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Photoactivatable small-molecule inhibitors for light-controlled TAM kinase activity

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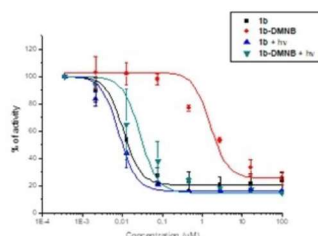
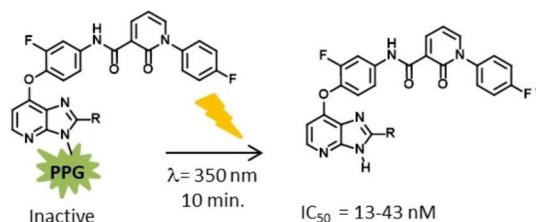
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Supporting information for this article is given via a link at the end of the document.

Abstract: The TAM kinase family arises as a promising therapeutical target for cancer therapy, auto-immune, and viral diseases. In this study, we report the first photoactivatable caged inhibitors of Tyro3 and Mer. This strategy enables spatial and temporal control of the biological activity of the inhibitor upon irradiation with UV light. We describe the design, the synthesis, the photocleavage properties, and the inhibitory activity of four Tyro3 and Mer photoactivatable small molecules. The proof of concept on the TAM kinase family was achieved *in vitro*, since irradiation by UV light restored the full inhibitory activity of two prodrugs.

Graphical Abstract:



TAM-light: The design, synthesis, photocleavage properties and inhibitory activities of the first four photoactivatable small molecules for controlling TAM kinase activity with light are reported

Since the approval of the tyrosine kinase inhibitor imatinib (Gleevec) in 2001, protein kinases have been recognized as valuable therapeutic targets. Almost 48 small-molecule kinase inhibitors have been approved by the Food and Drug Administration and reached the market for the treatment of malignancies and non-malignancies.[1] Despite this success, the poor drug selectivity involving side-effects and resistance issues are still major drawbacks. Achieving selectivity proves to be challenging since most of the pharmacological targets are constitutively expressed in both healthy and diseased tissues leading to uncontrolled drug activity in time and space throughout the organism. In order to circumvent the issues surrounding poor drug selectivity, the development of innovative therapeutic approaches for dynamically controlled drug activity would be of significant benefit for the patients.

Various strategies have been developed for triggering drug release by external stimuli, including light which is a particularly attractive source of stimulation.[2] Indeed, light can be readily available and focused, allowing the precise temporal and spatial definition of the stimulus. Photodynamic therapy is the most developed and applied approach combining light and therapeutic agents. Beyond that, the photopharmacology approach that relies on photochemical processes including photoswitches and photoremovable protecting groups (PPG) is an ever-growing field.[3],[4] The PPG strategy consists of covalently attaching a photoremovable protecting group on the active molecule to block one of its key positions, essential for its interaction with the protein. Hence, the PPG renders the molecule temporarily inactive, however upon irradiation, it is cleaved and the again active drug is released.[5] Despite the tremendous amount of biologically active compounds which has been controlled by either photoremoval or photoswitch, the kinase field has just started to be explored.[6] Only a few photoswitchable kinase inhibitors have been described in the literature with examples of inhibitors for Ret kinase[7], protein kinase C[8], MEK1[9], VEGFR- 2[10], or BRAFV600E.[11] The PPG strategy was also applied to a few kinase inhibitors with photoactivation examples for PKA[12], Rho kinase[13], Ret kinase[14], VEGFR-2 protein[15], and CKI[16]. The group of Peifer also developed photoactivatable versions of two marketed kinase inhibitors, imatinib[17] and vemurafenib.[18] Amongst the 518 proteins of the human kinome[19], the TAM family consists of three transmembrane tyrosine kinases: Tyro3, Axl, and Mer.[20] These receptors are overexpressed in many different types of cancer, such as myeloid leukemia, colon, liver, prostate, and breast cancers.[21] They are also involved in multiple sclerosis, other autoimmune reactions and viral infections (Zika, Ebola, Dengue virus and SARS-Cov-2).[22],[23] Only a few inhibitors have been specifically designed for these receptors, with a major interest for Axl and Mer, highlighting the potential of these biological targets from a drug discovery perspective.[24] As part of an ongoing project aiming to develop novel TAM kinases inhibitors, we recently published a small library of 2,6-disubstitutedimidazo[4,5-*b*]pyridines displaying highly potent TAM activity in the nanomolar range (Figure 1A).[25] Based on these

