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Citation for published version:

Digital Object Identifier (DOI):
10.1021/acs.bioconjchem.8b00481

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Bioconjugate chemistry

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Drug “Clicking” on Cell-Penetrating Fluorescent Nanoparticles for In Cellulo Chemical Proteomics

Teresa Valero,†,‡,⊥ Antonio Delgado-González,§,⊥ Juan Diego Unciti-Broceta,‡,§,# Victoria Cano-Cortés,‡,⊥ Ana M. Pérez-López,⊥ Asier Unciti-Broceta,⊥,* and Rosario M. Sánchez Martín,*,†,⊥

†Cancer Research UK Edinburgh Centre, MRC Institute of Genetics & Molecular Medicine, University of Edinburgh, Edinburgh EH4 2XR, United Kingdom
‡GENYO, Centre for Genomics and Oncological Research, Pfizer/University of Granada/Andalusian Regional Government, PTS Granada, Avda. Ilustración 114, 18016 Granada, Spain
§R&D Department, NanoGetic SL, Health Science Technological Park (PTS), Avenida de la Innovación 1, Edificio BIC, 18016 Granada, Spain
⊥Department of Medicinal & Organic Chemistry, Faculty of Pharmacy, University of Granada, Campus de Cartuja s/n, 18071 Granada, Spain

Supporting Information

ABSTRACT: Chemical proteomics approaches are widely used to identify molecular targets of existing or novel drugs. This manuscript describes the development of a straightforward approach to conjugate azide-labeled drugs via click chemistry to alkyne-tagged cell-penetrating fluorescent nanoparticles as a novel tool to study target engagement and/or identification inside living cells. A modification of the Baeyer test for alkynes allows monitoring the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction, guaranteeing the presence of the drug on the solid support. As a proof of concept, the conjugation of the promiscuous kinase inhibitor dasatinib to Cy5-labeled nanoparticles is presented. Dasatinib-decorated fluorescent nanoparticles efficiently inhibited its protein target SRC in vitro, entered cancer cells, and colocalized with SRC in cellulo.

INTRODUCTION

Drug discovery efforts across industry and academia have generated extensive libraries of small molecule compounds that are currently used in high-throughput screening campaigns. One such test employs cell assays to search for compounds that elicit phenotypic responses in particular disease models in a target agnostic manner. This approach, so-called phenotypic screening, is aimed at accelerating the earlier stages of drug discovery and, potentially, favor the finding of first-in-class inhibitors.1−3 After a phenotypic hit or lead is found, one of the main challenges is to elucidate the mechanism of action responsible for the observed pharmacological effect,4 as the assays only provide information on compounds’ activity in the cell model of choice without informing on the target.5 Consequently, target identification (ID) studies are often performed to support subsequent lead optimization campaigns.

Among the strategies developed for target ID, chemical proteomics enable us to interrogate the full proteome for direct drug–target interactions. Most chemical proteomics approaches combine affinity chromatography with advanced mass spectrometry to enrich and facilitate target identification from complex biological samples.6 An alternative approach has been proposed to directly visualize drug–target interactions in cells using drug-labeled nanodevices and fluorescent immunochemistry.7 Regardless of the method, an essential step in these strategies is the conjugation of the drug to the solid support, which typically requires individual optimization of the coupling process for each drug.8 Therefore, the development of versatile, robust conjugation methods is essential to facilitate the performance of target ID studies both in cell lysates and in native biological environments.

Herein, we present a straightforward procedure for the conjugation of azide-tagged drugs to miniaturized solid supports by click chemistry, along with an analytical method to rapidly determine whether the coupling reaction has been successful. As a proof of concept of the strategy, the synthesis, characterization, and biological validation—in vitro and in cellulo—of a novel cell-penetrating fluorescent nanodevice...
decorated with the tyrosine kinase inhibitor (TKI) dasatinib is reported.

RESULTS AND DISCUSSION

Selection and Development of Azide-Tagged Dasatinib. Dasatinib is a potent promiscuous kinase inhibitor approved by the FDA for the treatment of chronic myeloid leukemia (CML) after acquired resistance to imatinib. Dasatinib’s therapeutic effect is mediated by inhibition of the constitutively active fusion protein BCR-ABL, which is the product of an aberrant chromosomal translocation and responsible for CML oncogenesis. Nevertheless, dasatinib also inhibits a wide range of kinases including the nonreceptor tyrosine kinase SRC and its family members (LCK, HCK, YES, FYN, FGR, BLK, LYN, FRK), receptor tyrosine kinases (KIT, PDGFR, DDR1/2, c-FMS, ephrin receptors), and TEC family kinases (TEC and BTK), representing a good example of a promiscuous drug with potential use against numerous clinical disorders. The high potency of dasatinib, its interesting polypharmacological properties, and its easily functionalized chemical structure made it an optimal candidate to develop and test our strategy.

