients.[5−7]

However, in cancer treatment, using a single drug directed toward just one target/mechanism is usually inadequate and overtime disease management usually requires drug combinations targeting multiple cancer pathways to sustain efficacy.[8−10] Choosing the right combination of chemotherapeutic agents is crucial for maximizing progression free survival while minimizing side effects.

Inflammation has received considerable attention for its role in the progression and aggressiveness of many cancers including NSCLC. Many studies have shown the efficacy of NSAIDs in animal models of cancer prevention, partly due their ability to block cyclooxygenase (COX) activity.[11−19] Mechanistically, cyclooxygenases including COX-1 and especially COX-2 have been shown to be important in many stages of oncogenesis. A case in point is the USA FDA approval of the NSAID, celecoxib (Celebrex, a selective COX-2 inhibitor), for use in patients with familial adenomatous polyposis. Also, many studies have shown the efficacy of COX-2 inhibitors in various animal models of cancer. Mechanistically, COX-2 is highly expressed in many tumor and stromal cells and PGE2, a major product of COX-mediated arachidonic acid metabolism, is believed to be a contributing component of angiogenesis in lung cancer. Therefore, a combination drug that targets both EGFR and cyclooxygenases may be a more comprehensive and effective treatment modality for the treatment of lung cancer.

In this study we present a series of esterase hydrolyzable NSAIDs conjugated with erlotinib, which may have potential as a new class of chemotherapeutic agents that offers simplified dosing regimen with improved efficacy.

The preparation of conjugated NSAIDs with erlotinib **10a−f**, **11a−g**, and **21a−g** is outlined in Schemes 1 and 2. Compound **7** was obtained from commercially available 2-amino-4,5-dimethoxybenzoate (**1**) and elaborated according to the procedure previously described.[20,21] Then the 6-OH of compound **7** was reacted with 1-bromo-2-methoxyethane in the presence of K_2CO_3 to generate **8**. The desired conjugated derivatives **10a−f** were obtained by reacting **8** with **9a−f**, which had been prepared by esterification the corresponding acid with 2-bromoethanol in

