Light-Controlled Cellular Internalization and Cytotoxicity of Nucleic Acid-Binding Agents: Studies in Vitro and in Zebrafish Embryos

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Light-controlled cellular internalization and cytotoxicity of nucleic acid-binding agents. Studies in vitro and in zebrafish embryos

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Abstract

We have synthesized oligoarginine conjugates of selected DNA-binding agents (a bisbenzamidine, acridine and thiazole orange) and demonstrated that the DNA binding and cell internalization properties of such conjugates can be inhibited by appending a negatively charged oligoglutamic tail through a photolabile linker. Irradiation with UV light releases the parent octaarginine conjugates, thus restoring their cell internalization and biological activity. Preliminary assays using zebrafish embryos demonstrates the potential of this prodrug strategy for controlling in vivo cytotoxicity.

Keywords

DNA recognition; peptides; fluorescence; supramolecular chemistry; caged compounds

The development of efficient nucleic acid binding agents and stains has been a long-standing research goal at the interface between chemistry and biomedicine.[1] Indeed, cancer chemotherapy still relies largely on small DNA-binding molecules, including intercalators, minor groove binders and alkylating agents.[2] We have recently demonstrated that the DNA binding affinity and selectivity of minor groove binders can be enhanced by tethering peptidic moieties derived from transcription factor fragments. [3] Interestingly, derivatization of DNA binding agents with octaarginine cell-penetrating peptides (CPPs) results in conjugates that display increased DNA affinity, [4] as well as more efficient cell internalization. [5 , 6] Unfortunately, the clinical potential of most DNA-binding agents is seriously compromised by their adverse side effects, and thus there is a great interest in the development of new derivatives with a higher site selectivity and reduced toxicity.[7] One strategy to face this challenge consists of transforming known nucleic acid binders into non-active prodrugs that can be selectively activated in a spatio-temporal manner. In this context,

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it has been demonstrated that appropriate derivatization of DNA-binding agents with labile appendages allows to regulate their oligonucleotide binding properties with external inputs, such as light,[8] or chemical stimuli.[9]

Herein we report a new strategy for controlling the activity of nucleic acid binders based on the temporary suppression of their cell internalization, as well as their nucleic acid binding ability. More specifically, we demonstrate that tethering a polyglutamic tail to conjugates between nucleic acid binders and a octaarginine sequence inhibits their cell entrance and leads to a considerably decrease in their citotoxicity. Importantly, when the connection is carried out through a semipermanent photolabile linker, it is possible to restore the active parent conjugates by irradiation with UV light. [10] Preliminary studies with zebrafish embryos demonstrates that the strategy can be applied *in vivo*.

As representative nucleic acid binders for designing our conjugates we chose a bisbenzamidine (**BBA**), which is known to insert in the minor groove of AT-rich sites, and two intercalators, acridine (**ACR**) and thiazole orange (**TO**). **TO** is known to display a significant fluorescence emission enhancement upon binding to DNA (~1000-fold) or RNA (~3000-fold).[11] These nucleic acid-binding units are attached to the N-terminus of a oligocationic domain (Arg_8) tethered to an oligoanionic (Glu_8) tail through a photolabile 3-amino-3-(2-nitrophenyl)-propionic acid (ANP) group (Fig. 1).[12,13] The linker also includes a β-turn promoting sequence (Pro-Gly),[14] which might favor the interaction between the Glu_8 and Arg_8 units, thus avoiding the formation of higher order species through intermolecular association. As positive controls of the photorelease reaction, we also synthesized the conjugates between each of the DNA binders and the octaarginine tag (**BBA–R_8**, **ACR–R_8** and **TO–R_8**).

The core peptides **R_8E_8** and **R_8** were prepared following standard Fmoc/tBu solid-phase synthesis protocols (Fig. 1).[15] The TO and ACR units were attached to the peptide chain in the solid phase, and the BBA conjugates were assembled through a "*click*" reaction in solution (See the supporting information). The conjugates were purified by reverse phase HPLC and confirmed as the desired products by ESI-MS and MALDI.