results, we decided to develop a photoactivatable version of our hits to explore them as valuable tools in the ever growing field of photopharmacology. We chose to apply the PPG strategy over the photoswitch approach, since the former seems more versatile to us and often allows a more significant difference in the inhibitory activity between the active molecule and the caged compounds.

In this communication, we first describe the design of the photoactivatable inhibitors based on molecular modeling. Then, we detail the synthesis of the inhibitors bearing the PPGs, also called caged inhibitors (Figure 1B). Finally, we present the photophysical properties of the synthesized compounds, along with their biological activities, in an enzymatic assay, on Tyro3 and Mer.

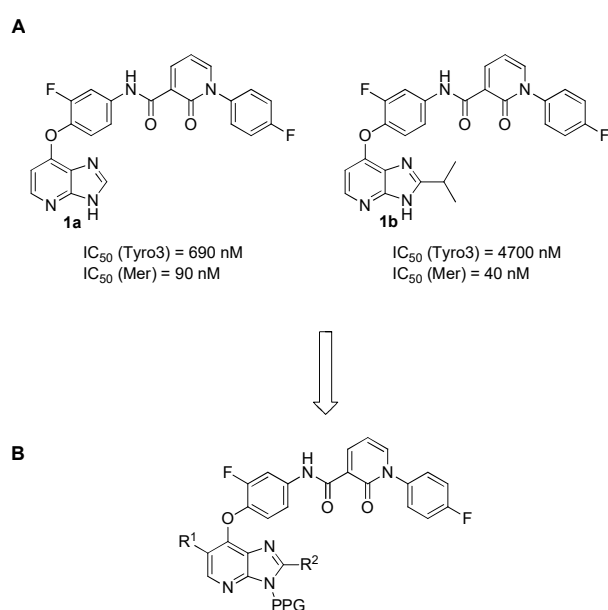


Figure 1. A) In-house TAM inhibitors. B) Caged inhibitors.

To determine which positions of our inhibitors **1a** and **1b** need to be blocked by PPGs, docking experiments of the inhibitors were performed within the crystal structures of the TAM proteins. Only the active conformations of the proteins were considered for docking in order to be comparable to the experimental settings, in which the enzymatic tests are performed on the activated proteins (PDB IDs: 3QUP, and 3BRB, for Tyro3, and Mer, respectively).[26] The ATP pocket of these kinases was targeted, which is very well conserved for the three TAM proteins, with a difference of only one residue (Ala591 in Tyro3, is replaced with Ile650 in Axl and Met598 in Mer). The docking poses of the inhibitors revealed a strong hydrogen bond between the N3 of the imidazopyridine and a methionine residue of the hinge region (Figure 2). Another strong interaction takes place between the nitrogen of the amide pyridone chain and an asparagine residue. Therefore, both positions would be considered suitable for bearing a PPG that should

temporarily inactivate the parent compound. However, we chose to attach a PPG to the N3 nitrogen of imidazopyridine for two reasons: first, for synthesis facility since the imidazopyridine's nitrogen can be easily alkylated as the last step allowing versatility with respect to the nature of the photolabile group; second, this N3 atom is well buried inside the cavity so that the protein is not expected to accommodate a PPG at this position leading most likely to the desired loss of biological activity.

