Bioconjugate Chemistry

Communication

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Thiophene-Based Compounds as Fluorescent Tags to Study Mesenchymal Stem Cell Uptake and Release of Taxanes

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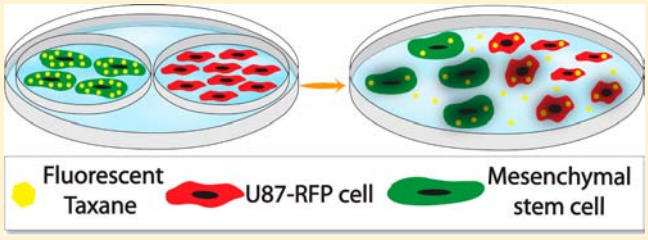
Supporting Information

ABSTRACT: Human mesenchymal stem cells (hMSC) are multipotent cells that display the unique ability to home and engraft in tumor stroma. This remarkable tumor tropic property has generated a great deal of interest in many clinical settings. Recently, we showed that hMSC represent an excellent base for cell-mediated anticancer therapy since they are able to internalize paclitaxel (PTX) and to release it in an amount sufficient to inhibit tumor cell proliferation. In order to shed light on the dynamics of drug uptake and release, in the present paper we describe the synthesis of two novel thiophene-based fluorophore−paclitaxel conjugates, namely PTX-F32 and PTX-F35, as tools for *in vitro* drug tracking. We aimed to study the ability of these novel derivatives to be efficiently internalized by hMSC and, in a properly engineered coculture assay, to be released in the medium and taken up by tumor cells. In order to ensure better stability of the conjugates toward enzymatic hydrolysis, the selected oligothiophenes were connected to the taxol core at the C7 position through a carbamate linkage between PTX and the diamino linker. Antiproliferative experiments on both tumor cells and stromal cells clearly indicate that, in good correlation with the parent compound, cells are sensitive to nanomolar concentrations of the fluorescent conjugates. Moreover, in the coculture assay we were able to monitor, by fluorescence microscopy, PTX-F32 trafficking from hMSC toward glioblastoma U87 tumor cells. Our work paves the way for novel possibilities to perform extensive and high quality fluorescence-based analysis in order to better understand the cellular mechanisms involved in drug trafficking, such as microvescicle/exosome mediated release, in hMSC vehicle cells.



Human mesenchymal stem cells (hMSC) are multipotent cells that can self-renew and at the same time differentiate into multiple lineages. During the past several years, hMSC have generated a great deal of interest in many clinical settings, including regenerative medicine, immune modulation, and tissue engineering.[1] Moreover, hMSC have the unique ability to migrate along chemokine gradients to sites of inflammation/injury and to tumor's stroma, making them useful tools for the targeted delivery of therapeutics to cancer cells.[2−4] To this end, hMSC have been engineered to express proteins that induce tumor cell death,[4,5] or more recently to carry prodrugs,[6] nanoparticle loaded drugs,[2] photosensitizers,[7] and conventional anticancer drugs[8] directly into the tumor.

With regard to the latter application, we recently reported on the ability of hMSC to internalize paclitaxel (PTX) and to release it in an amount sufficient to inhibit tumor cell proliferation.[8] The experiment on tumor cells was conducted by using the 24 h-conditioned medium obtained from hMSC loaded with the drug.

Fluorescent tagged drugs are useful tools to visualize drug trafficking dynamics (cell uptake and release) through fluorescence imaging. Probe brightness and photostability are crucial features to this aim. Moreover, drug conjugation with the fluorochrome should be easily achievable and the steric hindrance generated by the fluorophore should not completely withdraw and/or modify drug activity.

Two of the three reactive hydroxyl groups can be suitably used for paclitaxel derivatization, e.g., C2′ and C7 (Figure 1). The reactivity of these positions follows the sequence C2′ > C7. According to the literature on PTX derivatives,[9,10] the free 2′-hydroxyl group (or an easily hydrolyzable moiety at that position) is required for effective microtubule binding.[11] On the other hand, literature reports describe the general maintenance of cytotoxic activity upon C7 esterification.[12]

Based on these considerations, we selected the commercially available Flutax-2[13] (Figure 1) to attempt the study of PTX trafficking from hMSC toward tumor cells through fluorescence imaging. Flutax-2 is modified at the C7 position with the dye