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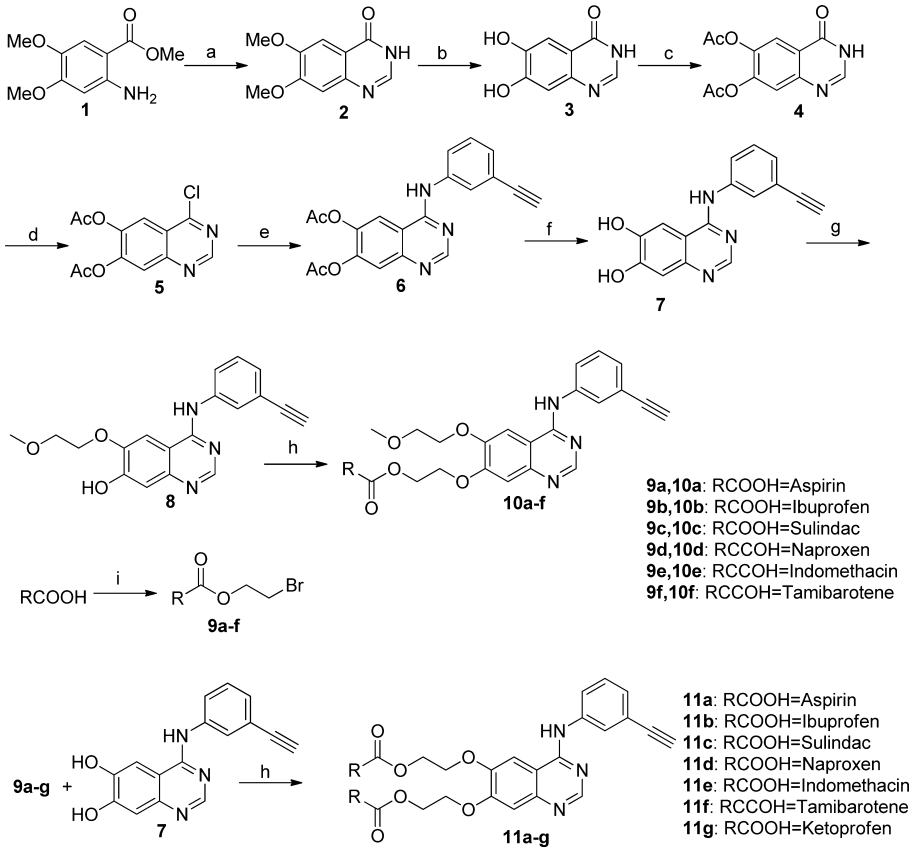
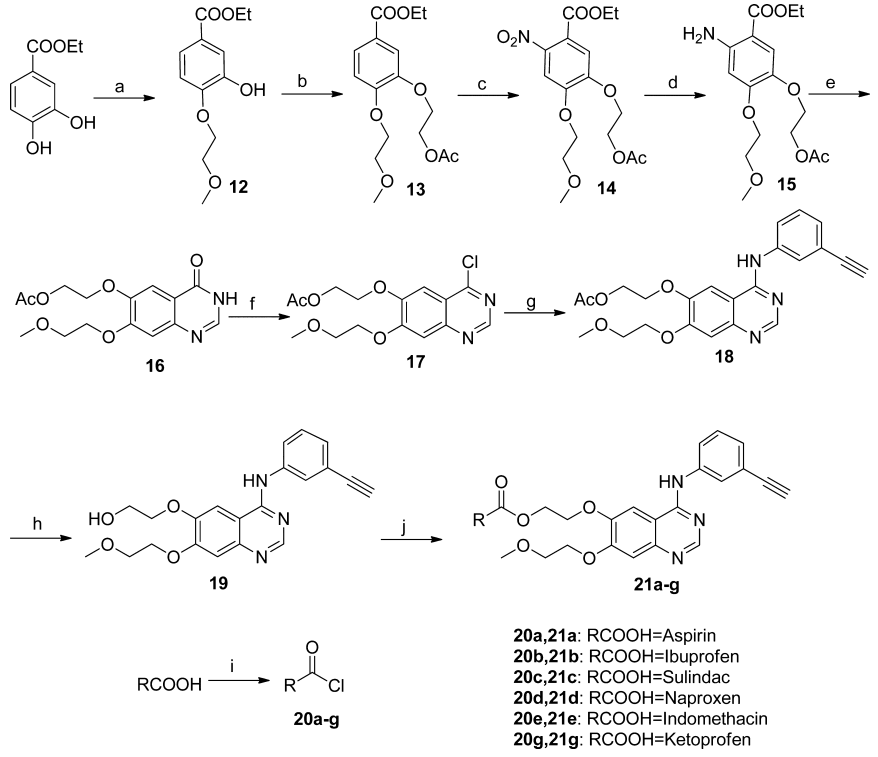
Scheme 1. Synthesis of Compounds 10a−f and 11a−g[a]



[a]Reagents and conditions: (a) HCO_2NH_4, $HCONH_2$, 140 °C, 24 h; (b) HBr, Ac_2O; (c) Ac_2O, py; (d) $POCl_3$, $CHCl_3$; (e) 3-ethynylaniline, i-PrOH, reflux; (f) NH_4OH; (g) 1-bromo-2-methoxyethane (1.0 equiv), K_2CO_3, DMF, 20 °C; (h) **9a−f**, K_2CO_3, DMF; (i) 2-bromoethanol, $SOCl_2$.

Scheme 2. Synthesis of Compounds 21a−g[a]



[a]Reagents and conditions: (a) 1-bromo-2-methoxyethane, NaH, DMF; (b) 2-bromoethyl acetate, K_2CO_3, DMF; (c) conc. HNO_3, conc. H_2SO_4, HOAc; (d) Fe, HOAc; (e) $HCOONH_4$, $HCONH_2$; (f) $POCl_3$, $CHCl_3$; (g) 3-ethynylaniline, i-PrOH, reflux; (h) NaOH, MeOH; (i) $COCl_2$, DCM; (j) **9a−f**, Et_3N, DCM.

