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Evaluation of a 7-methoxycoumarin-3-carboxylic acid ester derivative as a fluorescent, cell-cleavable, phosphonate protecting group

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Abstract

Cell-cleavable protecting groups often enhance cellular delivery of species that are charged at physiological pH. Although several phosphonate protecting groups have achieved clinical success, it remains difficult to use these prodrugs in live cells to clarify biological mechanisms. Here we present a strategy that uses a 7-methoxycoumarin-3-carboxylic acid ester as a fluorescent protecting group. This strategy is applied to synthesis of an HMBPP analogue to assess cellular uptake and human V γ 9V δ 2 T cell activation. The fluorescent ester displays low cell toxicity (IC₅₀ >100 μ M) and strong T cell activation (EC₅₀ = 0.018 μ M) relative to the unprotected anion (EC₅₀ = 23 μ M). The coumarin-derived analogue allows no-wash analysis of biological deprotection, which reveals rapid internalization of the prodrug. These results demonstrate that fluorescent groups can be applied both as functional drug delivery tools and useful biological probes of drug uptake.

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Keywords

Antigens; Butyrophilin; Coumarin; Phosphorus; Prodrugs

A variety of drugs and other biologically interesting molecules contain phosphate or phosphonate substructures.^[1] However, the negative charge that such compounds bear at physiological pH frequently acts as a barrier to cellular entry.^[2] As such, development of cell-cleavable protecting groups that increase in vivo absorption is of great interest, with several phosphonate “prodrugs” recently achieving clinical status.^[3] While cell-cleavable phosphoester^[4] and phosphonamidate^[5] protecting groups effectively increase cellular uptake,^[6] it remains challenging to use these compounds to assess biological mechanisms in real-time because concentrations of compounds in live cells and their uptake rates cannot be readily assessed.

The small isoprenoid (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (**1**, HMBPP, Figure 1) is a metabolic intermediate found in bacteria and other microorganisms.^[7] HMBPP synthesis is required for bacterial growth^[8] and it also functions as a potent pathogen-associated molecular pattern (PAMP) that stimulates an immune response from human V γ 9V δ 2 T cells.^[9] However, the mechanisms of HMBPP immunostimulation are currently a topic of intense debate^[10] and novel activators of V γ 9V δ 2 T cells are desirable.^[11] Therefore, development of HMBPP analogues which can contribute to understanding its immunostimulatory mechanism is warranted.^[12]

Here, we present a pH-sensitive, fluorescent, cell-cleavable phosphonate protecting group, the oxymethyl ester of 7-methoxycoumarin-3-carboxylic acid, to effectively quantify cellular uptake of a phosphonate HMBPP analogue. Compounds containing this group display potent activation of V γ 9V δ 2 T cells. Moreover, this reporter may also provide a means by which cellular uptake of a variety of phosphate and phosphonate drugs can be readily assessed with some precision.

The initial goal of this synthetic effort was to generate a fluorescent prodrug. We have applied the commonly-used^[3] non-fluorescent pivaloyloxymethyl (POM)^[13] protecting group to both phosphonates^[14] and bisphosphonates^[15] and observed strong gains in cellular potency, but lacked ways to easily quantify uptake in live cells. Here, we focused on a derivative of the HMBPP analogue (*E*)-4-hydroxy-3-methyl-but-2-enyl phosphonate (**2**, C-HMBP). We viewed C-HMBP as an ideal molecule to use in this regard because: 1) it has strong biological activity in a system that requires intracellular delivery; 2) it displays only weak cellular toxicity; 3) the mechanism(s) of action of its butyrophilin binding partner BTN3A1 remain unclear; and, 4) the POM-protected analogues **3** and **4** already were available for use as biological controls. Thus, we would be readily able to assess changes in potency/toxicity of the protecting strategy. Furthermore, in contrast to other phosphonates that require full deprotection for biological activity, this compound is biologically active as the mono-acid analog **8**. Therefore, rates of mono-hydrolysis of varied protecting strategies can be directly compared in cellular assays without the complexities of di-hydrolysis.