Having at hand the desired samples, we studied the photocleavage reaction. Thus, we irradiated 200 μM solutions of **BBA–R_8E_8**, **ACR–R_8E_8** and **TO–R_8E_8** in water with a handheld transilluminator for 20 min (300-375 nm).[16] HPLC analysis of aliquots of the irradiated mixtures showed the disappearance of the starting conjugates, and the formation of the expected photocleavage products, **BBA–R_8**, **ACR–R_8** and **TO–R_8**, as well as the **E_8**–nitrosoketone byproduct (see Fig. 2 for the case of the **TO–R_8E_8**, and the supporting information for the other conjugates).

We were then in position of studying the cellular internalization of the conjugates before and after irradiation. Toward this end we took advantage of the extraordinary fluorogenic properties of TO when binding to nucleic acids.[11] Thus, HeLa cells were incubated with 10 μM of **TO–R_8** in serum-free medium for 30 min at 37 °C. Analysis by fluorescence microscopy showed a bright intracellular red emission mainly localized in the cell nucleoli and cytoplasm, which is consistent with an efficient uptake of the TO conjugate and its

enhanced emission upon interacting with nuclear oligonucleotides as well as with cytoplasmic RNA. Furthermore, we could also confirm the relevance of the octaarginine tag for the cell entrance, since the unconjugated dye (**TO**) is very poorly uptaken (see the supporting information). Importantly, incubation of the cells with 10 μM **TO–R_8E_8** under the same conditions did not show any red emission from the TO (Fig. 3, middle row, left), which is consistent with the expected reduced internalization of the Glu_8 conjugate. However if the cells are irradiated for 10 min, then the resulting staining pattern is superimposable with that obtained from **TO-R_8** (Fig. 3, bottom row, left).

Once demonstrated that the attachment of a Glu_8 tail to the TO-oligoarginine hybrid effectively inhibits its cell internalization, and that it can be recovered by irradiation with UV light, we studied the potential influence of this appendage in the cytotoxicity of the conjugates. The studies of cellular viability were carried out with HeLa 229 cells using the sulforhodamine B assay (see the supporting information). [17] Interestingly, while thiazole orange (**TO**) and acridine (**ACR)** didn't appreciably affect the cell viability, their conjugation to the octaarginine unit promoted a considerable increase in the cytotoxicity, so that **TO–R_8** and **ACR–R_8** present IC_{50} values of 36 ± 1 μM and 54 ± 2 μM, respectively (Table 1). In agreement with previous reports,[18] the octaarginine unit displays very low toxicity (<50% cell growth inhibition), which confirms the synergistic effect in activity resulting from combining the DNA intercalators with octaarginine units. More importantly, the **R_8E_8** conjugates showed a drastically reduced toxicity to the point that their IC_{50} values could not be measured under the standard assay conditions (<50% CGI). Irradiation of the conjugates **ACR–R_8E_8** and **TO–R_8E_8** for 10 min (300-375 nm), before incubation, restored their cytotoxicity to values comparable to those of the Arg_8 conjugates (IC_{50} 63 ± 1 μM and 44 ± 1, respectively). We also confirmed that the irradiation itself had a negligible cytotoxic effect (<50% CGI). In the case of the bisbenzamidine, the isolated BBA unit is already cytotoxic, and its derivatization with the octaarginine tail did not significantly affect its inherent activity (Table 1).[19] However, the **R_8E_8** conjugate presented a drastically reduced cytotoxicity (<50% CGI), which could again be restored after irradiation (IC_{50} 27 ± 1 μM).

Taken together, the fluorescence microscopy and cytotoxicity studies suggest that the activity of the R_8 conjugates arises from their octaarginine-promoted internalization, and is most probably associated to the interaction with intracellular nucleic acids. To gain more information on this interaction, we performed electrophoretic mobility shift binding assays (EMSA) under non-denaturing conditions using double stranded oligonucleotides. [20] As expected, addition of increasing concentrations of **BBA–R_8** to a target A/T-rich dsDNA led to clear retarded bands consistent with the formation of the expected **BBA–R_8**/DNA complexes (Fig. 4a). However, the conjugate bearing the glutamic tail (**BBA–R_8E_8**) failed to promote the appearance of slower-migrating bands, indicating that the Glu_8 tail is strongly destabilizing the binding to the DNA (Fig. 4b). Incubation of the **BBA–R_8E_8** and **BBA–R_8** conjugates with an alternative DNA lacking the required A/T-rich region did not induce any new bands, thus confirming that the sequence selectivity of the interaction, in consonance with the expected behavior of the bisbenzamidine (see the supporting information).**9**, [21] On the other hand, the acridine conjugate **ACR–R_8** also led to clear retarded bands, which did not appear in the case of its Glu_8 derivative (Fig. 4c and 4d, respectively). **TO-R_8** also

showed interaction with the DNA, however in this case the bands are fainter and we observe partial precipitation at higher concentrations, probably because of the lower intrinsic affinity of **TO** for DNA (Fig. 4e).[22] In any case, it is also clear that the presence of the Glu_8 unit inhibits the interaction (Fig. 4f).