To introduce an azide “handle” in dasatinib for CuAAC reactions while retaining the essential kinase binding elements of its structure, the terminal hydroxyl group of the hydroxyethylpiperazinyl moiety was modified. The role of this moiety is to impart water solubility to the compound and it is displayed outside the protein in the SRC-dasatinib cocrystal structure. The substitution of the OH of dasatinib by N₃ was efficiently carried out using a reported procedure with minor modifications. Briefly, chlorination of that position was achieved by treating dasatinib with mesyl chloride and triethylamine. The corresponding chloro derivative dasatinib-Cl (2) was then reacted with sodium azide to obtain derivative dasatinib-N₃ (3) (Scheme 1; see full synthetic details in the SI). Cell viability assays against breast cancer MDA-MB-231 cells demonstrated that 3 retained the antiproliferative properties of the parent drug 1 (see SI).

Synthesis of Dasatinib-Decorated Cy5-Labeled Cell-Penetrating Nanospheres. In cellulo chemical proteomics has the potential to provide more biologically relevant results by studying drug−target interactions in the native cellular environment. It also provides a method to study target engagement, a relevant validation step in drug discovery programs. The development of facile and reliable strategies to conjugate drugs and fluorescent labels on devices with the capacity to penetrate cells without inducing toxicity is very attractive in this field. 500 nm cross-linked polystyrene nanospheres with demonstrated capacity to safely penetrate a wide variety of cell types were redesigned for the goals of this project (Scheme 1). NH₂-NP500 (4) was synthesized and functionalized according to previous reports. To increase biocompatibility, prevent unspecific protein binding, and maximize the freedom of the conjugated drug molecules, 4 was PEGylated before and after incorporation of the dye. By sequential coupling and orthogonal deprotection steps (Fmoc/Dde deprotection strategy), bifunctionalized doubly-PEGylated nanoparticles (NPs) were labeled with the far-red fluorescent dye Cy5 (NH₂-Cy5-PEG-NP500, 9). Then an alkyne handle was incorporated onto the NPs by conjugation of propiolic acid to generate Alk-Cy5-PEG-NP500 (10). Finally, reaction of 10 and 3 in the presence of Cu and

Scheme 1. Synthesis of Dasatinib-Decorated Fluorescently-Labeled Cell-Penetrating Nanoparticles
ascorbic acid\textsuperscript{23} gave rise to dasatinib-decorated Cy5-tagged nanospheres, DAS-Cy5-PEG-NP\textsubscript{500} (11) (see synthesis and loadings in the SI). Number of NPs per unit of volume was calculated by spectrophotometry according to previous reports\textsuperscript{24} (see calculations in the SI).

Analytical Determination of Dasatinib Incorporation onto the Solid Support. Although CuAAC reactions are typically described as virtually quantitative even under mild conditions\textsuperscript{25,26} click reactions on solid phase typically need optimized conditions to reach these standards\textsuperscript{27,28}. To confirm the effective coupling of 3 to the alkyne-tagged NPs 10, a series of analytical methods were attempted: (i) detection of unreacted alkynes on the solid support by modification of the Baeyer test for alkynes; (ii) zeta potential characterization of NPs at different steps of the synthesis; (iii) validation by NMR and mass spectrometry of the functionalization strategy by solid phase synthesis using an amino-functionalized resin (Rink amide resin).