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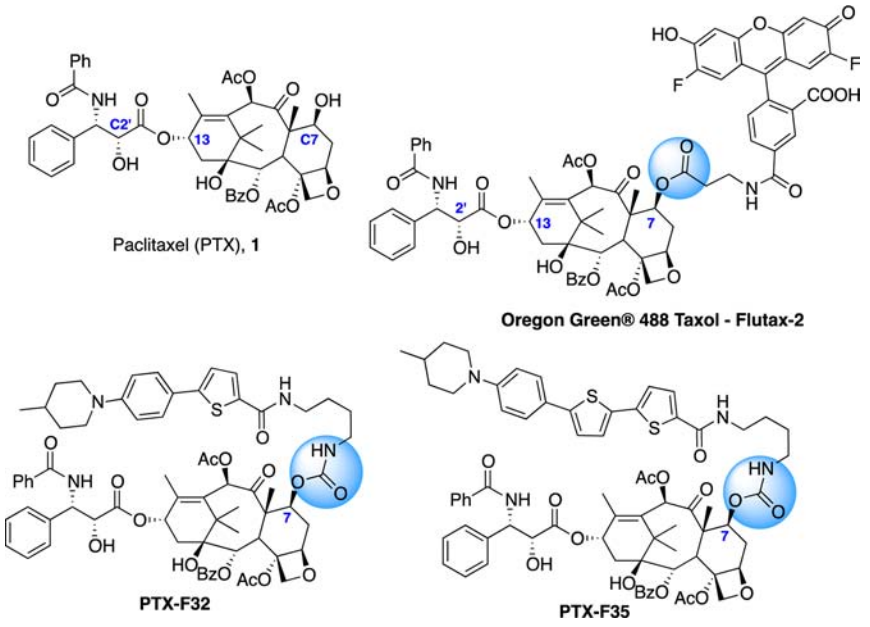
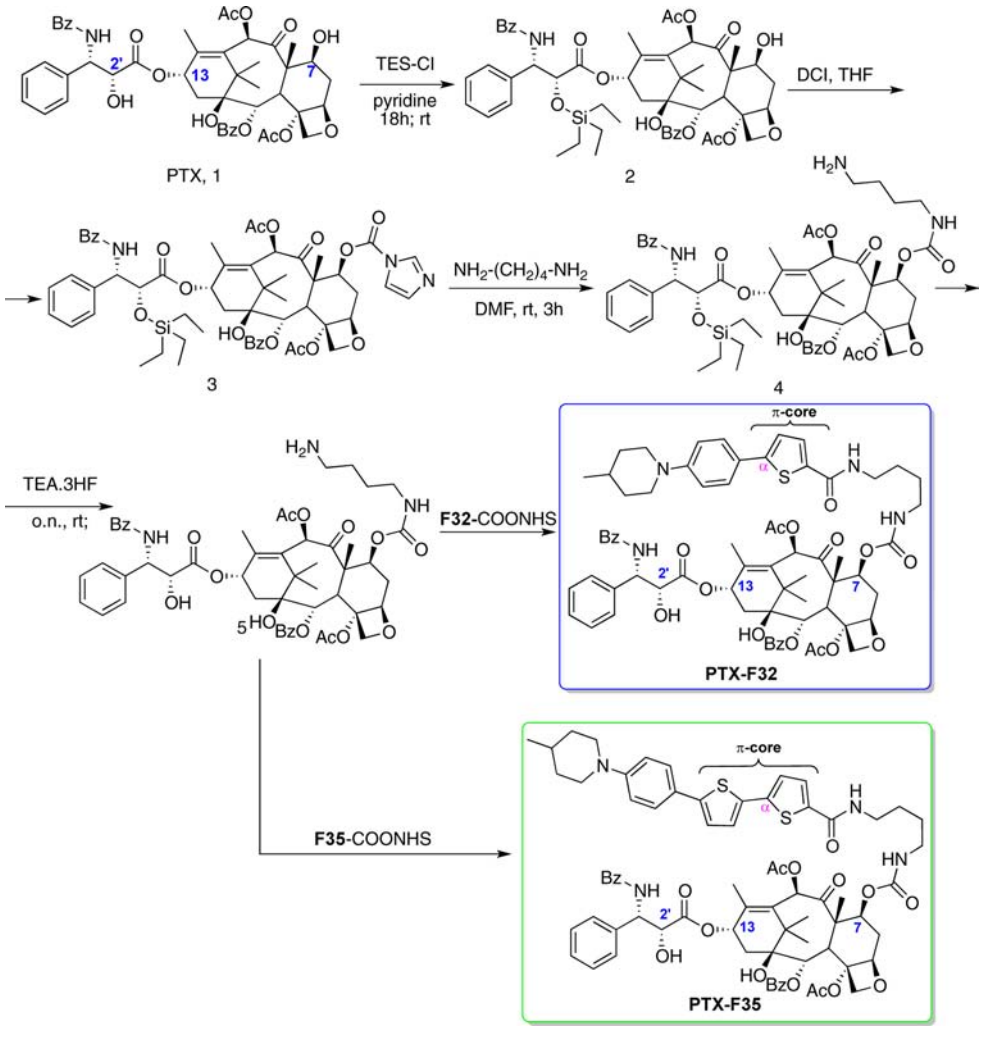


