



# Triphenylamines Induce Cell Death Upon 2-Photon Excitation

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## Abstract

Photodynamic therapy (PDT) is a promising therapeutic method for several diseases, in particular for cancer. This approach uses a photosensitizer, oxygen, and an external light source to produce reactive oxygen species (ROS) at lethal doses to induce cell death. One drawback of current PDT is the use of visible light which has poor penetration in tissues. Such a limitation could be overcome by the use of novel organic compounds compatible with photoactivation under near-infrared light excitation. Triphenylamines (TPAs) are highly fluorescent compounds that are efficient to induce cell death upon visible light excitation (458 nm), but outside the biological spectral window. Interestingly, we recently showed that TPAs target cytoplasmic organelles of living cells, mainly mitochondria, and induce a high ROS production upon 2-photon excitation (in the 760-860 nm range), leading to a fast apoptosis process. However, we observed significant differences among the tested TPA compounds in terms of cell distribution and time courses of cell death-related events (apoptosis vs necrosis). In summary, TPAs represent serious candidates as photosensitizers that are compatible with 2-photon excitation to simultaneously trigger and imaging cell death although the relationship between their subcellular localization and the cell death mechanism involved is still a matter of debate.

## Keywords

photodynamic therapy, advances in optical probes, imaging cellular stress, activatable probes, cell death, fluorescence imaging

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Photodynamic therapy (PDT) is a minimally invasive therapeutic procedure that has been approved for several indications, in particular for certain types of cancer.<sup>1</sup> The PDT approach involves a photosensitizer (PS), light, and oxygen. It is based on the cellular uptake of the PS, followed by illumination at an appropriate wavelength that is able to excite the PS. An ideal PS should present nontoxicity in the absence of light or moderate toxicity upon daylight excitation. Subjected to photon absorption, the PS reaches an excited high-energy state and reacts with biomolecules or molecular oxygen to produce reactive oxygen species (ROS) following type I photochemical reaction, or with molecular oxygen at the ground state (<sup>3</sup>O<sub>2</sub>) to generate singlet oxygen (<sup>1</sup>O<sub>2</sub>), following type II reaction.<sup>2</sup> Reactive oxygen species produced during photochemical reactions are then able to react with large biomolecules, such as proteins, DNA, and lipids, subsequently leading to cell death.

Most of the standard PS are characterized by 1-photon activation using wavelengths in the red part of the visible spectrum (600-750 nm), corresponding to optimum light penetration by minimizing light scattering and tissue absorption. However, the absorption by tissues is expected to be much lower in the

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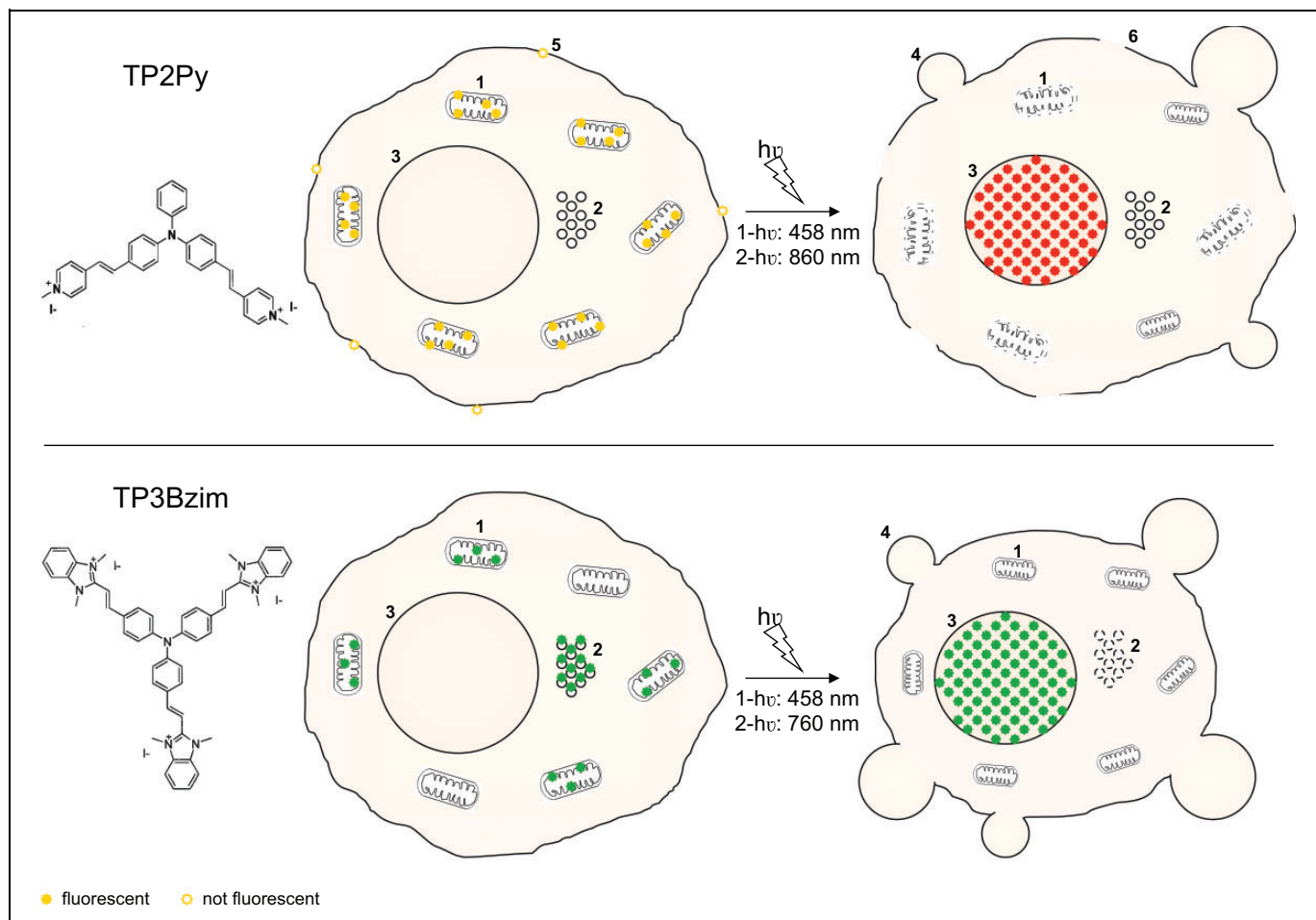
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**Figure 1.** Model for the photo-induced cell death by triphenylamines (TPAs; top, TP2Py; bottom, TP3Bzim). Before illumination (left), fluorescence signals of TPAs are localized in the cytoplasm of living cells: mainly at the mitochondria level (1) for TP2Py and, in both mitochondria and late endosomes (2) for TP3Bzim. Upon illumination (right), both TPAs translocate into the nucleus (3). This translocation process is accompanied by the appearance of plasma membrane blebs (4), a hallmark of cell death. Both TPAs lead to apoptosis but TP2Py leads also to a proper necrotic effect. To explain this necrotic effect and taking into account that the common mitochondrial localization of both compounds and the late endosome localization of TP3Bzim are associated with apoptosis (see text), we hypothesize that a subpopulation of nonfluorescent TP2Py compounds could be localized at the plasma membrane level (5) and thus could be responsible for direct photodamage of the membrane (6) causing necrosis.