At this point we considered pertinent to run a preliminary test on the potential of the strategy for *in vivo* applications. Zebrafish are considered an excellent model organism in biomedical research, [23] and there are recent examples of their application in photochemical activation studies.[24,25,26] Therefore, we decided to test the viability of our strategy to control *in vivo* toxicity analyzing the effect in the mortality of zebrafish embryos. Specifically, we compared toxicity effects of **TO-R_8**, and **TO-R_8E_8**, as well as **TO-R_8E_8** after UV irradiation. In the study, mortality values were corrected using the Abbott transformation ([(X–Y)/(100–Y)] × 100).[27] Control experiments on the effect of the irradiation in the fish embryos demonstrated that under our experimental setup, the values of mortality are under 20 % after one min of irradiation. Consistent with the *in vitro* studies, **TO-R_8** showed the highest dose-dependent mortality values *in vivo*, with its effect increasing within a narrow range (from 10.8 % of mortality at 10 mg/L to 89.2 % at 30 mg/L, see the supporting information). Using concentrations of 40 mg/L and above, the mortality even reaches 100 %. Importantly, and also in agreement with our hypothesis and the *in vitro* results, **TO-R_8E_8** showed much lower mortality effects, with almost plane dose dependent response in the range analysed.

When the fish incubated with **TO-R_8E_8** are irradiated for one minute with the above lamp, we observed a clear increase in the mortality, which must be associated with the release of the highly toxic **TO-R_8**. Unfortunately, the application of short irradiation times to avoid UV-induced mortality limits the amount of photocleavage, and therefore we could not reach the mortality values of the original **TO-R_8**. While these results showcase the limitations of current photolabile groups, which typically require the application of harmful short-UV light for photolysis,[28] they also demonstrate the viability and potential of the approach for on-demand *in vivo* activation. Ongoing efforts are therefore directed towards the adaptation of this strategy to the use of long-wavelenght photolabile linkers.[29,30]

Owing to the presence of a fluorogenic dye (TO) in our conjugates, we also analyzed by confocal microscopy the potential differences in fluorescence patterning, using anesthetized 96 hpf embryos just immediately after the toxicity analysis. As can be observed in Fig 5 right, there is a clear difference in the distribution of fluorescence between **TO-R_8** and **TO-R_8E_8**, which is fully consistent with an efficient cellular uptaken by the first. **TO-R_8E_8** emits a very weak signal, mainly concentrated in the outer face of the zebrafish epidermis. Importantly, after one min UV irradiation of the fish treated with **TO-R_8E_8** we observe a pattern that resembles that observed after incubation with **TO-R_8**, which is fully consistent with partial release of the active **TO-R_8** species.

In summary, we have demonstrated that the incorporation of octaarginine sequences to different small molecule DNA binders leads to a considerable increase in their cytotoxicity, consistent with a synergistic promotion of both cellular uptake and nucleic acid binding. More important, the activity of these conjugates can be inhibited by the attachment of a Glu_8

tail through a photolabile linker. Irradiation with UV light efficiently releases the parent compounds, thus restoring the cytotoxic activities. Preliminary *in vivo* assays using Zebrafish embryos are fully consistent with the *in vitro* results, and points towards the potential utility of the strategy for the development of new anticancer phototherapies.