Figure 1. Paclitaxel (PTX), Flutax-2, **PTX-F32**, and **PTX-F35** structures.

Scheme 1. PTX-F32 and PTX-F35 Synthesis



Oregon Green 488 through an ester bond (Figure 1). Unfortunately, in our hands, this compound did not provide the desired results in terms of both fluorescence stability and intracellular localization.[8] In fact, in our work we showed a

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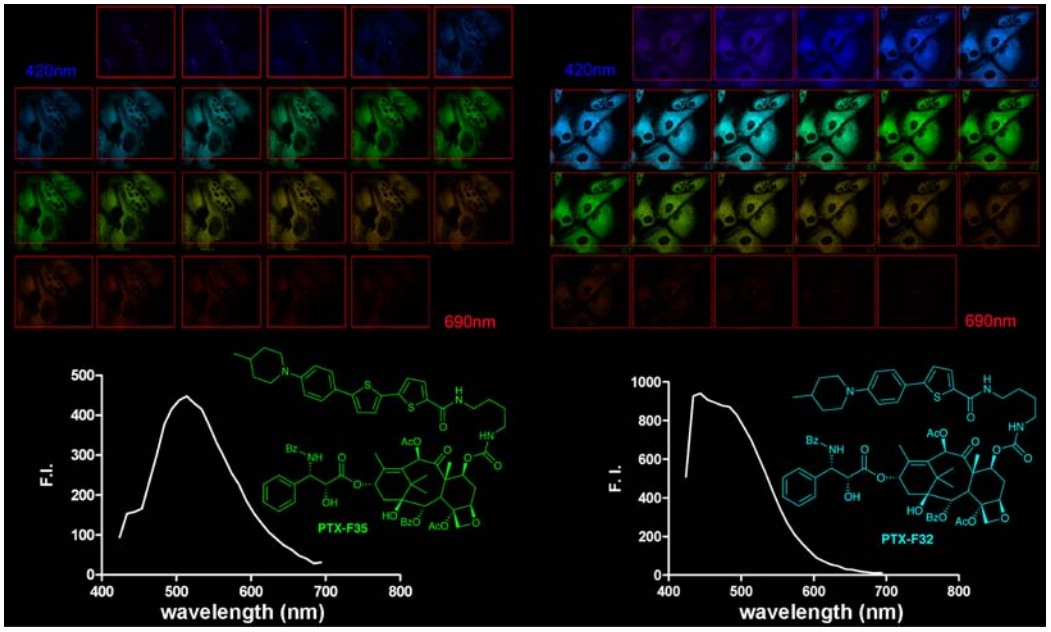

Figure 2. **PTX-F32** and **PTX-F35** spectral analysis performed with confocal laser microscopy and NIS-Elements AR software on loaded hMSC.