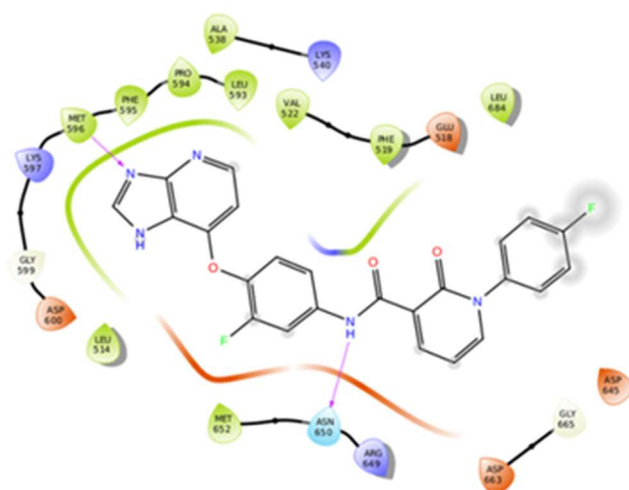
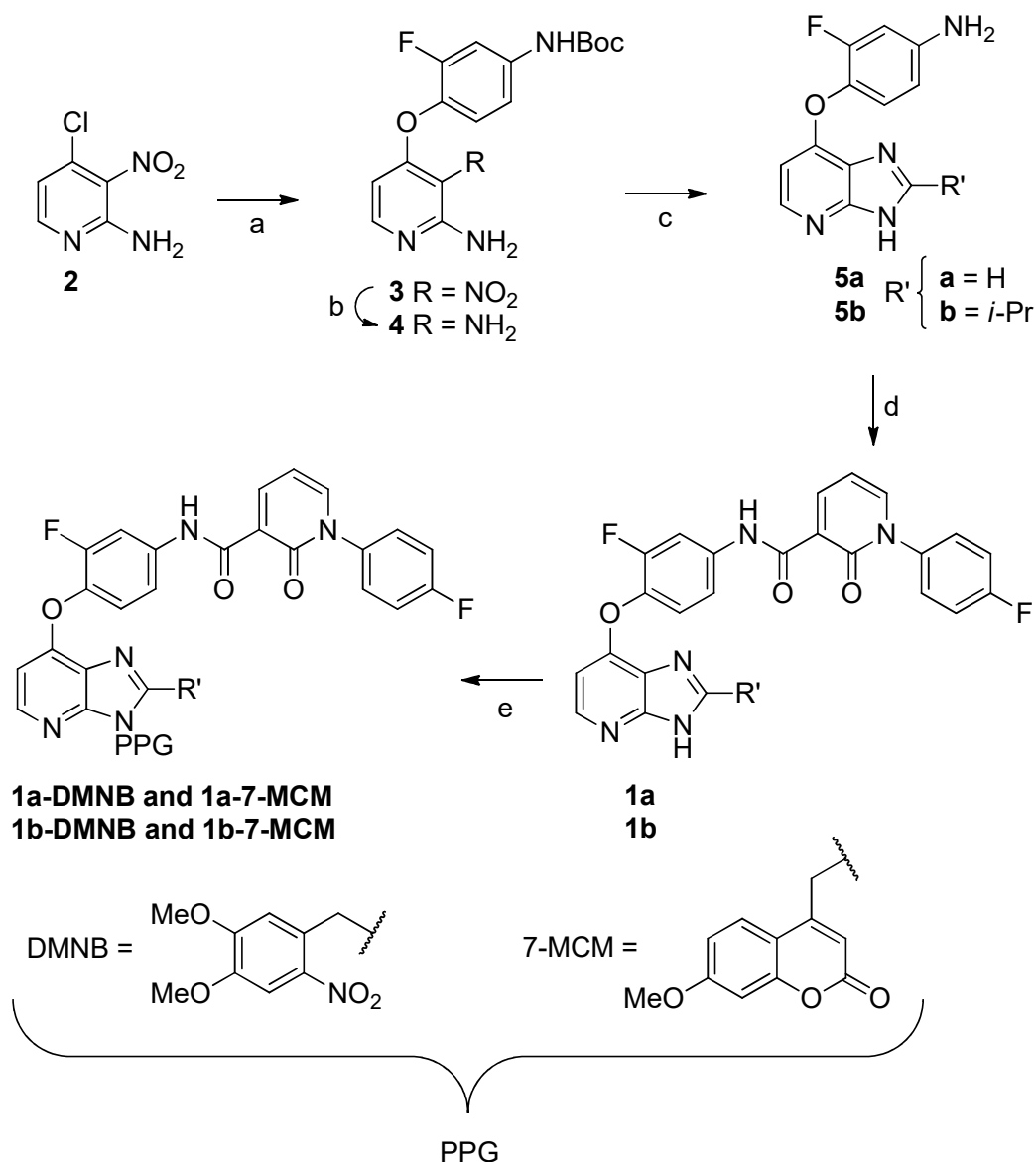


Figure 2. Model ligand interaction diagram of **1a** in the active site of Tyro3 crystal structure (PDB ID: 3QUP). Key ligand-protein interactions are shown (pink arrows = hydrogen bonds; grey dots = solvent exposure).