Experimental Section

Peptides were assembled in solid phase using Fmoc/tBu strategies and the small DNA binding agents were prepared using known procedures. The conjugations were carried out in solution using cooper catalyzed azide-alkyne cycloadditions for the incorporation of the BBA, and amide bonds for the other. All the processes, as well as the characterization data, are included in the supporting information. The photolysis experiments were carried out using a standard UV transilluminator from *UVPBioImaging, LCC* (95-0222-02: 8W, λ = 300-375 nm, 230V/50Hz); and the EMSA were carried out under standard conditions with a *BIO-RAS Mini Protean* gel system. Cytotoxicity studies were carried out with HeLa 229 (human cervical cancer) cells using a system based on cell staining with Sulforhodamine B. All the details are described in the supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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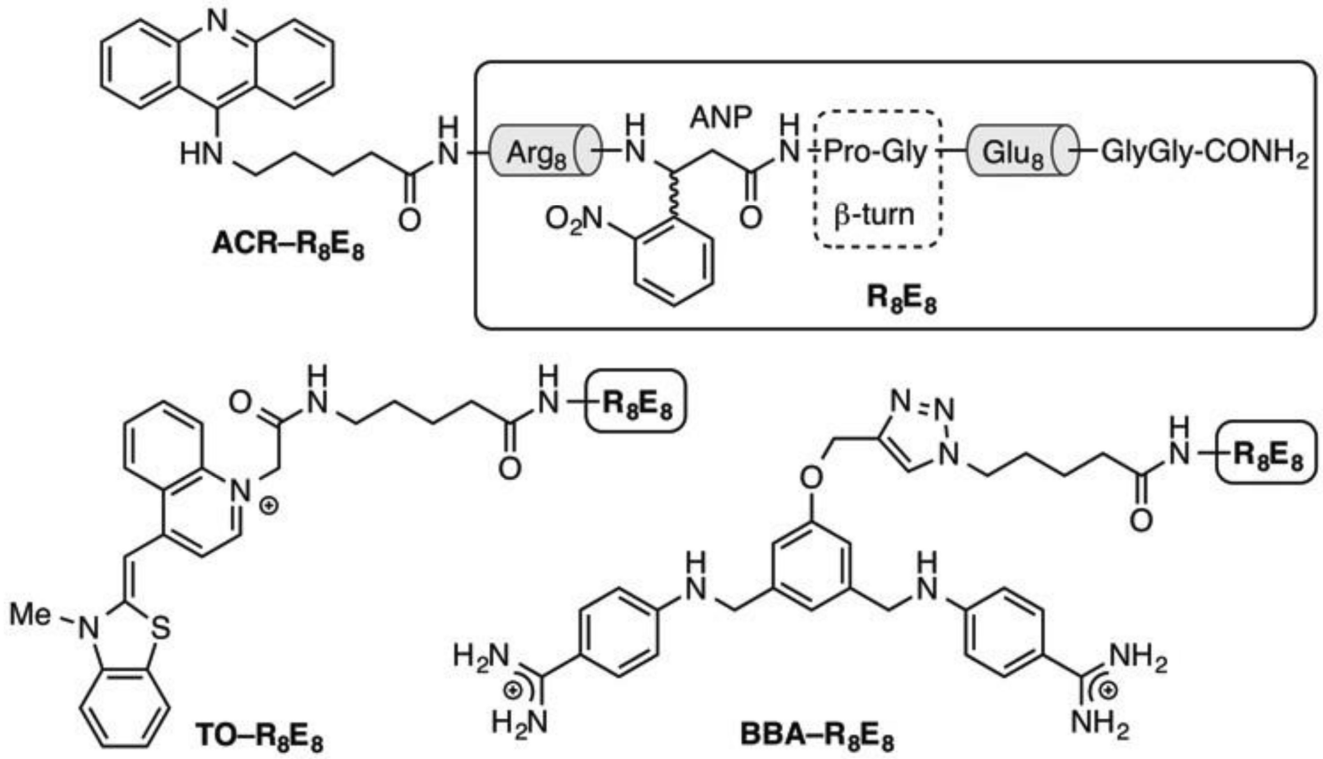

Figure 1.

Structures of the different conjugates used in this study: **BBA–R_8E_8**, **ACR–R_8E_8** and **TO–R_8E_8**.