The synthetic sequence (Scheme 1) began with preparation of 7-methoxycoumarin-3-carboxylic acid (**5**) by the known condensation of 2-hydroxy-4-methoxybenzaldehyde with Meldrum's acid.^[16] This carboxylic acid was treated with base and chloromethyl chlorosulfate according to the procedure of Graham et al.^[17] to obtain the chloromethyl ester **6**. To determine if this coumarin derivative would be cleaved within a cell to release a phosphoantigen, we prepared a derivative of the phosphonate **7**, which we have found does not itself stimulate V γ 9V δ 2 T-cells.^[14] To obtain the desired derivative, the dimethyl ester **7** was treated with one equivalent of DABCO to obtain the mono ester mono salt **8**.^[18] This salt was allowed to react with the chloromethyl ester **6** in the presence of NaI, conditions intended to form the more reactive iodomethyl ester *in situ*. In refluxing acetonitrile, this led to formation of the desired mixed ester **9** in reasonable yield. Compound **9** was fluorescent as expected, and readily detected with maximum excitation at 355 nm and emission at 405. Therefore it was examined in a variety of biological experiments to determine if it would function as a prodrug.

We determined the activity of compound **9** in a functional assay for its ability to stimulate proliferation of primary human V γ 9V δ 2 T cells (Figure 2). Peripheral blood mononuclear cells were treated with test compounds and expanded. In these assays, compound **9** was expected to undergo cellular metabolism leading to intracellular release of the carboxylic acid **5** and the biologically active compound **8** (Figure S1). Compound **4** was used as a control because it too would be expected to deliver active compound **8** with similar stoichiometry. Both compound **4** and compound **9** did function as V γ 9V δ 2 T cell agonists, causing a large expansion of the population of cells that express both CD3 and the V γ 9V δ 2 T cell receptor (Fig 2A). This is a key finding because it demonstrates that the coumarin-derived protecting group can effectively deliver a phosphonate payload, resulting in biological activity.

The maximal agonist activity (observed at 1 μ M) of compound **9** was not statistically different from that of POM analogue **4** (Figure 2B) in cells from the same donors. Dose response curves (Figure 2C) determined that compound **9** displays an EC₅₀ of 0.018 μ M while compound **8** displays an EC₅₀ of 23 μ M. Therefore the coumarin-carboxylate oxymethyl ester (CCOM) strategy offered a 1300 fold increase in activity. The potency of compound **9** compares favorably to the EC₅₀ value of 0.50 μ M that we obtained previously for compound **4** (Table S1), although availability of these primary human cells dictated the current set of compounds be tested in cells from different donors as earlier compounds. This data shows minimal differences between the CCOM and POM protecting groups of a matched pair of compounds, suggesting that the CCOM protecting group has similar prodrug functionality to that of the well-established and clinically-utilized POM protecting group.

Because the previous experiments utilized 3 day exposure, the assays may not have been sensitive enough to assess subtle differences in the rates of phosphonate release. Therefore, we sought to assess the activity of the novel compounds in a model of T cell mediated cytotoxicity which occurs with a shorter exposure of just 2 hours (Figure 2D). K562 cells that were pre-loaded with compound **4** and **9** were able to trigger V γ 9V δ 2 T cell mediated killing with similar efficacy.

The ideal prodrug protecting group would be non-toxic. We expected that to be the case for CCOM protection, as the activity of compound **9** in the T cell proliferation assay was similar to that of POM compound **4**. To establish the effects of the CCOM protecting group on cell viability, we evaluated growth inhibition in several cell lines. The carboxylic acid **5** alone was non-toxic to K562, Daudi, RPMI-8226, and Jurkat cells when they were exposed for 72 hours at concentrations up to 100 μ M (Table S2). Treatment of K562, Daudi, or Jurkat cells with compound **4** or **9** displayed no toxicity. Differences were observed between compound **4** and **9** in only in RPMI-8226 cells at the 100 μ M treatment (Figure S2), much higher than the EC₅₀ values for stimulation of V γ 9V δ 2 T cells.

We next determined whether compound **9** would be a useful probe with which to determine rates of biological deprotection (Figure 3). Compounds **5** and **9** were readily detected by spectrofluorimetry in aqueous solutions. Importantly, the fluorescence intensity of compound **5**, but not the prodrug **9**, was strongly dependent upon pH of the solution (Figure 3A), correlating with the expected protonation status of the carboxylic acid in compound **5**. At pH 6 the intensity of compound **5** was less than 10% of its intensity at pH 3 (Figure 3A). Therefore, the CCOM prodrugs would be expected to rapidly lose fluorescence intensity during metabolism at neutral pH, as the carboxylate is released from the prodrug and remains in the deprotonated form.