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Table 1. Inhibition of EGFR, COX-1, and COX-2

	enzyme inhibition IC_{50} value (μM)		
compd	EGFR	COX-1	COX-2
erlotinib	0.0005 ± 0.0004	>10	>10
aspirin	>10	>10	>10
ibuprofen	>10	>10	1.1 ± 2.27
sulindac	>10	16 ± 10.975	8.8 ± 2.72
naproxen	>10	0.18 ± 0.82	0.28 ± 0.21
indomethacin	>10	0.11 ± 0.05	0.41 ± 0.06
ketoprofen	>10	0.067 ± 0.05	0.061 ± 0.05
10a	0.005 ± 0.0006	>10	>10
10b	0.009 ± 0.0143	44.67 ± 20.61	>10
10c	0.031 ± 0.0034	>10	>10
10d	0.072 ± 0.0200	24.48 ± 14.71	>10
10e	0.027 ± 0.0015	11.34 ± 21.40	>10
10f	0.032 ± 0.0037	>10	>10
11a	0.034 ± 0.0068	39.57 ± 12.97	>10
11b	>10	>10	>10
11c	0.027 ± 0.0019	>10	>10
11d	0.39 ± 0.0480	>10	>10
11e	0.033 ± 0.0028	31.38 ± 0.31	>10
11f	0.88 ± 0.2820	>10	>10
GJT-0383	0.001 ± 0.0003	>10	>10

the presence of $SOCl_2$. Analogues **11a−g** were prepared by a similar experimental procedure.

Ethyl 3,4-dihydroxybenzoate was selectively alkylated with 1-bromo-2-methoxyethane to afford **12**, which was then converted to compound **13** in the presence of 2-bromoethyl acetate and K_2CO_3. Nitration of **13** with concentrated HNO_3 and H_2SO_4 in acetic acid gave **14**, which was converted into aniline **15** by reduction with iron in acetic acid. Quinazolin-4(3*H*)-one **16** was prepared by condensation of **15** and $HCOONH_4$ in $HCONH_2$. Treatment of **16** with $POCl_3$ in $CHCl_3$ gave 4-chloroquinazoline **17**, which was treated with 3-ethynylaniline in isopropyl alcohol to afford 4-anilinoquinazoline derivative **18**. Hydrolysis of the acetyl group of compound **18** was performed using sodium hydroxide in methanol to provide the key intermediate **19**. The desired conjugated compounds **21a−g** were prepared by reacting **19** with the corresponding NSAID acyl chloride using Et_3N as the base in DCM.

To assess the potency of the conjugated compounds versus the individual noncoupled molecules, selected compounds were tested against EGFR tyrosine kinase, COX-1 and COX-2 enzymes (Table 1). As expected, the conjugated compounds showed less activity in their coupled state compared to the corresponding unconjugated molecules. The noncoupled compounds effectively blocked their target enzymes with IC_{50} values consistent with previous reports.[22−26] These data suggest that the conjugated compounds are likely acting as pro-drugs for their parent molecules. Release of the parent molecules from these pro-drugs may be advantageous from a safety perspective as the plasma levels of fully active drugs may be more precisely managed using this strategy. In addition, it has been reported that esterases have elevated activity in tumors suggesting that activation of the drug conjugates may be enhanced at sites of disease relative to normal tissues.[27,28]

To assess the efficacy of the conjugated compounds as anticancer agents, a series of coupled molecules were evaluated for their antiproliferative activity against two tumor cell lines HCC827 and A431. As hoped, multiple conjugated drugs demonstrated excellent activity in blocking tumor cell proliferation (Table 2). Coupled compounds **10a**, **10b**, **10c**, **10d**, **10f**, **21a**, **21c**, and **21g** were particularly active at blocking proliferation of HCC827 cells. The efficacy potency of these compounds was comparable to erlotinib, which was used as a positive control. SAR analysis showed that NSAIDs conjugated via linkage to C-6 OH versus linkage to C-7 OH of the quinazoline nucleus possessed superior *in vitro* anticancer activity. It is important to point out that tumor cell-based assays are not adequate to assess the synergistic activity between erlotinib and the specific NSAID as cells grown under these conditions lack a significant inflammatory component. Future studies will focus on testing candidate conjugated drugs in animal models of cancer.