The Baeyer test is routinely used in chemistry laboratories to detect the presence of double or triple carbon−carbon bonds based on the capacity of KMnO\textsubscript{4} to oxidize alkenes and alkynes. Aqueous reduction of KMnO\textsubscript{4} (violet color) produces MnO\textsubscript{2} (blackish/brown color), a change that is detectable by spectrophotometry in the range of 250 to 500 nm.\textsuperscript{24} Therefore, we anticipated that this method could be used to detect unreacted alkynes on the NPs and thereby assess the efficacy of the drug incorporation reaction onto the solid support. DAS-Cy5-PEG-NP\textsubscript{500} (11) were treated with an aqueous solution...
of KMnO₄ and absorbance spectra analyzed, with the test of NH₂-Cy5-PEG-NP₅₀₀ (9) and Alk-Cy5-PEG-NP₅₀₀ (10) under the same conditions being used as negative and positive controls, respectively (see full details in the SI). After exploring different reaction conditions and careful spectroscopic analysis to improve the method, the measurement of the changes in the absorbance values at 420 nm after 5 min reaction in comparison to the unreacted KMnO₄ solution was found to be optimal. As shown in Figure 1a, analysis of the reaction of the positive control Alk-Cy5-PEG-NP₅₀₀ resulted in a significant increment of absorbance at 420 nm, a change that was undetectable for NH₂-Cy5-PEG-NP₅₀₀ (which do not contain triple bonds). Importantly, the intensity of the signal was significantly reduced in DAS-Cy5-PEG-NP₅₀₀ as it would be expected after conjugation with 3 by CuAAC. This simple analytical test served to verify that the click reaction took place at a relatively high yield, yet some unreacted alkynes were still present in the structure of DAS-Cy5-PEG-NP₅₀₀ most probably being those less-accessible alkynes found in the interior of the polymeric framework of the NPs. An estimation of the CuAAC reaction yield can be performed with this spectrophotometric method, giving a value of 63.6%. The presence of unreacted alkynes suggests that sterically constrained environments may affect the reaction efficacy.

Chemical moieties displayed at the outer layer of NPs influence the surface charge and consequently the zeta potential of these colloidal structures. To further validate the results obtained from the Baeyer test, the conjugation of dasatinib to the NPs was also assessed by measuring zeta potential of NH₂-Cy5-PEG-NP₅₀₀, Alk-Cy5-PEG-NP₅₀₀, and DAS-Cy5-PEG-NP₅₀₀. Figure 1b shows a displacement of zeta potential distribution to the negative range when alkynes were substituted by alkynes, whereas upon drug conjugation the presence of the drug on the NP displaces the zeta potential to less negative values, providing further qualitative evidence of the success of the CuAAC reaction.

As a final proof, rink amide resins (NH₂-RINK, typically used in solid phase peptide synthesis) were treated with 3 using the same reaction conditions used for the functionalization of the NPs (see Scheme 1). Following release of the corresponding triazole-modified dasatinib derive from the solid support by linker cleavage in acidic conditions, NMR analysis confirmed the efficacy of this conjugation reaction (see SI).

Biological Assays. To assess if the biological activity of the drug is maintained after conjugation onto the NPs, DAS-Cy5-PEG-NP₅₀₀ were incubated with recombinant SRC and the activity of the kinase analyzed using a luminescence commercial kit. NPs without dasatinib (NH₂-Cy5-PEG-NP₅₀₀) were used as negative control. As shown in Figure 2a, a concentration dependent reduction in SRC activity was observed in the presence of DAS-Cy5-PEG-NP₅₀₀ with a 50% of inhibition observed at 2,041 × 10⁵ NPs (51 × 10⁹ NP/µL). By calculating the amount of drug per NP, the IC₅₀ was estimated to be 33.4 nM.

Cross-linked polystyrene NPs have been previously shown to rapidly enter a wide range of cell types. To prove that the presence of dasatinib does not interfere with the internalization process, cell uptake into MDA-MB-231 cells was analyzed by flow cytometry (Figure 2b and SI) and time-lapse imaging (Movie S1). Cells exhibiting fluorescence in the Cy5 channel were considered “nanofected” and multiplicity of nanofection 50 (MNFS50) values were calculated as the ratio of nanoparticles per cell necessary to “nanofect” 50% of the cell population. As shown in Figure 2b, MNFS50 values of DAS-Cy5-PEG-NP₅₀₀ only were slightly inferior when compared to the unclicked control NH₂-Cy5-PEG-NP₅₀₀, although no differences in internalization efficiency were observed at ≥300 NPs/cell.