We then adapted this assay to a 384-well-plate format for use in a plate reader equipped with a 355/40 nm excitation filter and a 405/10 nm emission filter. As expected, the fluorescence intensity of compound **9** in PBS was much higher than that of compound **5** in PBS (Figure 3B). Additionally, the fluorescence of compound **5** was further reduced when incubated in human plasma (Figure S3). The loss of fluorescence in plasma was not due to metabolism of the coumarin, as analysis by thin layer chromatography of plasma extracts under UV light confirmed that this group had not been destroyed and further spectral analysis did not identify a shift in the excitation or emission spectra. Therefore, the esterase-mediated hydrolysis of compound **9** could be readily assessed by quantification of the two-step (release followed by quenching) loss of fluorescence following exposure to various biological matrices. Importantly, this measurement could be done directly in a single plate without washing or further assay steps.

In the presence of human plasma the concentration of compound **9** decreased over time (Figure 3C) with 2nd order kinetics. The half-life was determined to be 6 minutes in this assay. In order to confirm that the loss of fluorescence of compound **9** was due to hydrolysis of the CCOM-phosphonate ester and not fluorescence destruction, we extracted the end products and analyzed by thin layer chromatography (Figure 3D). As expected, compound **9** was fully hydrolyzed to yield the free carboxylic acid in a time dependent manner. Loss of the prodrug occurred at the same time as the appearance of the free acid. The enzymatic release occurred with a half-life of 7.8 minutes in this assay. The rate of hydrolysis in plasma is similar to some bis-POM compounds [19]. This is an important finding because it indicates that the presence of a bicyclic aromatic group does not prevent enzymatic hydrolysis of the phosphonate ester nor does it permit rapid non-enzymatic degradation.

We used compound **9** to examine internalization into K562 cells in PBS. No loss of fluorescence was observed in PBS (Figure 3E). Surprisingly, K562 cells could rapidly decrease the extracellular concentration (Figure 3F). This occurred with first order kinetics and a half-life of 55 minutes. While our current data cannot rule out the possibility that the K562 cells secreted an esterase, we believe these findings demonstrate the striking efficacy of phosphonate ester prodrugs to deliver their payload. Within hours effectively all of the drug can pass into the cells.

In conclusion, we report the synthesis of a novel fluorescent pH-dependent cell-cleavable protecting group and its application to an HMBPP analogue. These results show that bulky fluorescent systems can be tolerated by cellular esterases without loss of activity or generation of toxicity relative to the commonly-used POM protection. Fluorescent prodrugs^[20] are useful to detect the speed of cellular uptake in a way that is much faster and requires significantly smaller volumes of cells and materials relative to radiolabeling^[21] or HPLC^[22] approaches, making them amenable to high-throughput applications. While our current results are focused on release of the mono-acid form, we predict a parallel strategy could be used to modify phosphonates to release the di-acid form of compounds that require full deprotection for biological activity.

Experimental Section

Biological Procedures: Cells were expanded from human peripheral blood mononuclear cells in fresh T cell media (RPMI-1640, 10% heat-inactivated FBS, 1x HEPES, pyruvate, non-essential amino acids, penicillin/streptomycin, beta-mercaptoethanol) and added to 6-well plates. Cells were stimulated with test compounds for three days. Cells were washed twice then cultured for another eleven days after compound removal. Human interleukin 2 (5 ng/mL) was supplemented every three days. Experiments were performed at least three times independently using at least two different blood donors. In lysis assays, cells that had been expanded with 10 μ M HMBPP and purified by negative selection (Miltenyi) were used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
HMBPP and some biologically active analogues.

Figure 2.

Compound **9** is a potent Vγ9Vδ2 T cell agonist. A) PBMCs were stimulated with compound **9** and phenotyping was performed to quantify the percentages of cells expressing both the Vγ9Vδ2 TCR and the pan T cell marker CD3. Compound **9** was evaluated at a concentration (1 μM) previously shown to be maximal for compound **4**. Data is representative from 3 independent experiments. B) Quantification of Vγ9Vδ2 T cell proliferation in response to compounds **4** and **9**, n=3. C) Dose response curves for compound **9**. D) Induction of Vγ9Vδ2 T cell mediated K562 lysis by compound **4** or **9**.

Figure 3.

A) Fluorescence spectra of compound **9** and compound **5** in water or citrate buffers at various pH values. B) Intensity of 10 μM compound **9** and **5** in PBS pH 7.6. C) Metabolism in plasma was determined by plate reader or D) thin layer chromatography. E) Stability in PBS. F) Rate of uptake into K562 cells.

Scheme 1.
Synthesis of the fluorescent phosphoantigen **9**.