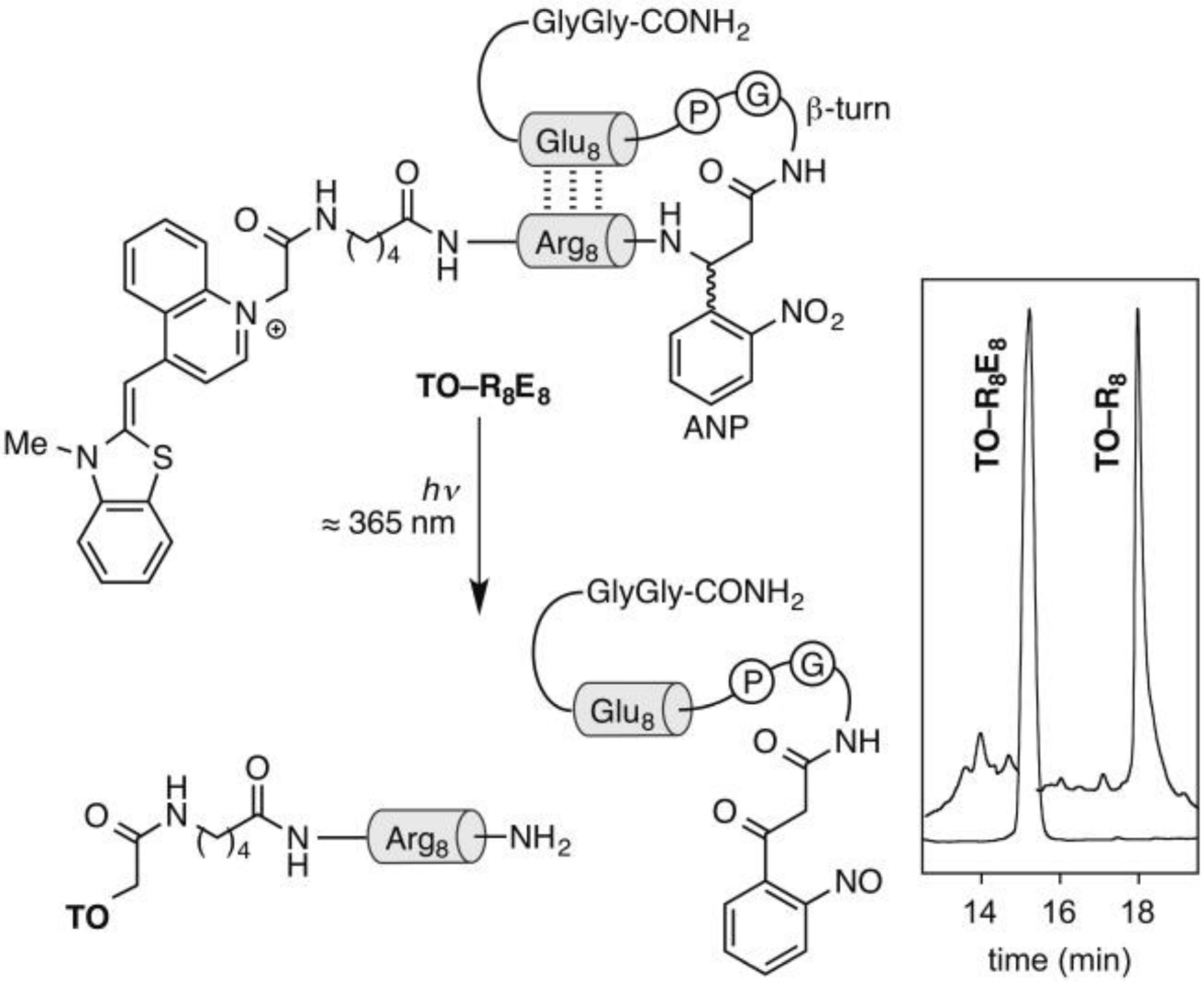

Figure 2.

Left: photocleavage reaction of the **TO–R_8E_8** conjugate. *Right*: Stacked HPLCs of the UV promoted reaction showing the trace of the reaction crude after 20 min. Irradiation was performed using a transilluminator at 300-375 nm.