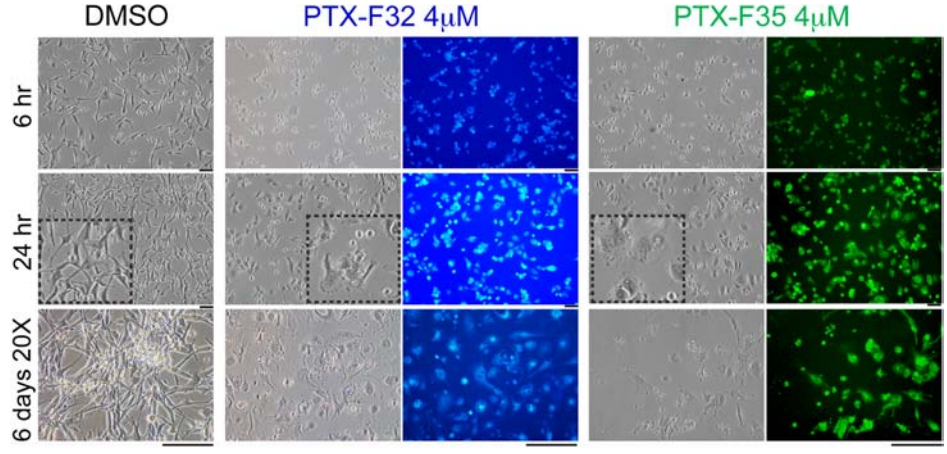

Figure 3. Representative pictures of U87-RFP tumor cells treated with 4 μM PTX-F32 (blue) and 4 μM PTX-F35 (green) and observed from 6 h after drug loading until 6 days after. Scale bar is 50 μm.

main Golgi staining by Flutax-2 instead of a microtubule network distribution. This behavior could be due to the low enzymatic stability of the ester linkage connecting Oregon Green with PTX, which in turn could compromise its proper visualization in our hMSC-tumor cell migration experiments.

Thus, we envisioned the need to design and synthesize novel fluorescent PTX derivatives. To this end we selected oligothiophenes as the fluorescent probes, which are chemically stable molecules characterized by a common fluorescent skeleton that can be suitably functionalized for several different applications including organic electronics[14,15] and biomedical applications.[16] They display intense absorption bands whose wavelength can be tailored to fall anywhere from the UV to the deep-red spectrum by carefully choosing the number of aromatic rings and by introducing suitable substituents at the α positions (Figure 1).[17] Thiophene-based fluorophores (TbF) have already been used for the efficient labeling of proteins, DNA, and cells,[18] while to our knowledge, no reports describe the conjugation of anticancer drugs with TbF. Recently, we reported the synthesis of TbF bearing electron donors and electron-acceptor moieties able to interact through the π-conjugated core (Figure 1).[17] These compounds are characterized by an efficient emission in the blue-orange interval and can be conveniently used to label biologically active molecules.

In this view, we first protected the C2′-hydroxyl of the taxol side chain with triethylsilyl chloride (TES-Cl) and, after activation of the C7-hydroxyl group with carbonyl diimidazole, the C2′-OTES-C7-imidazolyl taxol derivative (**3**) was transformed in the corresponding C2′-OTES-C7-(4-aminobutyl carbamoyl)-taxol derivative (**4**) using 1,4-diaminobutane. 2′-OH TES deprotection followed by fluorophore amidation, e.g., **F32** and **F35**,[17] afforded the desired fluorescent taxoids, **PTX-F32** and **PTX-F35** (Scheme 1), in acceptable overall yields (see ESI for experimental details). The normalized absorption and emission spectra of **PTX-F32** and **PTX-F35** have been recorded in CH_2Cl_2 and are depicted in Figure S3. Confocal microscopy spectral analysis detected comparable emission spectra profile in hMSC loaded for 24 h with the two compounds (Figure 2). A carbamate linkage between PTX and the diamino linker was intentionally selected because of its

