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Mechanistic insights into the activation of ester prodrugs of **666-15**

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ABSTRACT

cAMP-response element binding protein (CREB) is an oncogenic transcription factor implicated in many different types of cancer. We previously reported the discovery of **666-15** as a potent inhibitor of CREB-mediated gene transcription. In an effort to improve the aqueous solubility of **666-15**, amino ester prodrugs **1** and **4** were designed and synthesized. Detailed chemical and biological studies of **1** and **4** revealed that a small portion of the prodrugs were converted into **666-15** through intermediate **3** instead of a long-range *O,N*-acyl transfer reaction that was initially proposed. These results provide unique insights into the activation of these ester prodrugs.

cAMP-response element-binding protein (CREB) is a nucleus resided transcription factor involved in numerous biological processes including cellular proliferation, differentiation and memory formation.[1] It belongs to a large family of basic leucine zipper (bZIP)-containing transcription factors including c-Jun, c-Fos and c-Myc. The bZIP domain in the C-terminus can homodimerize to bind the cognate DNA sequence of 5′-TGACGTCA-3′ called cAMP-response element (CRE).[2] While this binding is thought to be constitutive in the cells, CREB's transcription activity is not turned on until it is phosphorylated at Ser133 by various protein serine/threonine kinases. The first protein kinase known to phosphorylate CREB is protein kinase A (PKA).[3] Since then, many other protein kinases have been shown to be able to phosphorylate CREB to turn on its transcription activity, which include protein kinase B (PKB/Akt), ribosomal S6 kinase ($p90^{RSK}$) and mitogen-activated protein kinases (MAPKs).[4] Phosphorylation of CREB at Ser133 is very dynamic to allow cells to respond to extracellular and intracellular signals. There are protein phosphatases that can dephosphorylate CREB. The protein phosphatases that are known to dephosphorylate CREB include protein phosphatase 1 (PP1),[5] protein phosphatase 2A (PP2A),[6] and phosphatase and tension homolog (PTEN).[7] This dynamic and reversible phosphorylation of CREBs allows its transcription activity to be tightly regulated under normal cellular homeostasis.

In the cancer cells, however, the kinases that can phosphorylate CREB are often mutated or overexpressed to confer their oncogenic activity. As a consequence, the positive signals to drive CREB phosphorylation in cancer cells are increased. On the other hand, the protein phosphatases known to dephosphorylate CREB are tumor suppressor proteins that are often inactivated or deleted in cancer cells, resulting in a decrease of signals to turn off CREB-mediated gene transcription. Due to the dysregulation of both positive and negative signals to CREB in cancer cells, CREB has been shown to be consistently upregulated and activated in many different cancer tissues including breast, lung, prostate, kidney, brain, pancreas and blood.[4,8–12] Because of this upregulation in many cancer tissues, CREB has been pursued as a potential cancer drug target. Consistent with this idea, genetic inhibition of CREB using both shRNA and dominant negative CREB mutants has been shown to produce profound anti-cancer effect in multiple preclinical cancer models.[4,9,12] Encouraged by these promising results, we initiated development of small molecule inhibitors of CREB-mediated gene transcription.[13–15] We previously developed **666-15** as a potent inhibitor of CREB-mediated gene transcription.[16,17] Kikuchi group recently reported a photo-caged version of **666-15** to allow potential spatio-temporal control of CREB inhibition.[18]

Despite **666-15**′s potent CREB inhibitory activity, its aqueous solubility needs further improvement.[19] In an effort to improve the aqueous solubility of **666-15**, we designed amino ester compound **1** as a traceless prodrug for **666-15** based on a long-range *O,N*-acyl transfer reaction that we previously described (Scheme 1).[20] It was anticipated that the primary amino group in **1** will nucleophilically attack the ester bond to form amide **666-15** in high yield at physiologically relevant buffers as observed before for a closely related congener compound **S1** (pathway A in Scheme 1 and Scheme S1).[20] Surprisingly, we found that only a small amount (< 10%) of **1** was converted into **666-15** in biologically relevant buffers at pH = 7.40.[19] Instead, the majority of compound **1** was converted into **653-47** and **2** (Scheme 1).[19] This unexpected conversion of compound **1** into **653-47** and **2** was likely