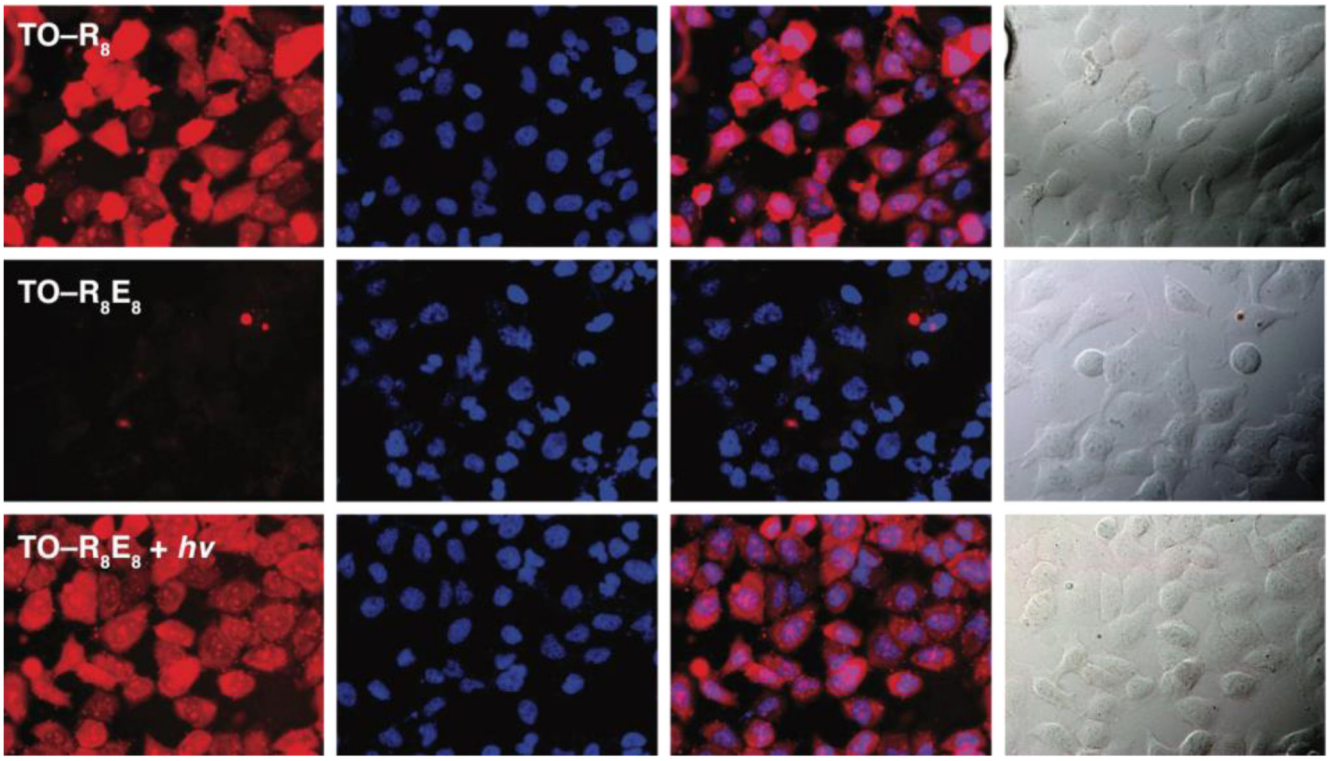

Figure 3.

Fluorescence microscopy of HeLa cells after 30 min incubation with Hoechst 3325 (3 μM) and **TO-R_8 or TO-R_8E_8** (both 10 μM) before and after uncaging. From left to right: red channel, blue channel, merge of both channels and brightfield images. *Top row*: 10 μM **TO-R_8** after 30 min incubation. *Middle row*: **TO-R_8E_8** in the same conditions. *Bottom row*: **TO-R_8E_8** irradiated with UV light for 10 min, and after 30 min of incubation. All micrographs taken at 400X, ISO 400 and with an exposition time of 200 ms; exc. 530-550 nm/em. 590 nm.