We chose two of the most described PPGs in the literature, the 4,5-dimethoxynitrobenzyl (DMNB) and the (7-methoxycoumarin-4-yl)methyl (7-MCM).[27] Their interest relies on their properties for two-photon uncaging, permitting the photoactivation with light at wavelengths more compatible with the biological environment.[28] The previous synthetic path for inhibitors **1a** and **1b** proved not suitable and was therefore fully re-investigated. [25] Starting from a different commercially available precursor, our investigations allowed the shortening of the overall synthesis sequence from 6 to 4 steps. Starting from the 2-aminopyridine **2**, nucleophilic aromatic substitution with amino-protected-fluorophenol afforded compound **3** with a very good yield (Scheme 1). The nitro function was then reduced by hydrogenation catalyzed by palladium on activated charcoal. The imidazo[4,5-*b*]pyridine ring was formed by condensation with trimethylorthoformate or isobutyric acid to give **5a** and **5b** in respectively 65% and 55% yield. Condensation with a pyridone carboxylic acid derivative afforded **1a**, and **1b** with excellent yield. Finally, the two PPGs were introduced by nucleophilic substitution to give the desired caged inhibitors. It should be noted that the modest yields at this final step are caused by the lack of regioselectivity of the alkylation leading to regioisomers. Four caged inhibitors

(**1a-DMNB**, **1a-7-MCM**, **1b-DMNB** and **1b-7-MCM**) were obtained following this synthetic path.



Scheme 1. Synthesis of caged inhibitors. a) *tert*-butyl (3-fluoro-4-hydroxyphenyl)carbamate, *t*-BuOK, DMF, 70°C, 16 h, 85 % ; b) H₂, Pd/C, MeOH, rt, 16 h, 100 % ; c) R = H : trimethylorthoformate, HCl, rt, 16 h, 65 % ; R = *i*-Pr : isobutyric acid, PPA, 150°C, 4 h, 55 % ; d) 1-(4-fluorophenyl)-2-oxo-1,2-dihydropyridine-3-carboxylic acid, TBTU, DIPEA, DMF, rt, 16 h, 84 % ; e) PPG-Br, NaH, DMF, 0°C to rt, 16 h, 20-40 %.

To evaluate the photoactivatable properties of the four caged inhibitors, we first recorded their absorption spectra (see SI, Figure S1). The maximum absorbance was reached at 345 nm and 328 nm for the DMNB-protected and the 7-MCM-protected inhibitors, respectively. Therefore,

the use of the Rayonet RPR- 200 system, with a 350 nm lamp was optimal to perform the photocleavage experiments. We irradiated for 10 minutes 10 μ M solutions of the four caged inhibitors in 10% DMSO/ H₂O v/v. Under these conditions, the DMNB group was rapidly photocleaved, in less than 3 minutes, providing the active inhibitors **1a** and **1b** (Figure 3 for **1a** and see SI, Figure S2 for **1b**).