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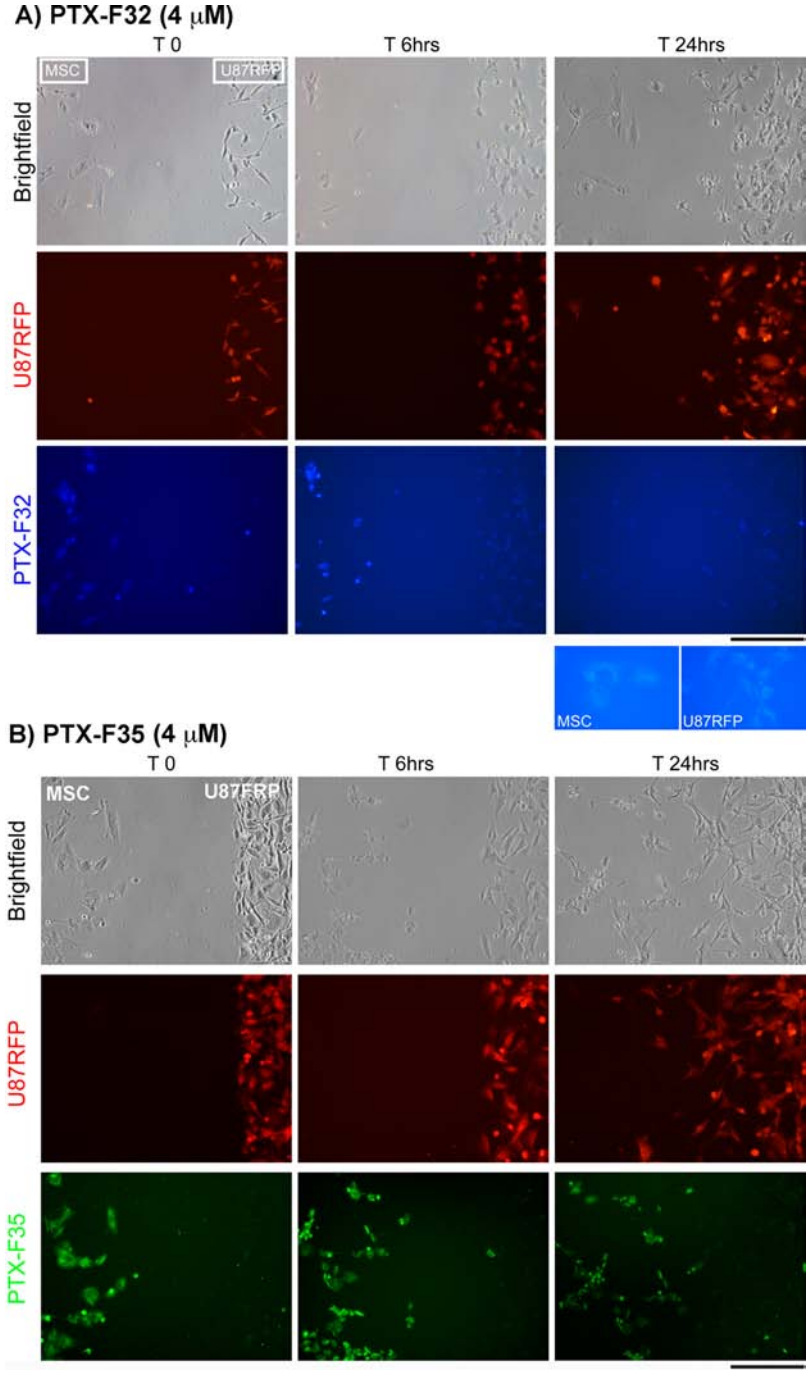

Figure 4. Co-culture assay between hMSC and U87-RFP tumor cells. Time-lapse epifluorescence imaging of hMSC primed for 24 h with PTX-F32 (panel A) and with PTX-F35 (panel B) co-cultured with U87-RFP cells. hMSC have been plated on the left inserts; U87-RFP on the right ones. Scale bar is 100 μm.

higher stability toward enzymatic hydrolysis as compared, for example, to the corresponding ester.[19] It is worth mentioning that optical properties of TbFs were not substantially altered upon conjugation with PTX,[17] and the observed hypsochromic-shift is in agreement with literature data.[20] In particular, large Stokes shifts are observed for the two novel derivatives, i.e., 108 and 142 nm for **PTX-F32** and **PTX-F35**, respectively, while they showed good photostability under constant illumination with a fluorescent microscope excitation source (data not shown).

We then evaluated the proliferation inhibition rate of our compounds on four different cell lines, e.g., human skin-derived fibroblasts (hSDFs), widely recognized as carriers for cancer therapy,[21] hMSC freshly isolated from two donors (hMSC1, hMSC2), human T-cell leukemia MOLT-4, and the glioblastoma U87-RFP cell line (Table S1). The generally lower activity of **PTX-F35** and **PTX-F32** compared to PTX can be ascribed to a slightly modified engagement of the taxanes to microtubules. However, our synthesized fluorescent conjugates showed that modification at the C7 position reduces but does not abrogate their antiproliferative activity. In fact, in good correlation with native PTX, both tumor cells and stromal cells proved to be sensitive to nanomolar concentrations of the fluorescent conjugates.

