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**Novel synthesis of fluorochrome-coupled zoledronate with preserved functional activity on gamma/delta T cells and tumor cells**

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# Electronic Supplementary Information

## Experimental Section

### Synthesis of FluorZOL (see Figure 2):

Zoledronate (**4**, (2-imidazol-1-yl)-hydroxy-ethylidene-1,1-bisphosphonic acid, disodium salt) was a kind gift from Novartis Pharma AG, Basel, Switzerland. 5(6)-Carboxyfluorescein succinimidyl ester was purchased from Sigma Aldrich (Munich, Germany). Thin layer chromatography was performed on silica gel plates (GF 254, Merck). Flash chromatography was performed on silica gel 60 (Merck, 230-400 mesh, particle size 0.040-0.063 mm). For the purification of the zoledronate derivative **8**, the Biotage – Isolera One™ flash system was employed using a reversed-phase KP-C18-HS column. $^{1}H$, $^{13}C$ and $^{31}P$ NMR spectra were recorded on Bruker DRX-500 and AV-600 spectrometers at 300 K. Chemical shifts are reported relative to internal tetramethylsilane ($\delta = 0.00$ ppm) or $D_2O$ ($\delta = 4.76$ ppm). Full assignment of NMR spectra was achieved with the aid of 2D NMR techniques. IR spectra were measured with a Perkin Elmer FT-IR Paragon 1000 (ATR) spectrometer. ESI mass spectra were recorded on Mariner instrument 5280. MALDI-TOF mass spectra were recorded on a Bruker Biflex III instrument with 19 kV acceleration voltage and an ionization laser at 337 nm. As matrix 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid were used.

***tert*-Butyl *N*-allyl carbamate (2)**[1] To a solution of allylamine **1** (1.75 g, 30.7 mmol) in dry $CH_2Cl_2$ (10 ml), a solution of $(Boc)_2O$ (6.54 g, 30.7 mmol) in $CH_2Cl_2$ (20 ml) was added at 0

°C and the reaction mixture was stirred at ambient temperature for 4 h. Then, it was diluted with $CH_2Cl_2$ (25 ml), washed with 5% citric acid (10 ml) and brine (10 ml). The organic phase was dried over $Na_2SO_4$, it was filtered and the filtrate evaporated under reduced pressure. Purification of the crude product by column chromatography (cyclohexane/ ethyl acetate, 1:4) gave 3.81 g (79%) of **2** as a white solid. mp 38 °C; $R_f$ =0.42 (cyclohexane/ethyl acetate,1:4); $^1$H NMR (300 MHz, $CDCl_3$): δ = 1.42 (s, 9H), 3.72 ($m_c$, 2H), 4.68 (bs, 1H), 5.18-5.06 (m,2H), 5.81 (m, 1H) ppm; $^{13}$C NMR (75 MHz, $CDCl_3$): δ 28.3, 43.0, 79.3, 115.6, 134.9, 155.8 ppm; ESI MS (*m/z*): $[M+Na]^+$ calcd for $C_8H_{15}NO_2$: 180.11; found 180.08; IR (ATR): ṽ = 3340, 2979, 1676, 1524, 1363, 1251, 1162 $cm^{-1}$.