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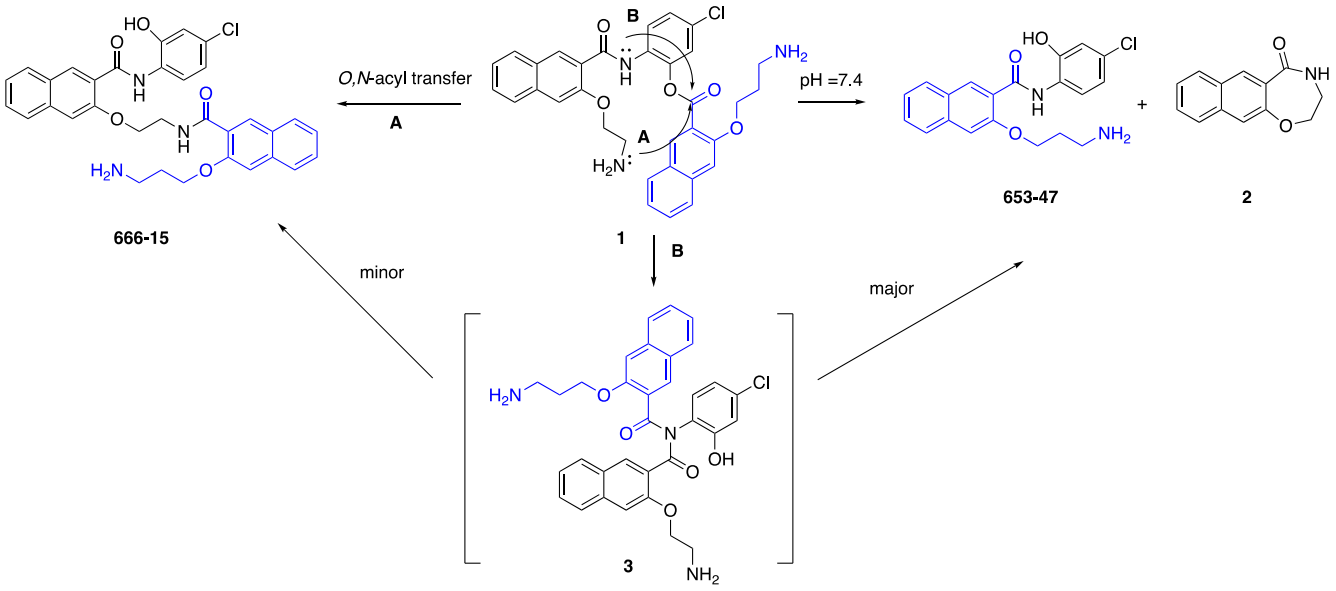


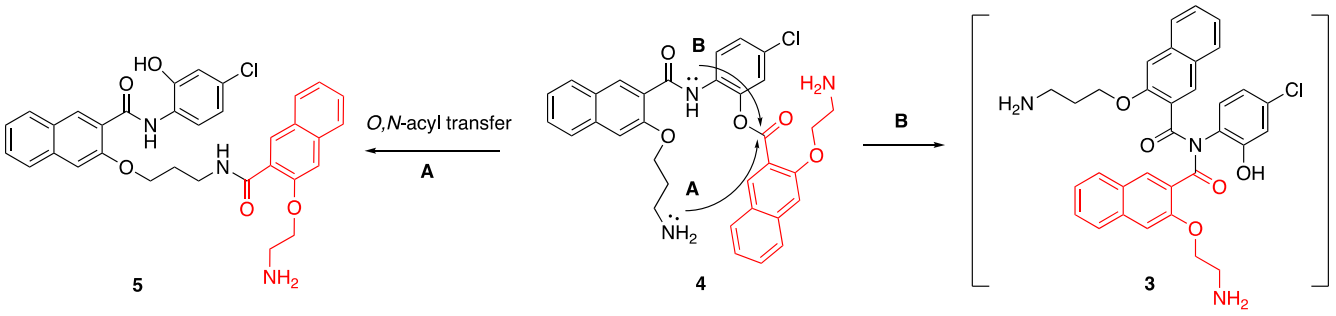
Scheme 1. Pathways for converting **1** to **666-15** and **653-47**.