To make sure the drugs were acting on their respective targets, namely, EGFR tyrosine kinase and COX-2, as a representative example, cells and media were collected from **10d** treated HCC827 cultures and analyzed for a reduction in both phosphorylation of EGFR and PGE2 levels by Western blot analysis and HPLC, respectively. As expected, a dose-dependent reduction of phosphorylation of EGFR (Figure 1) and PGE2 (IC_{50} 23 μM; data not shown) was observed indicating that **10d** is acting on its target/mechanism(s).

To assess the time-dependent hydrolysis of the conjugated compounds and the corresponding dissociation of the parent molecules, a pharmacokinetic analysis was performed using compound **10d** in cell culture. In the experiment, **10d** was added to culture media and then allowed to incubate with HCC827 tumor cells for up to 24 h. During the incubation, conditioned medium was collected at various time points and assessed for the coupled compound **10d**, and the parent

Table 2. Summary of the *in Vitro* Antiproliferative Activities of Conjugate Compounds against Two Human Cell Lines Including Epidermoid and Lung Adenocarcinoma

	IC_{50} (μM)			IC_{50} (μM)	
compd	HCC827	A431	compd	HCC827	A431
10a	0.1 ± 0.03	1.8 ± 0.49	**10b**	0.4 ± 0.23	2.2 ± 0.49
10c	0.3 ± 0.18	5.4 ± 1.83	**10d**	0.1 ± 0.04	3.2 ± 0.10
10e	0.9 ± 0.36	>10	**10f**	0.2 ± 0.05	>10
11a	0.9 ± 0.27	>10	**11b**	16.5 ± 18.35	NA
11c	3.5 ± 2.13	>10	**11d**	2.3 ± 0.32	NA
11e	1.6 ± 0.82	>10	**11f**	>10	NA
11g	2.1 ± 0.74	>10	**21a**	0.1 ± 0.04	>10
21b	>10	>10	**21c**	0.4 ± 0.29	4.8 ± 2.33
21d	0.5 ± 0.28	5.0 ± 0.21	**21e**	1.2 ± 0.46	>10
21g	0.3 ± 0.21	1.7 ± 1.35	erlotinib	0.029 ± 0.004	0.844 ± 0.02
GJT-0383	0.014 ± 0.003	1.27 ± 0.17			

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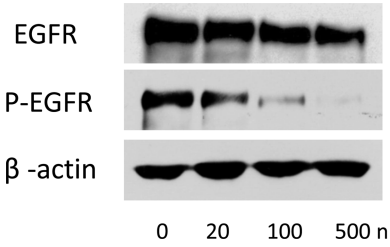

Figure 1. Western blot analysis showing the effects of **10d** at concentrations of 0 to 500 nM on the phosphorylation of EGFR in HCC827 cells.