***tert*-Butyl *N*-(2-oxiranylmethyl) carbamate (3)**[1] The Boc-protected amine **2** (1.00 g, 6.37 mmol) was dissolved in dry $CH_2Cl_2$ (50 ml) and *m*CPBA (2.80 g, 12.7 mmol) was added portion-wise over about 20 min at 0 °C. Then, stirring was continued at ambient temperature overnight (~ 16 h). The reaction mixture was diluted with $CH_2Cl_2$ (100 ml), then washed with 10% $Na_2SO_3$ (20 ml) and water (20 ml). The organic phase was dried over $Na_2SO_4$, it was filtered and the filtrate was concentrated under reduced pressure to get 1.00 g (91%) of the title epoxide **3** as colourless oil. $R_f$ = 0.18 (cyclohexane/ethylacetate,1:4); $^1$H NMR (500 MHz; $CDCl_3$): δ = 4.76 (bs, 1H), 3.51 (d, *J* = 12.1 Hz, 1H), 3.20 (ddd, *J* = 5.1 Hz, *J* = 6.3 Hz, *J* = 14.7 Hz, 1H), 3.07 ($m_c$, 1H), 2.76 (dd, *J* = 4.1 Hz, *J* = 4.7 Hz, 1H), 2.57 (dd, *J* = 2.7 Hz, *J* = 4.8 Hz, 1H), 1.43 (s, 9H) ppm; $^{13}$C NMR (125 MHz; $CDCl_3$) δ 155.9, 79.6, 50.8, 44.9, 41.7, 28.3 ppm; ESI MS (*m/z*): $[M+Na]^+$ calcd for $C_8H_{15}NO_3$: 196.11; found 196.06; IR (ATR): ṽ = 3348, 2977, 2931, 1694, 1516, 1366, 1247, 1162 $cm^{-1}$.

***N*$^3$-{3´´-[3´/4´-carboxy-2´-(3-hydroxy-6-oxo-xanthen-9-yl)benzoylamido]-2´´-hydroxypropyl}-*N*$^1$-(2-hydroxy-2,2-diphosphonoethyl)imidazolium (8)** The disodium salt of zoledronate (**4**, 200 mg, 0.629 mmol) was dissolved in water (4 ml, pH = 7.3) and the pH adjusted to 6.2 using 1 N aq HCl. Then, a solution of epoxide **3** (108 mg, 0.624 mmol) in MeOH (0.5 ml) was added and the reaction mixture stirred at 40 °C for 19 h. It was evaporated reduced pressure to get the crude coupling product **5** as a white solid (272 mg) which was used in the next reaction step without further purification. Thus, crude **5** (272 mg) was dissolved in a TFA-water mixture (1:1, 5 ml) and the reaction mixture was stirred at ambient temperature for 4 h. Then it was evaporated to dryness and the residue washed with diethyl ether (10 ml) and after another evaporation step amine **6** was obtained as a white solid (204 mg), which was used in next step without further purification.

Under exclusion of ambient ligh the intermediate **6** (200 mg) was dissolved in 0.1 N aq $NaHCO_3$ (l ml, pH 7.1) and the pH adjusted to 8.3 with 0.1 N aq NaOH. Then a solution 5(6)-carboxyfluorescein succinimidyl ester (**7**, 40 mg, 84.6 mmol) in DMF (200 µl) was added. Immediately the reaction mixture turned into a dark orange-red solution with some precipitation occurring. The pH was adjusted to 8.1 with 0.1 N aq NaOH to get a homogenous reaction mixture and then stirring was continued at ambient temperature for 3 h. The solvents were evaporated under reduced pressure to get the crude product, which was purified by reversed-phase column chromatography (methanol/water, water 0 % → 50 %) to yield 178 mg (21% over three steps) of the fluorescently labeled conjugate **8** as an orange-red amorphous solid. $^1$H NMR (600 MHz; $D_2O$): δ = 8.68 (s, 1H), 7.46-7.38 (m, 3H), 7.19 (d, *J* = 9.7 Hz, 2H), 7.16-7.11 (m, 2H), 6.64-6.59 (m, 4H), 4.83 ($m_c$, 1H), 4.58-4.55 (m, 2H), 3.82 (dd, *J* = 2.9 Hz, *J* = 12.8 Hz, 1H), 3.72-3.61 (m, 2H), 3.41 (dd, *J* = 6.6 Hz, *J* = 9.2 Hz, 1H) ppm; $^{13}$C NMR (150 MHz; $D_2O$) δ =180.4, 179.5, 163.2, 162.9, 159.1, 153.6, 136.7, 131.4, 123.3, 122.9, 121.2, 117.3, 115.4, 103.7, 77.8, 62.2, 52.3, 41.6 ppm; $^{31}$P NMR (202 MHz; $D_2O$) δ = 14.85, 14.14 ppm; MALDI-TOF MS (*m/z*): $[M+Na]^+$ calcd for $C_{29}H_{28}N_3O_{14}P_2$: *m/z* 725.10; found 725.27; IR (ATR): ṽ = 3350, 1678, 1676, 1393, 1204, 1130, 723 $cm^{-1}$.