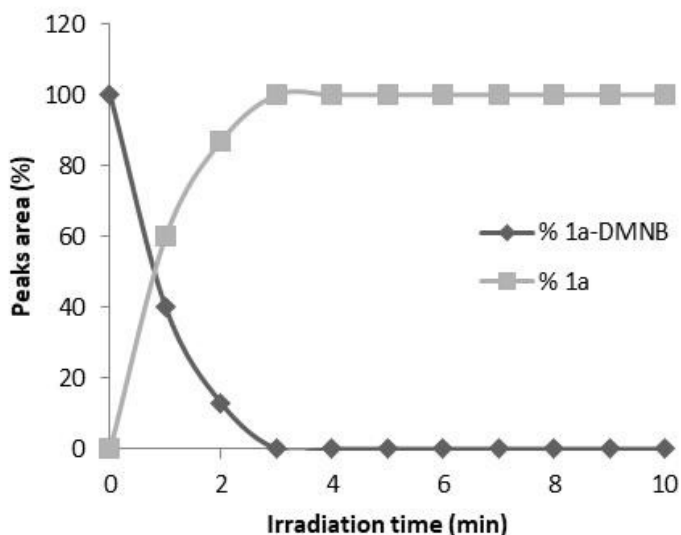
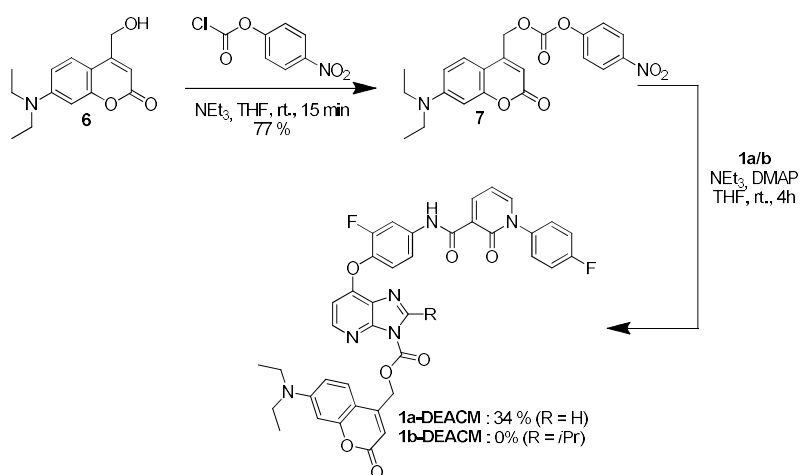


Figure 3. Release of parent inhibitor **1a** by irradiating the caged prodrug **1a-DMNB** with UV-light irradiation. 10 μ M solution of **1a-DMNB** in 10% DMSO/ H₂O v/v) was irradiated for 10 minutes with 350 nm light. The uncaging was examined by LC-MS analysis. The amount of **1a-DMNB** and the released parent inhibitor **1a** are plotted as the area under the LC peaks against irradiation time.

Conversely, the coumarin-caged compounds, **1a-7-MCM** and **1b-7-MCM**, could not be demasked upon irradiation in these conditions even after a prolonged time of 1 hour (see SI, Figure S3). Indeed, poor leaving groups, such as amine derivatives, are usually known to be resistant to photolysis. Therefore, the compounds **1a-7-MCM** and **1b-7-MCM** were modified by grafting the coumarin-PPG via a carbamate instead of a benzyl linkage. Indeed, carbamate linkage provides more efficient photocleavage since the liberated carbamic acid is unstable and undergoes decarboxylation to give the free amine.[27b] The 7- diethylamino-7-hydroxymethylcoumarin **6** was prepared according to the Klausen procedure.[29] The reaction with 4-nitrobenzyl chloroformate led to the formation of carbonate **7** in good yield (Scheme 2). This carbonate then reacted with the inhibitor **1a** to form [7-(diethylamino)coumarin-4-yl]methyl (DEACM) carbamate-caged compound **1a-DEACM** in 34% yield. Conversely, the reaction with **1b** could not proceed, probably due to the steric hindrance of the isopropyl group on the position 2 of the imidazopyridine moiety.



Scheme 2. Synthetic route for coumarin-carbamate-caged inhibitors.

Photocleavage experiments were then conducted on **1a-DEACM**. The maximum absorbance was reached at 360 nm, so we irradiated the compound in 10 μM solution in 10% DMSO/ H₂O v/v with 350 nm light. To our satisfaction, photodeprotection occurred completely in 2 minutes (see SI, Figure S4).