Target Engagement Study. To test the functionality of the nanodevices in cells, we next performed a target engagement assay. Triple negative breast cancer MDA-MB-231 cells were selected to this study as SRC protein is highly overexpressed in these cells. MDA-MB-231 cells were treated with DAS-Cy5-PEG-NP₅₀₀ for 24 h and target-NP localization studied by confocal microscopy. The presence of a Cy5 fluorescent moiety on the NPs along with their size (500 nm) enabled the visualization of NPs by confocal microscopy into living cells. Immunocytochemistry analysis was carried out to visualize the SRC protein in the cells using an anti-SRC primary antibody and a fluorescent secondary antibody (Dylight 405). Z-stack images were acquired to image the NPs distributed throughout the cytoplasm (Figure 2c,d). Upon immunocytochemistry, image analysis showed colocalization of DAS-Cy5-PEG-NP₅₀₀ with SRC protein in MDA-MB-231 cells. In contrast, the control NPs not decorated with dasatinib (NH₂-Cy5-PEG-NP₅₀₀) showed no colocalization with the target (see Supp. Inf.). These results were further confirmed by calculation of the Pearson’s correlation coefficient (Figure 2e), demonstrating linear correlation (positive value) between the localization of DAS-Cy5-PEG-NP₅₀₀ and SRC. In contrast, a negative value was obtained by analysis the localization of NH₂-Cy5-PEG-NP₅₀₀ and SRC. These results prove the capacity of DAS-Cy5-PEG-NP₅₀₀ to efficiently engage with a specific target in cellulo.

CONCLUSIONS

In conclusion, herein we have presented a novel drug-decorated nanoprobe with the capacity to penetrate mammalian cells and bind target proteins in the intracellular environment. The use of a conjugation strategy based on the efficient CuAAC reaction makes this approach accessible to many different drugs by simple incorporation of an azide handle. Caution, however, should be taken when assuming a quantitative yield for click reactions on miniaturized devices. To avoid false negatives, a rapid spectrophotometric test for click reactions on alkyne-tagged solid supports was developed based on the Baeyer test. This method can be used to estimate the yield of the coupling reaction by detecting unreacted alkynes on the solid support. As a proof of concept, fluorescent NPs were functionalized with dasatinib and the incorporation of the drug confirmed by different analytical methods. Dasatinib-decorated NPs induced a reduction in SRC activity in vitro, further demonstrating drug incorporation on the solid support, but also proving a correct spatial arrangement of the conjugate that maintained drug activity. These NPs were efficiently internalized into living MDA-MB-231 cells and, furthermore, demonstrated binding to the target protein (SRC kinase) in cellulo, proving the capacity of this novel nanodevice to detect drug-target interactions in complex living environments. The size of the nanoprobe (∼500 nm in σ) was selected based on the technical limitations of conventional fluorescence microscopy. Nevertheless, if high-resolution imaging techniques are available, the versatile methodology presented herein could be readily implemented on NPs of smaller size.
MATERIALS AND METHODS

General Chemistry Protocols. All commercially available chemicals were obtained from either Fisher Scientific, Matrix Scientific, Sigma-Aldrich, or VWR International Ltd. Reactions were performed under inert atmosphere (nitrogen) using anhydrous solvents. NMR spectra were recorded at ambient temperature on a 500 MHz Bruker Avance III spectrometer. Samples were dissolved in deuterated solvents commercially available from Sigma-Aldrich. 1H NMR spectra: chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane. The data is presented as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants as a J value in Hertz (Hz) and interpretation. The number of protons (n) for a given resonance is indicated as nH, and is based on spectral integration values. TLCs were run on Merck TLC Silica gel 60 F254 plates, typically 5 cm × 10 cm, and monitored using a 254 nm UV source. High resolution mass spectra were recorded by the MS Department of the University of Edinburgh on a Thermo MAT 900 XLPE high resolution, double focusing mass spectrometer. All the couplings were carried out on the Eppendorf Thermomixer comfort and washed after centrifugation in an Eppendorf mini centrifuge.

Conjugation of Dasatinib to NPs. 9 (1 mL; 1 equiv) was suspended in DMF (1 mL). Separately, propiolic acid (50 equiv) was dissolved in DMF and activated by the addition of oxyma (50 equiv) for 4 min at r.t. before the addition of DIC (50 equiv; Sigma-Aldrich) and mixed for 8–10 min at r.t. The solution mixture was then added to nanospheres and suspension mixed on the Thermomixer at 1400 rpm for 14 h at 25 °C to obtain akyne-functionalized nanospheres 10 after centrifugation and subsequently washing with DMF (3 × 1 mL) MeOH (3 × 1 mL), and deionized water (3 × 1 mL). 500 μL aliquots of 10 were washed, suspended in DMF, and centrifuged. Separately, a mixture of DMF:piperidine (4:1) was degassed and a solution of Cul (30 equiv), ascorbic acid (60 equiv), 3 (30 equiv) in 500 μL degassed DMF:piperidine (4:1) under nitrogen bubbling. This mixture was added onto the NPs while bubbling nitrogen, sealed, and mixed overnight in a rotary wheel. Dasatinib-labeled nanospheres, 11 were obtained by centrifugation and subsequently washed with DMF (3 × 500 μL), MeOH (3 × 500 μL), and deionized water (3 × 500 μL).