through an imide intermediate **3** (pathway B in Scheme 1). Imide **3** could then either be cleaved to generate **653-47** and **2** or rearranged to give **666-15** as a minor pathway. The unanticipated discovery of **653-47** from **1** became significant because we found that **653-47** was able to potentiate **666-15**'s inhibitory activity against CREB-mediated gene transcription even though **653-47** was inactive alone.[19]

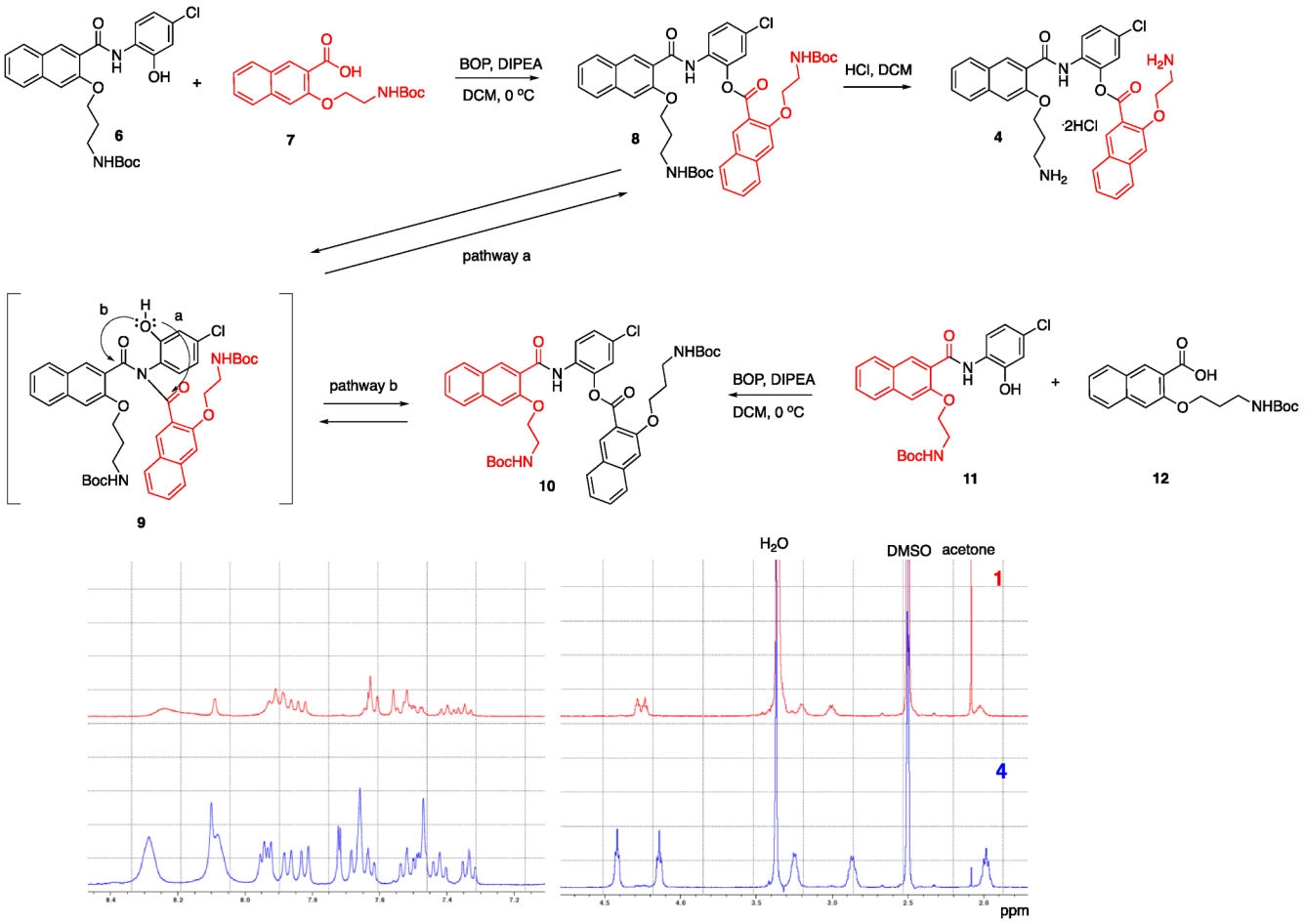
While the mechanism of activation of prodrug **1** invoking imide **3** is intriguing, further experimental evidence is still lacking. We hypothesized that the regioisomer amino ester **4** should be able to give the same intermediate imide **3** by pathway B (Scheme 2), which shall generate the same products **653-47** and **2** as the major products and **666-15** as a minor species. On the other hand, if the alternative direct *O,N*-acyl transfer was the major mechanism for the conversion (pathway A in Scheme 2), the anticipated product would be amide **5**. In this communication, we synthesized compound **4** and further studied its hydrolytical stability and biological activities to provide unique mechanistic insights into the activation of these designed ester prodrugs.