The prerequisites of photodeprotection are that 1) the caged molecule should not be deprotected under natural light, 2) the active molecule should not be degraded by UV light after its release from the caged molecule, and 3) the targeted protein keeps its activity intact after irradiation. First, the stability of the caged compounds to natural light exposure was investigated to make sure that no active inhibitor was released before starting the irradiation. Both DMNB and 7-MCM caged inhibitors showed excellent stability after 2h of natural light exposure in DMSO but also in the biological buffer (see SI, Figure S5). However, the study of the stability of **1a-DEACM** showed that photodecaging also occurs without UV irradiation. 1h exposure to the natural light of a 10 μM solution of **1a-DEACM** in DMSO or biological buffer led to 50% release of inhibitor **1a** (see SI, Figure S6). Consequently to these instability issues, the derivative **1a-DEACM** was not further explored.

Second, inhibitors **1a** and **1b** showed excellent stability after 1h irradiation at 350 nm (see SI, Figure S7).

Finally, the stability of the enzymes after irradiation was verified. For that, the enzymatic activity on the TAM family kinases was measured using ADP-Glo Kinase Assay from Promega, a luminescence-based assay measuring the amount of ADP formed from ATP conversion by the kinase. However, Axl presented a very low kinase activity of the purchased enzyme, regardless of the supplier or the batch, therefore, it was not suitable for reliable and reproducible results. In the following, our work focused on Tyro3 and Mer only.

Satisfyingly, the kinase activity of the TAM proteins was not altered by irradiation within 1 hour.

Then, in order to verify if the presence of the PPG leads to a loss of potency, the inhibitory activity of the parent compounds **1a** and **1b** and their caged derivatives on these TAM kinases was evaluated, without irradiation. After incubating each compound for 2 hours with the enzymes, their residual kinase activity was checked. Satisfyingly, the loss of potency of the caged molecules was well observed here. On Tyro3 (Table 1), compounds **1a-7-MCM**, **1b-DMNB**, and **1b-7-MCM** were totally inactive (no inhibition at 100 μ M). Compound **1a-DMNB** was 4-fold less active than the initial inhibitor **1a** (Table 1, IC₅₀ = 4393 nM vs 1333 nM for **1a**). On Mer (Table 2), both 7-MCM-caged molecules, **1a-7-MCM** and **1b-7-MCM**, displayed no inhibitory effect whereas **1a-DMNB** was 17-fold less active than the original inhibitor (IC₅₀ = 1968 nM vs 113 nM for **1a**), and **1b-DMNB** was 230-fold less potent than **1b** (IC₅₀ = 3365 nM vs 14.6 nM for **1b**). Note that the IC₅₀ values of **1a** and **1b** in Tables 1 and 2 are slightly different from their values in Figure 1 due to the difference in the biological test used.[25]

Compound	IC ₅₀ without irradiation	IC ₅₀ after irradiation
1a	1333 \pm 908 nM	560 \pm 183 nM
1a-DMNB	4393 \pm 573 nM	1055 \pm 50 nM
1a-7-MCM	> 100 μ M	ND
1b	1360 \pm 651 nM	457 \pm 254 nM
1b-DMNB	> 100 μ M	5535 \pm 2185 nM
1b-7-MCM	> 100 μ M	ND

Table 1. IC₅₀ values of inhibitors and caged compounds on Tyro3 without and with UV irradiation (ADP-Glo Kinase Assay)

We then investigated whether the inhibitory activity of the DMNB-protected compounds on Tyro3 and Mer could be restored under UV irradiation. The caged molecules, **1a-DMNB** and **1b-DMNB**, were incubated with the enzymes and irradiated for 10 minutes at 350 nm. The incubation was continued to reach 2 hours under natural light. For control, the related inhibitors **1a** and **1b** were also tested under the same conditions. For Tyro3 (Table 1 and SI Figure S8), the irradiated caged inhibitor **1a-DMNB** was significantly more potent than the non-irradiated one (IC₅₀ = 1055 nM vs 4393 nM), with IC₅₀ approaching that of **1a** (IC₅₀ = 560 nM). Similarly, the inhibitory activity of the irradiated compound **1b-DMNB** was substantially improved, with an IC₅₀ of 5535 nM. Note that the inhibitory activity of **1a** and **1b** was not significantly affected by the irradiation.