Baeyer Test for Alkenes. 1 μL of stock KMnO4 solution (2.5 mM) was added to 5 × 105 NPs or 0.5 mg Rink amide resins to a final volume of 10 μL in water. NPs and resins were then centrifuged at 13000 g for 5 min in an Eppendorf centrifuge and absorbance spectra were analyzed. 420 nm absorbance was represented as fold increase to control solution of KMnO4.

Dynamic Light Scattering and Zeta Potential. The hydrodynamic size and zeta potential of intermediate and final nanoparticle configurations were measured on a Malvern Zetasizer Nano-ZS (Malvern, Malvern Hills, UK) in molecular biology grade water in a disposable sizing cuvette for size measurements or clear disposable zeta cuvette for zeta potential measurements.

Kinase Activity Assay. Recombinant full-length human SRC activity was detected by luminescence using ADP-Glo Kinase Assay + SRC Kinase Enzyme System (Promega), according to manufacturer instructions in half area solid white 96-well plates at a total volume of 40 μL. The influence of a range of serial dilutions from 25 × 106 NPs, control NPs, or free drug on SRC activity (0.154 ng/μL) were analyzed by luminescence using a GloMax Multi (Promega) with an integration time of 0.5s and normalized to internal controls.

Cell Culture. MDA-MB-231 cells were cultured in DMEM (Gibco), supplemented with 10% (v/v) FBS (Gibco), 2 mM L-Glutamine (Gibco) in a standard incubator (95% humidity, 5% CO2), and subcultured twice per week. Serum-free medium was used for 6 h during incubations with NPs to prevent nanoparticle aggregation.

Uptake Efficiency. For flow cytometry assays, 5 × 105 MDA-MB-231 cells were seeded in 24-well plates and stabilized in an incubator for 18 h. Subsequently 3000, 1000, 300, 100, 30, 10, 3, or 0 of 9/cell or 11/cell were incubated in 500 μL of serum-free DMEM to evaluate cellular uptake. After 6 h, an equal volume of DMEM containing 2× FBS was added to all wells and cells were allowed to stabilize overnight. After washing with PBS, living cells were trypanized for 5 min, washed twice with PBS, and collected in 96 well plates with round-bottom. Cells were analyzed by flow cytometry with a BD Fortessa X-20 (Becton Dickinson & Co., NJ, USA) equipped with 4 lasers: Blue Laser (488 nm) FSC, Red Laser (640 nm), Violet Laser (405 nm), and a Yellow Green Laser (561 nm) with automated plate loader system BD HTS. The Cy5 emission fluorescence was detected in the APC channel (640–670/30 nm). The data were analyzed using BD FACS software version 8.0.1 for data acquisition and FlowJo (Flowjo, LLC, OR, USA) for data analysis. Gate thresholds were adjusted to non-nanofected cells and 5 nanofected cells and maintained throughout the experiments. Percentages of cells containing 9 or 11 were represented versus log (ratios NP/cell) and adjusted to a sigmoidal dose–response curve using GraphPad Prism Software. MNF50 values were calculated as the ratio NP/cell necessary to obtain a 50% of cells containing nanoparticles.