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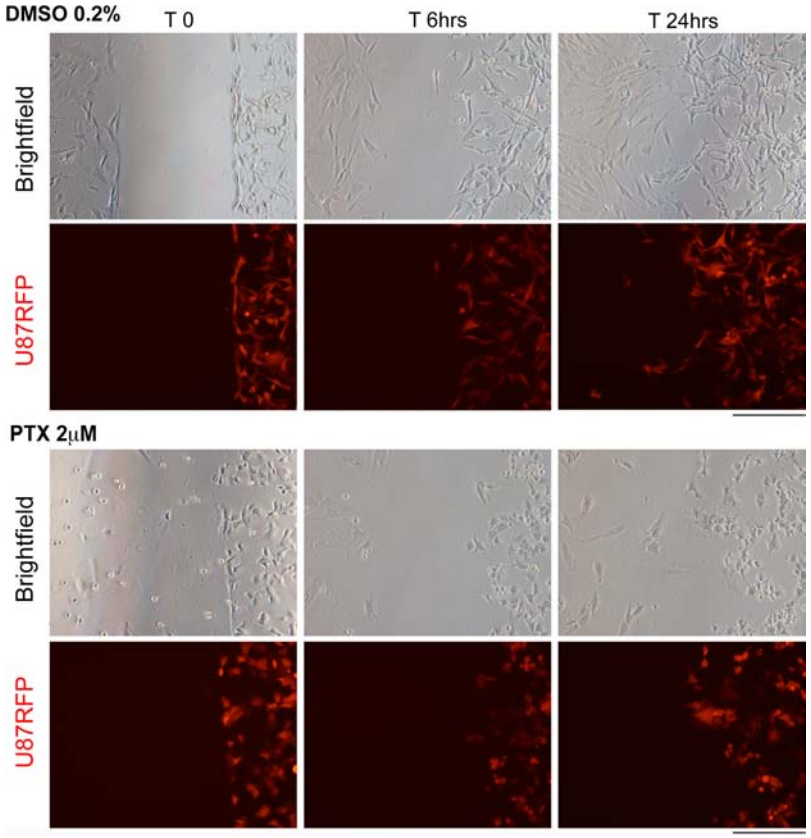

Figure 5. Co-culture assay between hMSC and U87-RFP tumor cells. Time-lapse epifluorescence imaging of hMSC primed for 24 h with vehicle DMSO 0.2% (top panels) and with 2 μM PTX (bottom panels) co-cultured with U87-RFP cells. hMSC have been plated on the left inserts; U87-RFP on the right one. Scale bar is 100 μm.

Fluorescence imaging was then used to investigate the compounds' behavior in tumor cells. To this end, we treated U87-RFP glioblastoma cells with 4 μM **PTX-F32** (blue in Figure 3), with 4 μM **PTX-F35** (green in Figure 3) and PTX 4 μM (Figure 3) as the control. Images were acquired starting from 6 h after the compound initial loading over time up to 6 days in cell culture. In spite of the results obtained with the MTT assay (Table S1), we used a higher PTX-F concentration in order to better visualize their intra- and subcellular localization with conventional fluorescence microscopy.[11] We observed an increase in fluorescence level within the incubation time from 6 h (29.2 ± 7.1 and 20.2 ± 6.5 mean fluorescence intensity **PTX-F32** and **PTX-F35**, respectively) up to 24 h (39.2 ± 13.3 and 34.5 ± 9.6 mean fluorescence intensity **PTX-F32** and **PTX-F35**, respectively). At this time point tumor cells were efficiently stained while their morphology appeared strongly affected (see magnifications in Figure 3). After several days, cells still showed a good intracellular fluorescence signal (see 6 days in Figure 3), while the morphology was highly compromised. These results demonstrate that our compounds retain an acceptable cytotoxic activity toward tumor cells, while their fluorescence is stable enough to perform long-time experiments. Fluorescence imaging was then used to investigate the compounds' trafficking between hMSC and U87-RFP tumor cells. For all the experiments described in the following section, hMSC are intended to be hMSC1, which resulted in lower sensitivity to PTX and PTX-F treatment (Table S1). To this aim, we first evaluated whether the novel PTX-F conjugates were efficiently internalized by hMSC. Accordingly, hMSC were exposed to **PTX-F32** and **PTX-F35** (4 μM) for 24 h and immunostained for confocal microscopy imaging. β-Tubulin staining demonstrated that **PTX-F32** distributes along the microtubule networks (white arrows in Figure S4) and in vesicle-like structures (white asterisks in Figure S4), and not only in Golgi apparatus as previously observed with Flutax-2.[8] Moreover, the internalization of the fluorescently labeled PTX derivatives did not strongly affect β-tubulin organization even if a rounded-shaped morphology was observed for treated cells (Figure S5).