We performed the same experiments on Mer (Table 2). The IC₅₀ of the irradiated inhibitor **1a** (21.0 nM) was slightly lower than that of the non-irradiated **1a** (113 nM), although the enzymatic activity of the enzyme itself was not affected by irradiation alone. This result supports the idea

that the irradiation sensitizes Mer to **1a**. Considering the caged molecules **1a-DMNB** and **1b-DMNB**, their activity was completely restored upon UV irradiation with 13.7 nM for **1a-DMNB** and 43.0 nM for **1b-DMNB** respectively. (Table 2 and Figure 4 for **1b-DMNB**, see SI Figure S9 for **1a-DMNB**).

Compound	IC ₅₀ without irradiation	IC ₅₀ after irradiation
1a	113 ± 6 nM	21.0 ± 11.3 nM
1a-DMNB	1968 ± 612 nM	13.7 ± 11.5 nM
1a-7-MCM	> 100 μM	ND
1b	14.6 ± 1.6 nM	10.2 ± 4.4 nM
1b-DMNB	3365 ± 955 nM	43.0 ± 43.3 nM
1b-7-MCM	> 100 μM	ND

Table 2. IC₅₀ values of inhibitors and caged compounds on Mer without and with UV irradiation (ADP-Glo Kinase Assay)

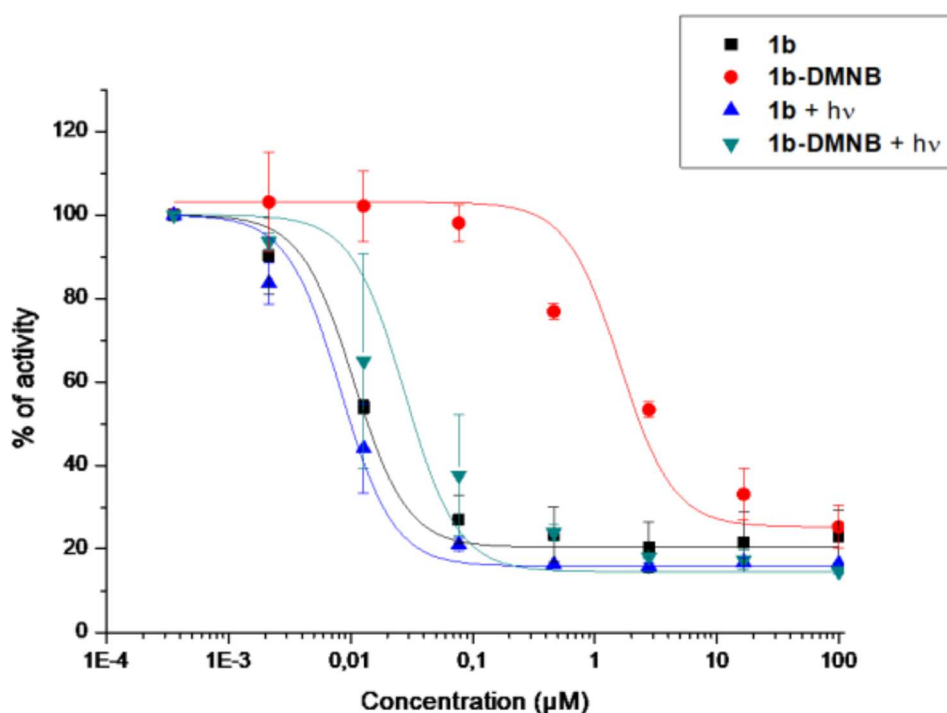


Figure 4. Enzymatic evaluation of **1b** and **1b-DMNB** on Mer with and without UV-light irradiation (350 nm, 10 minutes of irradiation). All biological experiments were duplicated, error bars represent standard deviation.

As shown above, the use of coumarin-based caging group lead to a complete loss of inhibitory activity whereas this was not systematically the case with the DMNB group. To better

understand these results, we performed docking experiments of our caged compounds in the active sites of Tyro3 and Mer. The docking experiments on Mer revealed that none of the four caged inhibitors enter into the active site of the protein. Similarly, the docking experiments on Tyro3 showed that molecules **1b-DMNB**, **1a-7-MCM** and **1b-7-MCM** do not enter into the ATP pocket. These results are in good agreement with the observed complete loss of activity of these compounds.