The synthesis of compound **4** is shown in Scheme 3. When we employed a typical amide coupling protocol with BOP or MsCl as the activation reagent, we observed the formation of a mixture of both desired amide **8** and undesired amide **10**, which were difficult to separate by conventional column chromatography. Changing to other coupling reagents including EDCI or DCC did not improve the reaction outcome. We previously reported that lowering the reaction temperature and decreasing the reaction time could significantly inhibit the formation of undesired isomer albeit at the expense of reduced reaction conversion.[19] By applying this revised protocol (BOP, 0 °C, 1 h), we were able to isolate pure compound **8** in 23% yield. Finally, deprotection of the Boc groups in **8** delivered designed compound **4** smoothly. While isomers **1** and **4** are structurally very similar, their ^{1}H NMR spectra are quite distinct from each other in both the aromatic region and aliphatic region (Scheme 3). Similar to compound **1**,[19] compound **4** also exhibited dramatically improved aqueous solubility in ddI H_2O (> 100 mg/mL) compared to **666-15**·**HCl** (< 0.5 mg/mL in ddI H_2O). Assessing the solubility of **1** and **4** in aqueous buffers at pH = 7.4 was hampered due to its instability at this pH (see below). That the preferential formation of **8** from **6** and **7** versus preferential formation of **10** from **11** and **12**[19] under these conditions suggests that the proposed imide intermediate **9** was not formed directly from **6** and **7** or **11** and **12**. Instead, it is more likely that **9** was formed after **8** or **10** was generated during the reaction. Decreasing the reaction time and temperature could potentially inhibit this rate-limiting step of formation of **9** from **8** or **10**.

With compound **4** in hand, we evaluated its stability and reaction conversion in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). To this end, compound **4** (200 μM) was incubated in the complete tissue culture media at 37 °C for different periods of time, when an aliquot was taken for HPLC analysis. Similar to compound **1**,[19] compound **4** was very unstable in the complete tissue culture media and rapidly converted into multiple species (Fig. 1A). The HPLC peaks generated from incubating **4** were the same as those generated from **1**. After 5 min of incubation at 37 °C, most of **4** was converted into **653-47** and **2**. Smaller amounts of **666-15** and **5** were also generated (Fig. 1). The individual peaks were identified by carefully comparing with the previously synthesized authentic samples (Fig. S1).[16,19] Under this incubation condition, **666-15** itself was found to be



Scheme 2. Proposed conversion of amino ester **4**.



Scheme 3. Synthesis of compound **4**.

very stable and unchanged even after 24 h incubation (Fig. S2). To further confirm the identities of the individual species generated from **4**, it was treated in phosphate buffer saline (PBS, pH 7.4) and then the reaction mixture was subjected to Boc protection to facilitate purification of compounds containing amino groups (Scheme 4). After careful chromatography separation and ^{1}H NMR analyses, this reaction gave an inseparable mixture of **13** and **14** (23%) in a ratio of 2:1 as determined by ^{1}H NMR, mono-Boc protected **15** (49%), doubly Boc protected **16** (10%) and cyclic amide **2** (45%). The discovery that both **1** and **4** produced the same composition of mixtures further supports that pathway B in Scheme 2 was the pathway to contribute to the formation of **666-15**[19] as opposed to pathway A involving a long-range *O*,*N*-acyl transfer reaction that we previously proposed.[20]