near-infrared (NIR) region which is considered as an optimal biological spectral window (700-950 nm).<sup>3</sup> Thus, to enhance the precision of PDT and target the deep part of tissues, 2-photon excitation PDT is of current interest. Unfortunately, most of the current PDT compounds require high excitation power because of their low 2-photon absorption cross-sections ( $\sigma^2$ ). Hence, there is a need for novel PDT compounds with larger  $\sigma^2$  values, characterized by easy synthesis, high chemical stability and that satisfy the biocompatibility criteria.<sup>4</sup>

Triphenylamines (TPAs) carry 2 or 3 vinyl branches terminated with pyridinium (Py) or N-methyl benzimidazolium (Bzim) groups<sup>5,6</sup> (Figure 1). They were initially designed for 2-photon absorption and their binding to the DNA minor groove in vitro leads to a considerable enhancement of their fluorescence emission. Accordingly, TPA-treated fixed cells present highly contrasting nuclear staining, using 1- or 2-photon microscopy. Nevertheless, the distribution of the

fluorescence signal of TPAs is dramatically different in living cells.<sup>6</sup> At initial times after incubation, TPAs do not stain the nucleus in living cells but instead, localize in the cytoplasm. Upon visible light irradiation ( $\approx 450$  nm), TPA-treated cells display a decrease of the cytoplasmic fluorescence intensity, accompanied by a strong increase of the fluorescence signal in the nucleus. Concomitant with this cellular relocation, TPAs are able to induce rapid and massive cell death as illustrated by cell shrinkage and membrane blebbing, even at submicromolar/low micromolar concentration range. Moreover, an external source of excitation is strictly required to trigger the subcellular delocalization of TPAs and cell death with very low dark and daylight cytotoxicities below 30  $\mu$ M. Furthermore, TPAs directly translocate to the nucleus of apoptotic cells which were pretreated with an other proapoptotic compound (eg, camptothecin).<sup>6</sup> Although, TPAs present interesting properties to simultaneously trigger and image cell

death, their 1-photon absorption bands (<510 nm) are far from the therapeutic spectral window corresponding to the red part of the visible spectrum. Nevertheless, TPAs display high fluorescence intensities in fixed cell nuclei by 2-photon imaging thanks to their  $\sigma^2$  that are found between 250 and 1080 GM (1 GM =  $10^{-50}$  cm<sup>4</sup>·s·photon<sup>-1</sup>·molecule<sup>-1</sup>).<sup>5</sup> Thus, if TPAs are able to display the same subcellular features in living cells upon 2-photon excitation (simultaneous nuclear translocation and cell death) like previously observed upon 1-photon excitation, they could present great interest for PDT using NIR excitation.