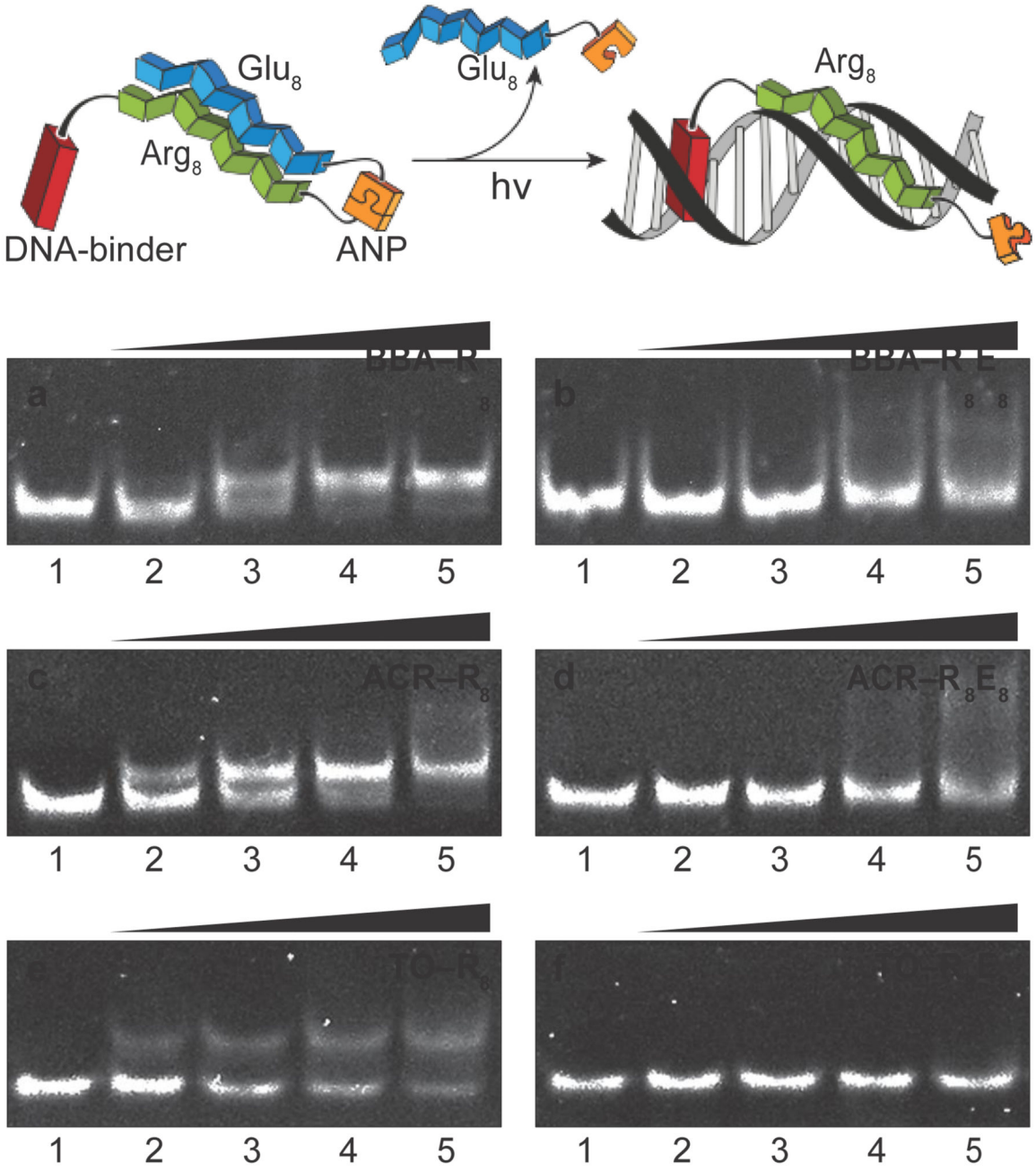

Figure 4.

EMSA showing the binding of the **BBA–R_8**, **ACR–R_8** and **TO–R_8** conjugates and their Glu_8 derivatives to dsDNA. DNA concentration is 50 nM in all lanes; lanes 2-5: 50, 100, 150 and 250 nM, of the conjugates in all gels (see the supporting information).