Conversely, **1a-DMNB**, the only caged molecule with a residual inhibitory activity on Tyro3, was able to adopt a satisfying pose in the active site of this protein (Figure 5). In this pose, the DMNB group enters deeply in the ATP pocket, where its nitro function establishes a hydrogen bond with the backbone of the methionine residue of the hinge region. The amide-pyridone moiety of **1a-DMNB** is also involved in three hydrogen bond with residues of the cavity and the terminal phenyl establishes a π -cation interaction with a lysine residue. All these stabilizing interactions with the residues of the cavity may explain the residual inhibitory activity observed for **1a-DMNB** on Tyro3.

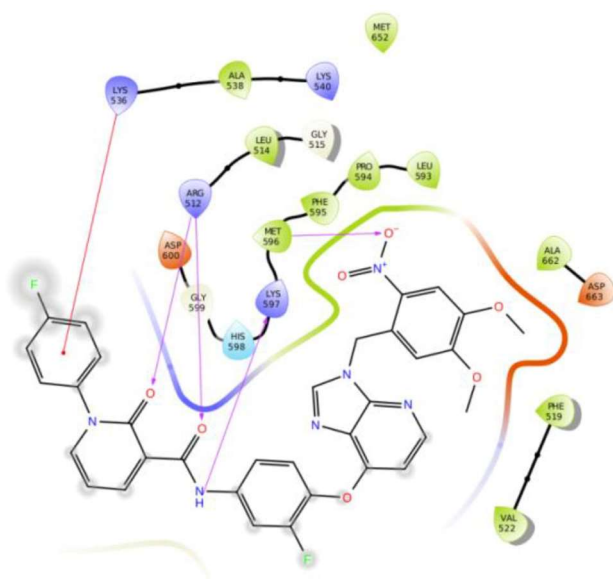


Figure 5. Model ligand interaction diagram of **1a-DMNB** in the active site of Tyro3 crystal structure (PDB code: 3QUP). Key ligand-protein interactions are shown (pink arrows = hydrogen bonds; grey dots = solvent exposure, red lines = π -cation interactions).

However, although **1a-DMNB** establishes more hydrogen bonds with Tyro3 than the parent inhibitor **1a** (Figure 2), it is less well embedded into the ATP pocket than **1a**, due to its larger size, resulting in a much larger hydrophobic surface exposed to the solvent. This may explain why **1a-DMNB** is less active than **1a**, as observed in Table 1. Note that, no satisfactory pose was found for **1b-DMNB** in the cavity of Tyro3, because **1b** is slightly bigger than **1a** due to the

presence of the isopropyl group. And no pose was found either for **1a-DMNB** in the cavity of Mer, despite the sequence similarity with the cavity of Tyro3, because the 3D structure of the cavities are different due to the different relative orientations of the N- and C-terminal domains of the two proteins (see SI, Figure S9). The modeling results show that, whereas the MCM group is bulky enough to prevent the molecule from binding to the ATP cavity, the size of DMNB is on the edge, depending on the size of the initial inhibitor or the conformation of the active site.

In conclusion, we presented the synthesis, the photochemical characterization, and the *in vitro* biological activity of the first photoactivatable inhibitors of the TAM kinase family. The presence of a PPG on the nitrogen N3 of the imidazopyridine induces a significant or a complete loss of the inhibitory activity. In addition, the nature of the PPG and the linkage have a relevant impact on the photostability and photocleavage properties highlighting in our case the DMNB group as the best photoactivatable group. Satisfyingly, the inhibitory activity of DMNB-protected inhibitors **1a-DMNB** and **1b-DMNB** could be substantially improved on Tyro3 and entirely restored on Mer upon a short time irradiation. This validates the application of photopharmacology concept on the TAM kinase family for the first time opening the way for further investigations of photoremovable small-molecules on this target class. We are currently investigating two-photon sensitive photactivatable protecting groups in order to adjust the phototherapeutic window compatible to tissue irradiation. Combining photopharmacology and TAM protein kinases will most certainly find significant medical applications in the future

Acknowledgments

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Keywords: photopharmacology • protein kinases • imidazopyridines • photocaging • small molecule inhibitors

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