Recently, we addressed this question and showed for the first time that TP2Py and TP3Bzim are compatible with both photo-induced and imaged cell death upon 2-photon excitation with optimal excitation wavelengths of 860 and 760 nm, respectively.<sup>7</sup> In the same study, we obtained deeper insight into the mechanisms engendered by photoactivation of TPAs. We showed that 1- or 2-photon excitation of TPAs leads to ROS production in a manner similar to that of conventional PDT compounds. Two-photon excitation of TPAs involves fluence values higher than those found for the 1-photon excitation process (188-750 and 15-144 J·cm<sup>-2</sup>, respectively). Nevertheless, these values remain compatible with noninvasive illumination conditions used *in vivo* and significantly below the photodamage threshold. Both TP2Py and TP3Bzim compounds present important characteristics to outspread the use of 2-photon PDT, due to their photochemical properties, their very low dark and daylight cytotoxicities, in addition to their large  $\sigma^2$  values.

However, we found different fates for these 2 compounds in living cells. Indeed, although TP2Py and TP3Bzim remain localized in the cytoplasm of living cells before photoactivation, the subcellular localization of these 2 compounds is not strictly identical. TP2Py mainly localizes to mitochondria, whereas TP3Bzim localizes to mitochondria and late endosomes. It is well-known that most of the PDT compounds targeting mitochondria induce apoptosis while other PDT compounds targeting lysosomes or plasma membrane usually lead to necrosis.<sup>8</sup> Surprisingly, upon light irradiation, while TP3Bzim induces apoptosis only, the mainly mitochondria-localized TP2Py seems to primarily lead to apoptosis but also to a substantial amount of necrosis. To date, the reason for the observed difference regarding the type of cell death photo-induced by TP2Py and TP3Bzim remains elusive but such a difference could be due to the differential subcellular localization of these compounds. Indeed, photoactivation of TPAs leads to ROS production that attack biomolecules localized near their production site due to their high reactivity and relative short half-life. The late endosomes-localized part of TP3Bzim could be involved or not in the induction of cell death after illumination. As described in the photochemical internalization technology, TP3Bzim could escape from late endosomes upon light excitation by causing severe membrane damage and could subsequently target several biomolecules or mitochondria before translocation in the nucleus. Not mutually exclusive with the previous mechanism, the photoactivation of TP3Bzim can cause disruption of late endosomes

membrane, thus causing cytosolic acidification by the release of intravesicular contents into the cytosol which is known to play a role in the apoptotic pathway.<sup>9,10</sup> Likewise, TP2Py could be present in late endosomes without being fluorescent in these organelles and generate the same mechanism which leads to apoptosis than TP3Bzim. However, if involved in the photo-induced cell death process, the part of late endosome-localized TPA compounds most likely accounts for apoptosis as also expected for the common part of mitochondria-localized compounds. But the most important difference between these 2 TPAs is that TP2Py induces apoptosis as TP3Bzim, but also proper necrosis. Taking into account that direct targeting of mitochondria by TPAs preferentially induces apoptosis, what could be the origin of the necrotic cell population specifically observed with TP2Py? As discussed above, it is unlikely that the mitochondria/late endosome partition explains for the proper necrosis process. To explain such a difference, we may suppose that TP2Py could be in or near the plasma membrane without being strongly fluorescent but with the ability to cause plasma membrane photodamage that leads to necrosis (Figure 1). Accordingly, we have previously shown that the emission of TP2Py was more sensitive to the subcellular environment/cellular compartment (yellow emission in the mitochondria vs red emission in the nucleus) compared to TP3Bzim.<sup>6</sup>

Regarding the structural determinants of photo-induced cell death by TPAs, we have shown that a neutral TPA derivative (TP3<sup>N</sup>Bzim) has no specific subcellular localization in living cells and was not able to trigger cell death under light excitation.<sup>6</sup> This demonstrates that the cationic charges of TP2Py and TP3Bzim are involved in their cytoplasmic localization and sequestration before illumination, at least for their common localization at the mitochondria level. The differences between the 2 compounds in terms of subcellular localization and the type of photo-induced cell death mechanism are likely due to differences in structural features. According to our current knowledge, the TPAs' subcellular localization could be determined by the number of vinyl branches or the nature of the electron acceptor group carried by the compound, which both differ in TP2Py and TP3Bzim. Alternatively and as commented above, the apparent differences in cell distributions of TP2Py and TP3Bzim may be due to their ability to differentially fluoresce depending on their surroundings (for instance in interaction with the membrane or free in organelles). Regardless of the exact nature of these mechanistic differences, the different fates and photo-induced effects of TP2Py and TP3Bzim in the cell context also suggest that these 2 compounds may internalize in cells by different uptake mechanisms: active/passive transport through the plasma membrane and endocytosis. Consequently, further investigations are required for a comprehensive understanding of the relationship between the initial localization of TPAs and the precise cell death cascade involved upon light illumination.

#### Declaration of Conflicting Interests

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