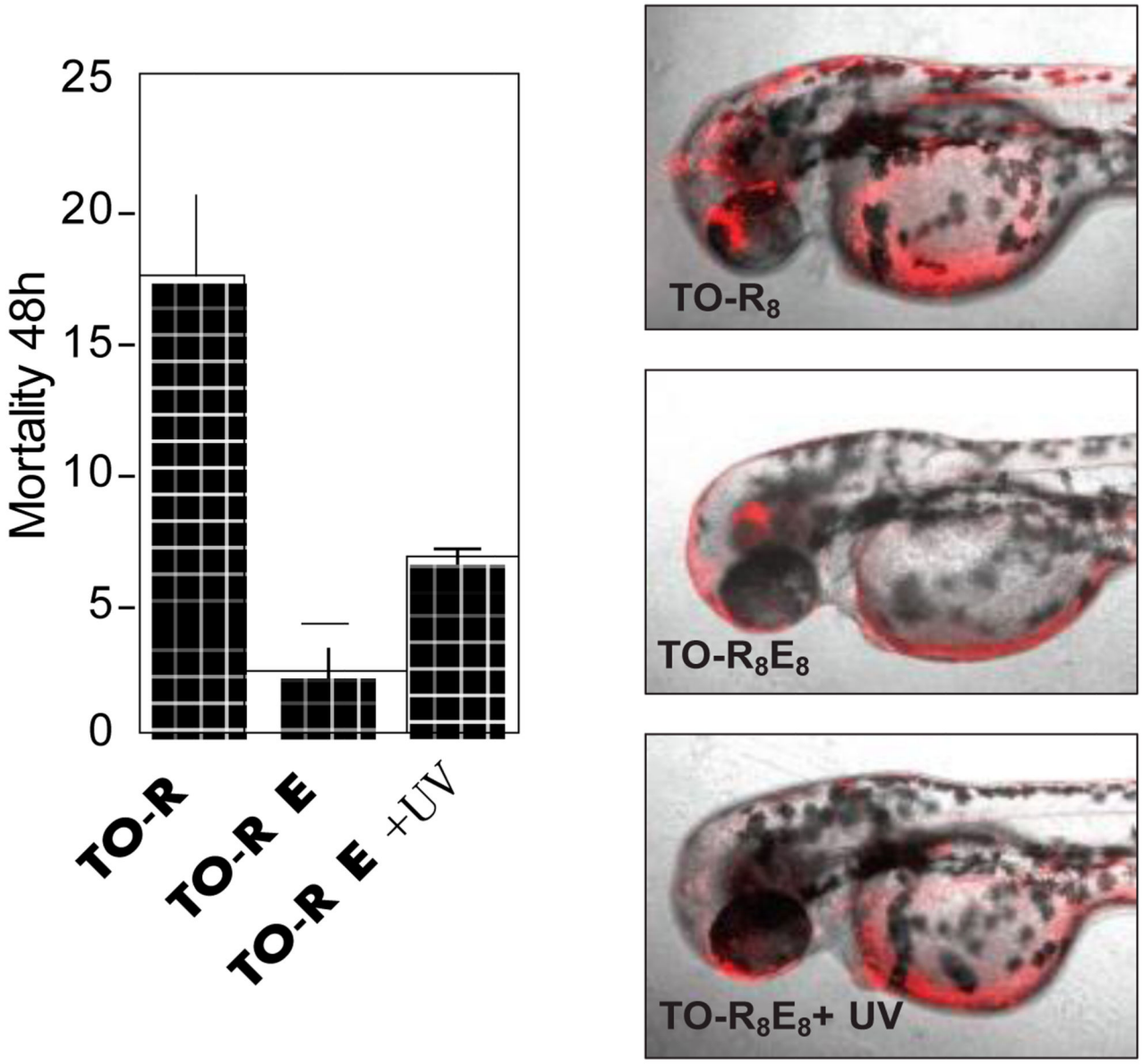

Figure 5.

Left. Mortality rates at 48 h in the *in vivo* toxicology analysis for Thiazole Orange (TO) conjugates. **TO-R_8E_8** and **TO-R_8E_8**+UV were analysed at the same concentrations. **TO-R_8E_8** did not promote any relevant mortality. *Right.* Confocal images of toxicity test survival embryos at 96 hpf. **TO-R_8** (30 mg/L); **TO-R_8E_8** (80 mg/L); **TO-R_8E_8**+UV (80 mg/L).

Table 1

Cell growth inhibition (CGI, IC_{50}, μM) of the small DNA binders (column 1); their octaarginine derivatives (column 2); and their light-sensitive Glu_8 conjugates before, and after irradiation with UV light for 30 min (columns 3 and 4, respectively). In all cases cisplatin (IC_{50} = 0.79 ± 0.01 μM) was used as a positive control. See supporting information for experimental details.

	RNA binder	**R_8 conjugate**	**R_8E_8 conjugate**	
			No irradiation	**irradiated**
TO	< 50% CGI	36±1	< 50% CGI	44±1
ACR	< 50% CGI	54±2	< 50% CGI	63±1
BBA	12±1	12±1	< 50% CGI	27±1