The biological activities of compounds **1** and **4** were further evaluated and compared. If both **1** and **4** were converted to the same mixture of species in the complete tissue culture media, they would be expected to have the same biological activities. To test this hypothesis, we first evaluated their activities in inhibiting CREB-mediated gene transcription using a cell-based CREB transcription reporter assay.[13] In this assay, HEK293T cells were transiently transfected with a plasmid pCRE-RLuc that would express *RENILLA* luciferase in response to the activation of CREB. Then the cells were treated with different concentrations of the compounds followed by stimulation of the cells with forskolin (Fsk)[13,21] to increase CREB phosphorylation. The results in Fig. S4A and Table 1 showed that both **1** and **4** were of equal potency in inhibiting CREB-mediated gene transcription (IC_{50} = 0.26 ± 0.097 μM for **1** and 0.25 ± 0.16 μM for **4**), which are consistent with the results that both **1** and **4** were converted to the same species under the assay conditions (Fig. 1A). We previously reported that CREB inhibitor **666-15** was very potent in inhibiting triple negative breast cancer (TNBC) cell growth (Table 1).[16] **666-15** also inhibited the expression of endogenous CREB target gene *c-Fos* in the TNBC cells (Fig. S3). TNBC is an aggressive subtype of breast cancer whose cells do not express estrogen receptor (ER), progesterone receptor (PR) or present amplification of human epidermal growth factor receptor 2 (HER2).[22] TNBC patients do not benefit from current targeted breast cancer therapies including ER-targeting (e.g. tamoxifen) and HER2-targeting agents (e.g. trastuzumab, lapatinib). To investigate the potential anti-TNBC effect of **1** and **4**, we evaluated their cell growth inhibitory activity in MDA-MB-231 and MDA-MB-468 cells, both of which are TNBC cells. The cells were treated with increasing concentrations of **1** or **4** for 72 h. Then the remaining viable cells were quantified using MTT reagent (methylthiazolyldiphenyl-tetrazolium bromide).[14] The concentration needed to inhibit the cell growth by 50% was designated as GI_{50}. As anticipated from the results in Fig. 1 showing that both **1** and **4** gave the same reaction mixture upon incubation in the tissue culture media, both of the compounds displayed the same antiproliferative activity in MDA-MB-231 and MDA-MB-468 cells (Fig. S4B-4C and Table 1). In MDA-MB-231 cells, the GI_{50}s were 0.54 and 0.67 μM for **1** and **4**, respectively. In MDA-MB-468 cells, both of the compounds showed higher potency with GI_{50} being 0.045 and 0.032 μM, which are on a par with **666-15**. In both of the cell lines tested, the compounds produced net cell killing effect at higher concentrations of the drugs (> 10 μM) (Fig. S4B-4C).[23] Since both compounds **1** and **4** were rapidly converted into **666-15** and **653-47** in the complete tissue culture media under our biological assay conditions, we attributed the potent CREB inhibition activities and breast cancer cell growth inhibition potencies seen with **1** and **4** to the synergistic effect between **666-15** and **653-47**.[19] It is interesting to note that both compounds **1** and **4** were about 10-fold more potent in MDA-MB-468 cells