**Activation of γδ T cells by FluorZOL**:

PBMC were obtained from blood of healthy donors by Ficoll-Hypaque density centrifugation. Informed consent was obtained from all donors. The studies were performed in compliance with the relevant laws and institutional guidelines, and were approved by the Ethics Committee of the Medical Faculty of University of Kiel. PBMC were cultured at $10^5$ cells per well in 96-well round bottom plates in RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum. Cell cultures were further supplemented with titrated concentrations of ZOL or FluorZOL and 50 U/ml IL-2. Bromohydrin pyrophosphate (BrHPP, kindly provided by Innate Pharma, Marseille, France) was used as a positive control for γδ T cell expansion.[2] After 7 days, the absolute number of viable γδ T cells per microculture well was measured by flow cytometry as previously reported.[3] Briefly, cells contained in microculture wells were stained with PE-labeled anti-Vδ2 monoclonal antibody (Biolegend, Fell, Germany). After washing steps, a defined number of „standard cells“ (fixed lymphocytes that had been stained with FITC-labeled antibodies directed against HLA class I [clone w6/32] and T-cell receptor αβ [clone BMA031] 0.2 µg/ml plus propidium iodide) were added and served as the standard to calculate absolute numbers of viable Vδ2 cells per well.[3] To generate short term lines of γδ T cells, PBMC ($1x10^6$ per ml) were cultured in the presence of 2.5 µM ZOL and 50 U/ml IL-

2. Il-2 was freshly supplemented every other day. After 12 to 14 days, these cultures contained >90% γδ T cells, and were used as effector cells in a Real Time Cell Analyzer (RTCA) and cytotoxicity assays.[4]

**Tumor cells and FluorZOL uptake**:

The following human tumor cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany): monocytic leukemia THP-1 (ACC 16), histiocytic lymphoma U-937 (ACC 5), Burkitt lymphoma Daudi (ACC 78), Burkitt lymphoma Raji (ACC 319), erythroleukemia K-562 (ACC 10). Breast carcinoma cell lines MDA-MB231 (ATCC-HTB-26) and MCF7 (ATCC-HTB-22) were obtained from Amercian Type Culture Collection (LGC Standards, Wesel, Germany). Pancreatic ductal adenocarcinoma cell lines Panc89, PancTu-I and Colo357 were kindly provided by Prof. H. Kalthoff (Institute of Experimental Oncology, University of Kiel). All cell lines were kept in RPMI 1640/10% fetal calf serum. To investigate the uptake of FluorZOL, tumor cells ($5x10^5$ per ml) were cultured for 4 and 24 hrs in 6-well culture plates in the absence or presence of titrated concentrations of FluorZOL. Thereafter, adherent cells were briefly (10 min) trypsinized, washed twice and resuspended in 1% paraformaldehyde. Trypsinization was omitted for suspension cells. Fluorescence was analyzed on a FACS Calibur flow cytometer (BD Biosciences, Heidelberg, Germany) using the CellQuestPro software. In some experiments, FluorZOL uptake was additionally investigated by ImageStream cytometry. To this end, MDA-MB231 cells were cultured for 20 hrs in the absence or presence of 20 µM FluorZOL and 10 µg/ml TRITC-dextran as described above. Dead cells were stained with the LIVE/DEAD fixable far red dead cell stain kit (Life Technologies) following the manufacturer's protocol. After washing, fluorescence images of 10.000 cells per sample were acquired with an Amnis Imagestream MK2 (EMD Millipore) employing 40x objective lenses. Brightfield images were acquired in Channel 1. FluorZOL, TRITC-dextran and the far red dye were excitated at 488 nm, 561 nm and 642 nm, respectively. The corresponding fluorescence was detected in Channel 2, Channel 3 and Channel 5, respectively. For analysis, the files were merged and fluorophores were compensated employing Amnis IDEAS software and a compensation matrix generated from single-stained samples. Only single, viable and focussed cells were included in the analysis. Cells with a RMS gradient >50 were considered focussed. Single cells were identified by size and aspect ratio based on brightfield images. Far red dye-negative cells were considered viable.