Confocal Microscopy Colocalization. MDA-MB-231 cells were seeded on 10 μm poly(l-lysine) precoated coverslips in 24 well plates (10 000 cells/well). In parallel, 6 × 105 of 9, 11, 5 or NP-free controls were incubated in 10 μL 5% BSA/H2O overnight at 4 °C to block unspecific binding (Supporting Information). NPs were thoroughly dispersed in a sonication bath for 15 min. Cells were then incubated in the presence or in the absence of 3000 NPs/cell in 500 μL of serum-free DMEM in duplicates. After 6 h, 500 μL of DMEM supplemented with 2× FBS were added to each of the wells and cells were kept in an incubator for another 18 h. NPs and cells were then fixed with paraformaldehyde (4% v/v) for 10 min and washed 3 times with PBS every 5 min. Cells were permeabilized for 15 min with 0.3% Tween/PBS and washed 3 times with PBS every 5 min. Coverslips were incubated in blocking buffer (0.3% BSA, 10% FBS, 5% nonfat dry milk, 0.1% Tween in PBS) for 30 min. Anti-Src Rabbit mAb (Cell Signaling Technology) was incubated in milk-free blocking buffer (0.3% BSA, 10% FBS, 0.1% Tween in PBS) for 2 h at a dilution of 1:400. After washing 3 times in 0.1% Tween/PBS, coverslips were incubated for 30 min with goat anti-rabbit IgG (H+L) secondary antibody, DyLight 405 conjugate (Thermo Fischer Scientific) in milk-free blocking buffer (0.3% BSA, 10% FBS, 0.1% Tween in PBS) at a dilution of 1:400. Coverslips were washed twice with 0.1% Tween/PBS, and mounted with Mowiol (Sigma-Aldrich) on a slide. NPs and SRC localization was imaged using a scanning confocal inverted microscope LSM 710 Axio Observer (Carl Zeiss, Jena, Germany).
images were acquired with a Plan-Apochromat 63x/1.4 OIL DIC M27 immersion objective and software ZEN 2010 (Carl Zeiss, Jena, Germany). Confocal images of 9, 11, S, or NP-free controls in cell lysates and cells were obtained in a sequential mode using the following settings: (1) a 405 nm diode excitation laser (30 mW) at 2% power, an emission of in a range of 410 to 488 nm with a main beam splitter MBS-488/543/633, and a pinhole of 1.0 Airy Unit (0.6 μm optical section); and (2) a 633 nm HeNe excitation laser (5.0 mW) at 15% power, an emission wavelength range of 636—742 nm with a MBS-488/543/633, and a pinhole of 1.0 Airy Unit (0.7 μm optical section). Transmitted images were obtained with a T PMT. Z-stack images were recorded in an average range of 6 μm, 13 slices, and a constant interval of 0.5 μm, according to NP size, to localize 500 nm nanoparticles throughout the cell volume. Images were processed with Software ZEN 2010 Black Edition (Carl Zeiss, Jena, Germany) to obtain overlap coefficient and Pearson’s Correlation Coefficient as colocalization coefficients. Both coefficients, crosshair diagrams, and mask images were obtained after calibration according to negative controls and maintained for all the experiments (SI). Unpaired t test statistical analysis of four independent experiments was performed using Graph Pad Prism 5.0 for Windows GraphPad Software, La Jolla California USA, www.graphpad.com.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.8b00481.

Full synthetic details, characterization spectra, results of control nanoparticles and resins, complementary biological data, confocal microscopy images (PDF)

Time-lapse imaging of cell uptake into MDA-MB-231 cells (AVI)

## AUTHOR INFORMATION

### Corresponding Authors

*E-mail: Asier.Unciti-Broceta@igmm.ed.ac.uk.
*E-mail: rmsanchez@ugr.es.

### ORCID

Asier Unciti-Broceta: 0000-0003-1029-2855

### Present Address

*VivaCell Biotechnology España S.L., Parque Científico Tecnológico de Córdoba (Rabanales 21). C/Astronoma Cecilia Payne, 14014 Córdoba (Spain).

### Author Contributions

All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This project was funded by FP7 cofund Talentia Postdoc under grant agreement 267226 and CEIBioTic CEI2015-MP-B548. AUB and AMPL are grateful to the EPSRC (EP/N021134/1) for funding. RSM is grateful to the Marie Curie Career Integration Grants within the Seventh European Community Framework Programme (FP7-PEOPLE-2011-CIG-Project Number 294142) and the Ministry of Economy and Competitiveness (BIO2016-80519-R) for funding. JDUB thanks Spanish Ministerio de Economia y Competitividad for a Torres Quevedo fellowship (PTQ-13-06046). ADG acknowledges scholarship from the Spanish Ministry of Education (grant FPU14/02181) and the University of Granada, PhD programme in Biomedicine. We are grateful to Dr. B. Rubio Ruiz for her insights in the synthesis of derivative 2. The authors thank SciToons team (scitoons.com) for preparing the graphical abstract.

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