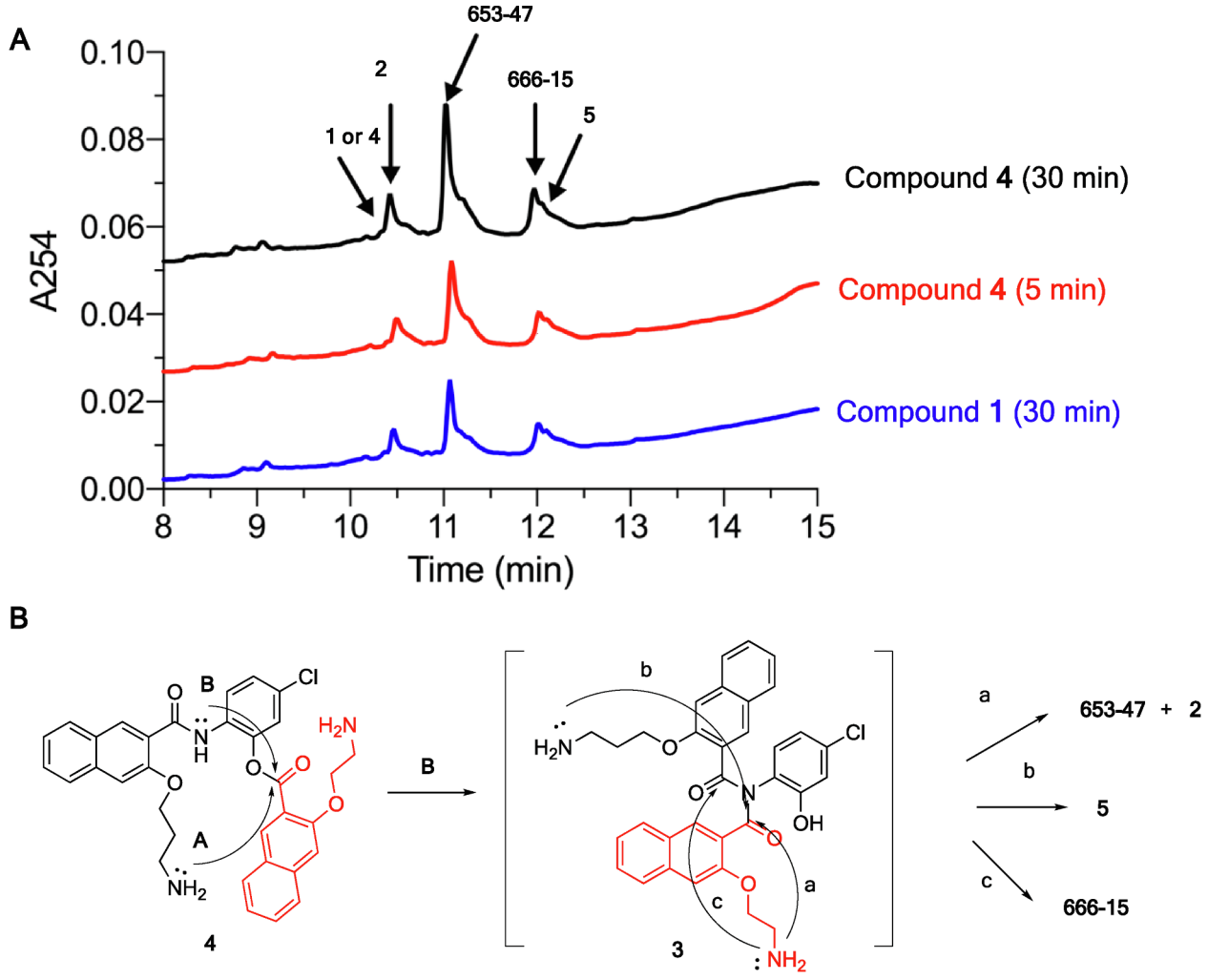
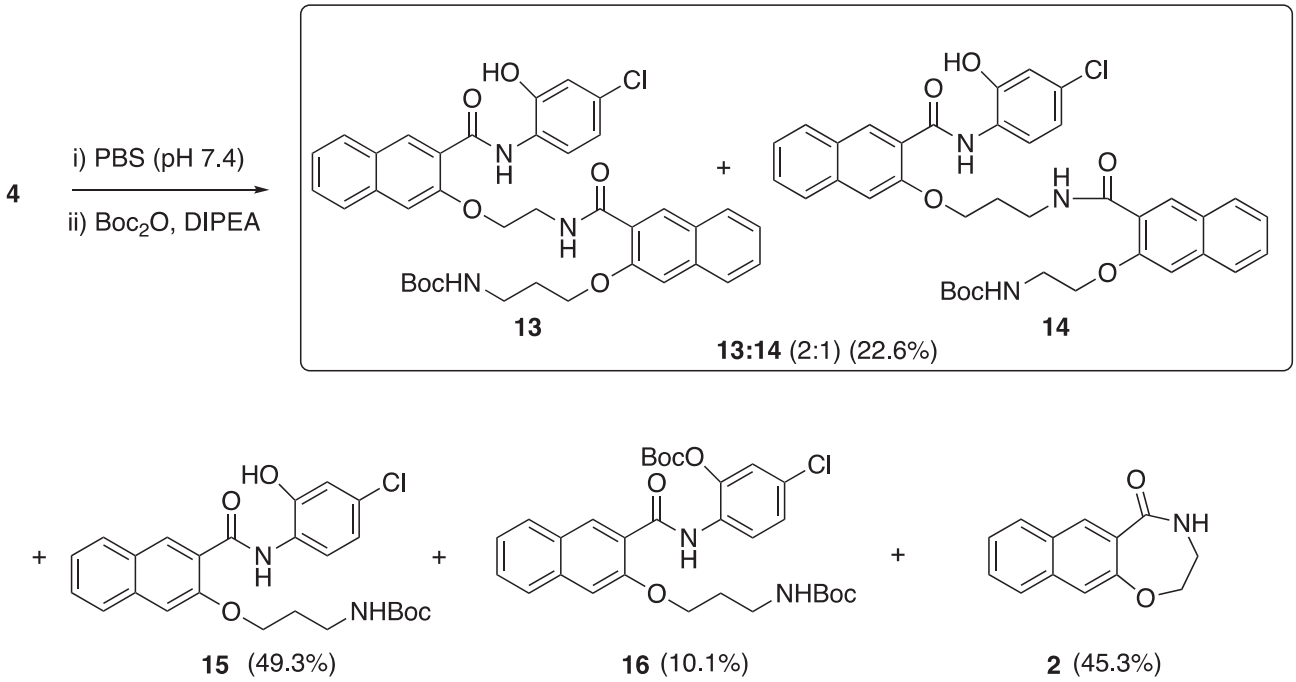