Finally, we optimized an *in vitro* assay to determine whether our fluorescent taxanes are valuable tools for visualizing their migration through cells. This model mimics the physiologic hMSC-tumor cells interaction in a 2D system. We therefore primed hMSC with 4 μM **PTX-F32** and **PTX-F35** for 24 h, and meanwhile, we co-plated U87-RFP cells, keeping them initially separated by inserts. After the 24 h of incubation, hMSC were washed several times to eliminate potential unloaded drugs, and inserts were removed, making hMSC free to migrate toward tumor cells (Figure 4). Time-lapse imaging was performed over 24 h. Non-conjugated PTX was used as the control (Figure 5), confirming it to be efficiently released from hMSC, since co-cultured tumor cells appear to be strongly affected (Figure 5).[8,21] As shown in Figure 4A, the time-lapse imaging of **PTX-F32** loaded hMSC revealed that the fluorescent taxane was released and uploaded by tumor cells within 6 h. After 24 h observation, U87-RFP cells become round-shaped and detach from plate differently from DMSO control and similarly to PTX loaded hMSC (Figure 5). It is worth noting that after 24 h both hMSC and U87-RFP are still equally stained (see magnifications of Figure 4A), but **PTX-F32** fluorescence results were

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much weaker compared to those at time 0. These data confirm that **PTX-F32** has been released from hMSC in the medium and taken up by tumor cells. The same experiment performed with **PTX-F35** loaded hMSC revealed that the compound is not completely released by the host cells within 24 h (Figure 4B). Right after incubation, hMSC start to suffer and detach, being unable to efficiently transfer the drug to U87-RFP cells that in turn are motile, proliferative, and only slightly fluorescent (Figure 4B). In order to verify that this behavior was not caused by the higher potency of **PTX-F35** compared to **PTX-F32** (Table S1), we decided to repeat the experiment using a lower concentration of **PTX-F35** (e.g., 2 μM instead of 4 μM). However, even under these conditions **PTX-F35** was only barely able to cross over the medium to reach U87-RFP cells, which after 24 h were faintly stained displaying only weakly compromised morphology (Figure S6).

In summary, we described the synthesis of novel thiophene based fluorophore−paclitaxel conjugates as promising tools for *in vitro* drug tracking. We demonstrated that such compounds could be effectively internalized in several cell lines. Further, in the case of **PTX-F32**, we were able to monitor, by fluorescence microscopy, the fluorescent taxane trafficking from hMSC toward tumor cells. The different behavior of **PTX-F35** loaded hMSC co-cultured with U87 cells could be ascribed either to a different mechanism of action or, more likely, to the specific nature of the thiophene fluorophore that might induce aggregate formation through π-stacking interactions. However, the high tunability of these fluorophores in terms of chemical and photochemical properties, paves the way to future investigations in the field. In view of the high expectations of hMSC use as drug carriers, our work suggests a novel possibility to perform extensive and high quality fluorescence-based analysis to better understand the cellular mechanisms involved in drug trafficking, such as microvesicle/exosome mediated release.[22]

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, spectroscopic data, and copies of $^{1}H/^{13}C$ NMR of new compounds; additional figures and tables on biological experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

S. Duchi, P. Dambruoso, and E. Martella equally contributed to this work.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PTX, Paclitaxel; hMSC, human Mesenchymal Stem Cell; Thiophene-based fluorophore, TbF

