

## Tubulin Photoaffinity Labeling Study with Biotin-Tagged Diketopiperazine-Based Anti-Microtubule Agents

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### Introduction

The introduction of anti-microtubule agents such as taxanes and *vinca* alkaloids has revolutionized cancer treatment and improved patient survival time. However, tumors become resistant to these drugs after long-term treatment. Hence, there is a significant need to develop novel anti-microtubule agents.

As one of such candidates, natural diketopiperazine, phenylalhistin (PLH, halimide) exhibiting colchicine-like anti-microtubule activity [1, 2], has been our focus. We have succeeded in total synthesis of PLH, and performed a structure activity relationship (SAR) study of its derivatives [3]. From this SAR study, a highly potent derivative NPI-2358 (**1**, IC<sub>50</sub> = 15 nM against HT-29 cells) was developed. Additionally, it was recently shown that NPI-2358 functions as a strong "vascular disrupting agents (VDAs)" to induce tumor-selective vascular collapse [4]. The therapeutic potential targeting the tumor vascular supply is now widely recognized. Therefore, NPI-2358 is a valuable compound for an antitumor drug, which is currently in Phase I clinical trial in the US.

Although NPI-2358 is suggested to recognize regions around the colchicine binding site on tubulin [5], its three-dimensional structure could not be superimposed over that of colchicine. In order to understand the precise binding mode of NPI-2358, we decided to develop its biologically active photoaffinity probe. Photoaffinity labeling is well-known as a powerful tool to provide valuable information about the topology of the ligand-binding site on the target proteins [6].

Here, we describe the design and synthesis of a photoaffinity probe and its photolabeling into tubulin.

### Results and Discussion

We have established the synthetic route of NPI-2358 and its derivatives. Until now, more than 100 analogues were prepared and screened by HT-29 cytotoxicity assay. Then, a SAR study was performed. As a result, several highly potent derivatives were developed. One of these compounds is KPU-244 (**2**), having a benzophenone structure, which is often used photoaffinity labeling as a photo-reactive group. In cytotoxicity assay, KPU-244 exhibited highly potent activity (IC<sub>50</sub> = 4 nM against HT-29 cells).

Therefore, we designed biotin-tagged KPU-244, KPU-244-B1 (**3**), as a photoaffinity probe [7], which can be photo-reactive and detected by an avidin-peroxidase system. In the synthesis of KPU-244-B1, a biotin-tag was successfully connected at the 4' position of the benzophenone moiety.

Table 1. Tubulin binding and cytotoxicity of compounds.

Compound	K <sub>d</sub> (μM)	IC <sub>50</sub> (HT-29 cells)
NPI-2358	1.46 ± 0.15	14.9 ± 3.8
KPU-244-B1	7.95 ± 1.22	N.D. <sup>a</sup>
colchicine	3.32 ± 0.31	16.2 ± 3.0

<sup>a</sup> N.D. : Not determined.

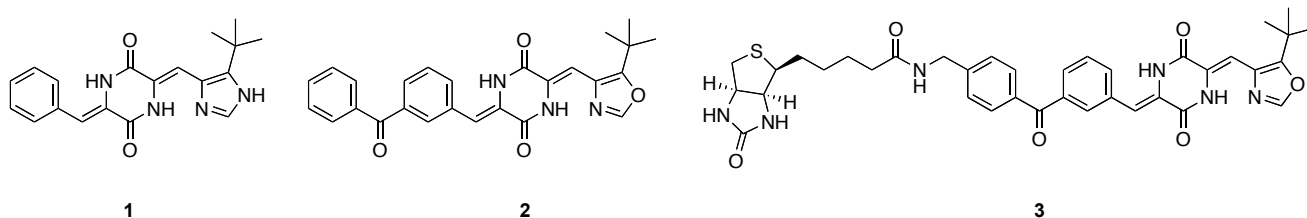


Fig. 1. Structure of NPI-2358 (**1**), KPU-244 (**2**) and KPU-244-B1 (**3**).

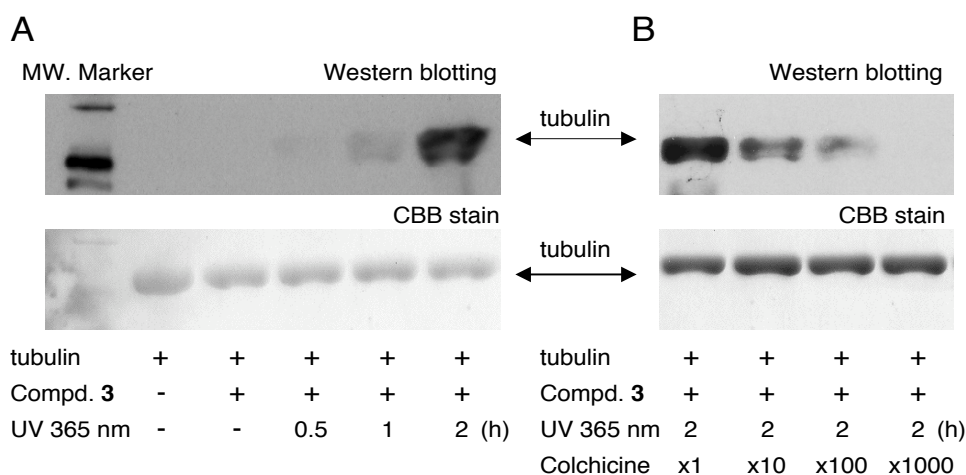


Fig. 2. Photoaffinity labeling of KPU-244-B1 to tubulin.

Firstly, to investigate the biological activity of biotin-tagged photoaffinity probe **3**, we performed a binding assay to tubulin according to the tubulin's intrinsic fluorescence change caused by the binding of compounds. The dissociation constant ( $K_d$ ) of KPU-244-B1 to tubulin (0.5  $\mu$ M) at 37  $^{\circ}$ C was calculated to be 7.95  $\mu$ M, which was about 2 and 5 times lower affinity than those of colchicines (3.32  $\mu$ M) and NPI-2358 (1.46  $\mu$ M), respectively. This result indicated that probe **3** at least possesses a significant binding ability to tubulin. Additionally, KPU-244-B1 (5  $\mu$ M) exhibited 30 % inhibition of the tubulin polymerization and cytotoxic activity against HT-29 human colon cancer cell lines with  $IC_{50}$  value of 0.91  $\mu$ M. These results suggest that KPU-244-B1 is able to function as the biologically active photoaffinity probe.

Therefore, secondly, tubulin was photo-irradiated at 365 nm in the absence or presence of KPU-244-B1 under physiological conditions. An aliquot of sample solution was applied to SDS-PAGE and electrically blotted onto nitro-cellulose membrane, followed by detection using the avidin-biotin system. A specific and irradiation-time-dependent labeling was observed (Fig. 2A). Non-specific labelling was not observed without photo-irradiation. Under photo-irradiation, irradiation time dependent labeling was observed. Additionally, this labeling was relatively selective to beta-tubulin over alpha-tubulin on condition that alpha and beta-tubulin were separated by SDS-PAGE (data not shown). Moreover, this labeling was also dose-dependently inhibited by a colchicine addition (Fig. 2B).

These results suggest that KPU-244-B1 specifically and covalently binds near to the colchicine binding site on beta-tubulin. It is also suggested that KPU-244-B1 recognizes not only the colchicine binding site, but also the interfacial space between alpha- and beta-tubulins. These results would lead to further understanding of the precise binding site of NPI-2358 and its depolymerization mechanism towards microtubules.

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