Fig. 1. Compound **4** was converted into **666-15**, **653-47**, **2** and **5** in complete tissue culture media. (A) The HPLC traces of the reaction mixtures from incubating **1** or **4** in complete tissue culture media at 37 °C for different periods of time. (B) Proposed reaction pathways for generation of **666-15**, **653-47**, **2** and **5** from **4**.



Scheme 4. Reaction conversion of **4** in PBS.

Table 1
The biological activities of **1**, **4** and **666-15**.[a]

Compound	CREB inhibition IC_{50} (μM)[b]	GI_{50} (μM)[c]	
		MDA-MB-231	MDA-MB-468
1	0.26 ± 0.097	0.54 ± 0.071	0.045 ± 0.025
4	0.25 ± 0.16	0.67 ± 0.15	0.032 ± 0.028
666-15[d]	0.081 ± 0.04	0.073 ± 0.04	0.046 ± 0.04

[a]The stock solutions of compounds **1** and **4** were made in DMF while **666-15** was dissolved in DMSO. The use of DMF was necessary for **1** and **4** because these two compounds were found to be unstable in DMSO. [b]The CREB inhibition IC_{50} refers to the concentration needed to inhibit the CREB-mediated gene transcription reporter assay in HEK293T cells by 50%. The IC_{50} values are presented as mean ± SD of at least two different experiments performed in triplicates. [c]The GI_{50} refers to the concentration needed to inhibit the cancer cell growth by 50% in an MTT assay after incubating the cells with the compounds for 72 h. The GI_{50} values are presented as mean ± SD of at least two different experiments performed in duplicates. [d]These values are from reference[16].

than MDA-MB-231 cells, suggesting that the extent of synergistic effect between **666-15** and **653-47** may be cell type-dependent, the mechanism of which remains to be established.

CREB has been investigated as a promising cancer drug target. We previously identified **666-15** as a potent CREB inhibitor with efficacious in vivo anticancer activity. In an effort to improve the aqueous solubility of **666-15** using prodrugs, we recently reported the design of prodrug **1**,[19] which revealed unexpected synergistic effect of the combination between **666-15** and **653-47**. This unexpected discovery also challenged our initial proposal of the transformation from **1** to **666-15** through a long-range *O*,*N*-acyl transfer process.[19,20] Instead, we proposed a modified mechanism of conversion through imide intermediate **3** (Scheme 1). In our initial proposal for the long-range *O*,*N*-acyl transfer process, the compound designed contained the same side chains that would make it hard to distinguish the two mechanisms of conversion (Scheme S1). In the current design of **1** and **4**, the differences in the side chains (2-carbon and 3-carbon) allowed us to provide new insights into the mechanism of conversion. Although the formation of imide intermediate **3** could be from the amide oxygen attacking the ester bond as shown in Scheme S2, our findings that both **1** and **4** generated same mixture suggested that **S4** and **S5**, if formed transiently, would be converted into **3**. In summary, the synthesis, hydrolysis studies, biological characterization of **1** and **4** fully support that the long-range *O*,*N*-acyl transfer process is an unlikely event and transformation via imide intermediate **3** is mostly plausible. These results provide unique insights into the activation of these ester prodrugs and further suggest new strategies to design prodrugs of **666-15**.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

The Supporting Information is available: Detailed experimental procedures, Scheme S1-S2, HPLC chromatograms, dose-response curves, qRT-PCR data, NMR spectra (PDF). Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127455.

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