**Modulation of tumor cell sensitivity to γδ T cell cytotoxicity by FluorZOL:**

Two independent assays were used to monitor killing of tumor celly by γδ T cells. The impedance of adherent PancTu-I and Colo357 tumor cells (5000 per well in 96-well micro-E-plate) as target cells was monitored with the Real Time Cell Analyzer (RTCA; ACEA, Bremen).[4] Tumor cells were pretreated for 24 hrs with 5 μM ZOL or FluorZOL or medium before 100.000 short-term γδ T cell line cells (see above) were added. BrHPP was added as a positive control for γδ T cell activation, and wells containing 1% Triton-X-100 served as control for maximal lysis. Whenever γδ T effector cells induced lysis of the tumor cells, the loss of impedance of tumor cells was recorded. The cells were monitored every minute for 6 hrs and, thereafter, every 5 min for up to additional 20 hrs. In an independent approach, γδ T cell-resistant THP-1 acute monocytic leukemia cells were pretreated for 20 hrs with 100 μM ZOL or FluorZOL or medium. After extensive washing, the THP-1 cells were labeled for 30 min at 37° C with 1 μl calcein AM (Life Technologies).[5] After washing, the labeled target cells were cocultured with short-term cultured γδ T cell lines (see above) at various effector:target (E/T) cell ratios. Wells containing 1% Triton-X-100 served as control for maximal lysis. After 4 hrs, 100 μl of supernatant were transferred to non-binding black μCLEAR® 96 well microplates (Greiner bio-one, Frickenhausen, Germany) and fluorescence intensities were measured using a Tecan microplate reader at 490 nm with reference filter at 530 nm. % specific lysis was calculated as follows: $OD_{experimental} - OD_{spontaneous}/OD_{maximal} - OD_{spontaneous}$ x 100.

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**Legends to Supplemental Figures**

**Supplemental Figure 1: Uptake of FluorZOL by additional tumor cells**. Adherent tumor cells Colo357, Panc89 (both pancreatic ductal adenocarcinomas), MCF7 and MDA-MB231 (both breast carcinomas) (top) and suspension cell lines Daudi (Burkitt lymphoma), K-562 (erythroleukemia), and THP-1 (acute monocytic leukemia) were cultured for 4 (left) and 24 hrs (right in the absence (thin grey line) or presence of 3.1 (dotted line), 12.5 (thin black line) or 50 μM (thick black line) FluorZOL. After washing, the cells were resuspended in 1% paraformaldehyde and fluorescence intensity was measured on a FACS Calibur flow cytometer.

**Supplemental Figure 2: Effects of ZOL and FluorZOL on tumor cell growth**. To investigate direct effects of FluorZOL on tumor cells, tumor cells were cultured at $5x10^4$ per microculture well in RPMI 1640/10% fetal calf serum and titrated concentrations of ZOL and FluorZOL as indicated. After 1, 2 and 3 days, the viability of tumor cells was quantified by the cleavage of the tetrazolium salt XTT following the recommendations of the manufacturer (Cell Proliferation Kit II; Roche Diagnostics, Mannheim, Germany). Results are presented as mean values of triplicate determinations of optical density (O.D). Qualitatively similar results were obtained in two additional experiments.