■ REFERENCES

(1) Caplan, A. I. (2007) Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J. Cell. Physiol. 213*, 341−7.
(2) Gao, Z., Zhang, L., Hu, J., and Sun, Y. (2013) Mesenchymal stem cells: a potential targeted-delivery vehicle for anti-cancer drug, loaded nanoparticles. *Nanomedicine 9*, 174−84.
(3) Studeny, M., Marini, F. C., Champlin, R. E., Zompetta, C., Fidler, I. J., and Andreeff, M. (2002) Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res. 62*, 3603−8.
(4) Kauer, T. M., Figueiredo, J.-L., Hingtgen, S., and Shah, K. (2012) Encapsulated therapeutic stem cells implanted in the tumor resection cavity induce cell death in gliomas. *Nat. Neurosci. 15*, 197−204.
(5) Loebinger, M. R., Eddaoudi, A., Davies, D., and Janes, S. M. (2009) Mesenchymal stem cell delivery of TRAIL can eliminate metastatic cancer. *Cancer Res. 69*, 4134−42.
(6) Gjorgieva, D., Zaidman, N., and Bosnakovski, D. (2013) Mesenchymal stem cells for anti-cancer drug delivery. *Recent Pat. Anticancer Drug Discovery 8*, 310−318.
(7) Duchi, S., Sotgiu, G., Lucarelli, E., Ballestri, M., Dozza, B., Santi, S., Guerrini, A., Dambruoso, P., Giannini, S., Donati, D., and Varchi, G. (2013) Mesenchymal stem cells as delivery vehicle of porphyrin loaded nanoparticles: Effective photoinduced in vitro killing of osteosarcoma. *J. Controlled Release 168*, 225−237.
(8) Pessina, A., Bonomi, A., Coccè, V., Invernici, G., Navone, S., Cavicchini, L., Sisto, F., Ferrari, M., Viganò, L., Locatelli, A., Ciusani, E., Cappelletti, G., Cartelli, D., Arnaldo, C., Parati, E., Marfia, G., Pallini, R., Falchetti, M. L., and Alessandri, G. (2011) Mesenchymal stromal cells primed with paclitaxel provide a new approach for cancer therapy. *PLoS One 6*, e28321.
(9) Nicolaou, K. C., and Valiulin, R. A. (2013) Synthesis and biological evaluation of new paclitaxel analogs and discovery of potent antitumor agents. *Org. Biomol. Chem. 11*, 4154−63.
(10) Gropeanu, R. A., Baumann, H., Ritz, S., Mailänder, V., Surrey, T., and Del Campo, A. (2012) Phototriggerable 2′,7-caged paclitaxel. *PLoS One 7*, e43657.
(11) Mastropaolo, D., Camerman, a, Luo, Y., Brayer, G. D., and Camerman, N. (1995) Crystal and molecular structure of paclitaxel (taxol). *Proc. Natl. Acad. Sci. U. S. A. 92*, 6920−4.
(12) Fu, Y., Li, S., Zu, Y., Yang, G., Yang, Z., Luo, M., Jiang, S., Wink, M., and Efferth, T. (2009) Medicinal chemistry of paclitaxel and its analogues. *Curr. Med. Chem. 16*, 3966−3985.
(13) http://www.lifetechnologies.com/order/catalog/product/P22310.
(14) Durso, M., Gentili, D., Bettini, C., Zanelli, A., Cavallini, M., De Angelis, F., Grazia Lobello, M., Biondo, V., Muccini, M., Capelli, R., and Melucci, M. (2013) π-Core tailoring for new high performance thieno(bis)imide based n-type molecular semiconductors. *Chem. Commun. (Camb.) 49*, 4298−300.
(15) Oliva, M. M., Casado, J., Raposo, M. M. M., Fonseca, A. M. C., Hartmann, H., Hernández, V., and López Navarrete, J. T. (2006) Structure-property relationships in push-pull amino/cyanovinyl end-capped oligothiophenes: quantum chemical and experimental studies. *J. Org. Chem. 71*, 7509−20.
(16) Duca, M., Dozza, B., Lucarelli, E., Santi, S., Di Giorgio, A., and Barbarella, G. (2010) Fluorescent labeling of human mesenchymal stem cells by thiophene fluorophores conjugated to a lipophilic carrier. *Chem. Commun. (Camb.) 46*, 7948−50.
(17) Sotgiu, G., Galeotti, M., Samorí, C., Bongini, A., and Mazzanti, A. (2011) Push-pull amino succinimidyl ester thiophene-based fluorescent dyes: synthesis and optical characterization. *Chemistry 17*, 7947−52.
(18) Zambianchi, M., Barbieri, A., Ventola, A., Favaretto, L., Bettini, C., Galeotti, M., and Barbarella, G. (2007) Testing oligothiophene fluorophores under physiological conditions. Preparation and optical characterization of the conjugates of bovine serum albumin with oligothiophene N-hydroxysuccinimidyl esters. *Bioconjugate Chem. 18*, 1004−9.

(19) Simplício, A. L., Clancy, J. M., and Gilmer, J. F. (2008) Prodrugs for amines. *Molecules 13*, 519−547.
(20) Piacenza, M., Zambianchi, M., Barbarella, G., Gigli, G., and Della Sala, F. (2008) Theoretical study on oligothiophene N-succinimidyl esters: size and push-pull effects. *Phys. Chem. Chem. Phys. 10*, 5363−73.
(21) Pessina, A., Coccè, V., Bonomi, A., Cavicchini, L., Sisto, F., Ferrari, M., Ciusani, E., Navone, S., Marfia, G., Parati, E., and Alessandri, G. (2013) Human skin-derived fibroblasts acquire in vitro anti-tumor potential after priming with Paclitaxel. *Anticancer Agents Med. Chem. 13*, 523−30.
(22) Lai, R. C., Chen, T. S., and Lim, S. K. (2011) Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. *Regen. Med. 6*, 481−92.