Scheme 3. Hydrolysis of Compound 10d

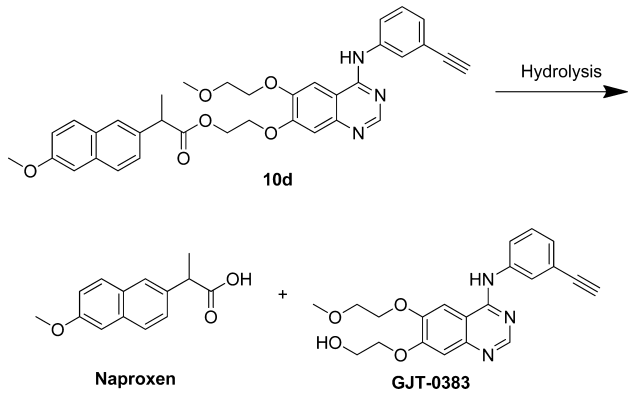

Table 3. Hydrolysis of Compound 10d in HCC827

time (h)	**10d** (μg/L)	GJT-0383 (μg/L)	naproxen (μg/L)
0	3707 ± 4.714	105.0 ± 2.160	91.20 ± 0.8832
0.25	3567 ± 26.24	158.3 ± 4.110	108.4 ± 8.536
0.5	3427 ± 12.47	224.3 ± 4.922	128.7 ± 9.672
2	3233 ± 12.47	704.7 ± 3.682	293.0 ± 39.91
4	3150 ± 21.60	1277 ± 18.86	382.0 ± 29.34
8	2923 ± 33.00	2363 ± 9.428	849.0 ± 87.41
24	2137 ± 20.55	4397 ± 123.9	2017 ± 65.49

molecules naproxen and erlotinib. In the case of erlotinib, following hydrolysis of compound **10d** an additional hydrogen atom is left behind giving the molecule a slightly higher molecular mass and referred to as GJT-0383 in this study (Scheme 3). To make sure GJT-0383 is as potent as erlotinib, the compound was tested for activity against EGFR tyrosine kinase. Both GJT-0383 and erlotinib showed comparable IC_{50} values (Table 1).

As can be seen in Table 3, there is a time-dependent hydrolysis of **10d** resulting in the release of both GJT-0383 and naproxen overtime. Within 24 h approximately 50% of compound **10d** was hydrolyzed demonstrating that the conjugated drug can be processed in a cell based system. With the loss of compound **10d** there is a corresponding increase in the concentrations of GJT-0383 and naproxen. At time 0 a small amount of GJT-0383 and naproxen was detected suggesting some hydrolysis during storage of **10d**.

In the cell based experiments, active serum was used bringing up the possibility that esterases present in the serum are partly or mostly responsible for the hydrolysis of **10d** overtime. In order to address this, **10d** was incubated with both active versus heat inactivated whole serum and measuring the hydrolysis overtime. It was observed that **10d** was effectively hydrolyzed overtime in the active serum, but in contrast, little or no hydrolysis of **10d** occurred in the heat inactivated serum presumably due to the denaturing of the esterases contained in the serum (Figure 2). Based on these data it appears that a major proportion of the activation of **10d** observed in HCC827 and A431 cells is mediated by serum esterases.

In this study we describe a new series of conjugated compounds that may be used as a potentially simplified approach for treating cancers where both EGFR overexpression and inflammation are involved. Future studies will focus on the *in vivo* efficacy of the more potent coupled compounds in animal models of colon and lung cancer where inflammation is believed to be a significant contributing factor to disease progression.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00286.

Complete experimental details along with synthesis and characterization of Erlotinib−NSAID conjugates (PDF)

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

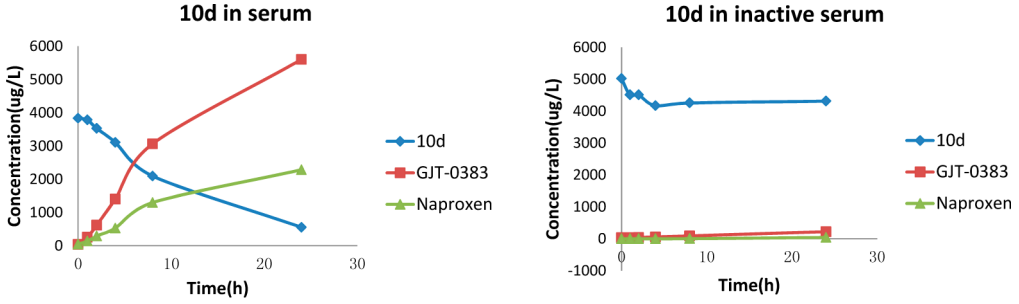

Figure 2. Hydrolysis of **10d** in active versus heat inactivated serum.

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