Supporting Information

Targeting translation activity at the ribosome interface with UV-active small molecules

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Chemical synthesis of 3PA, 3PB and 3PC

Conversion 1 + 2 → 3 (Scheme 1)

A solution of 100 mL (d 1.015 g/mL) of BOC anhydride (100.86 gr di-tert-butyl-dicarbonate 2) in CHCl₃ was added in 2 h to a solution of the diethanamine 1 (7.5 gr) in CHCl₃ (343ml) maintaining a temperature in the range of 0-5 °C; the final solution (443 mL) was allowed to warm to 15-18 °C and kept overnight at the same temperature. The crude reaction was then washed with a saturated solution of NaHCO₃ (2x300 mL) and with saturated brine (2x300 mL), the organic phase was dried on MgSO₄, evaporated in vacuo at 40 °C to provide 6.9 g of pure mono-NBOC derivative 3 (80.6% yield).

NMR of 3 (CDCl₃) : 5.21 (brs, -N\textsubscript{HCO}, 1H), 3.62 (s, -OCH\textsubscript{2}CH\textsubscript{2}O-, 4H), 3.55 (brt, 5.2 Hz, -OCH\textsubscript{2}CH\textsubscript{2}NHCO, 2H), 3.52 (t, 5.1 Hz, -OCH\textsubscript{2}CH\textsubscript{2}NH\textsubscript{2} , 2H), 3.32 (q, , 5.2 Hz, -CH\textsubscript{2}-NHCO, 2H), 2.88 (t, 5.2Hz, -CH\textsubscript{2}-NH\textsubscript{2} , 2H), 1.44 (s, (Me)\textsubscript{3}-BOC, 9H).

Conversion 3 + 4 → 5 (Scheme 1)

A solution of equimolar amount of amine 3 (1.938 g) and 3-(prop-2-ynyloxy)propanoic acid 4 (1.00 g), in acetonitrile (40 mL) and DMAP (2 equivalent) followed by addition of EDC (1 equivalent) was stirred at 19-21 °C overnight, after which it was evaporated in vacuo at 40 °C, stirred in ether (50mL) and re-acidified to pH 1 with 2M HCl (aq). Then the solution was extracted with EtOAc (50mL), DCM (2x 50mL), EtOAc (50mL). The organic extracts were combined, washed with sat NaHCO₃ , dried (MgSO4) and evaporated in vacuum at 40 °C to provide 5 (oil, 2.00 g, yield 71.5%)

NMR of 5 (CDCl₃): δ 6.40 (brs , NHCO, 1H), 5.03 (brs, NHCO, 1H), δ 4.17 (d, 2.4 Hz, -OCH\textsubscript{2}-alkyne, 2H), 3.81 (t, 6.0 Hz, alkyneCH\textsubscript{2}-OCH\textsubscript{2}-, 2H), ), 3.61 (s, -OCH\textsubscript{2}CH\textsubscript{2}O-, 4H), 3.55 (q, 5.2 Hz, -OCH\textsubscript{2}CH\textsubscript{2}NHCOOBoc, 2H), 3.48 (t, 5.1 Hz, -OCH\textsubscript{2}CH\textsubscript{2}NHCO, 2H), 3.32 (q, 5.2 Hz, -CH\textsubscript{2}-NHCO, 2H), 2.50 (t, 5.5Hz, -CH\textsubscript{2}-CONH, 2H), 2.45 (t, 2.4 Hz, alkyne-H, 1H), 1.44 (s, (Me)\textsubscript{3}-BOC, 9H).
Conversion 5 $\rightarrow$ 6 (Scheme 1)

A stirred a solution of 5 (2 g) in dichloromethane (40 mL) was added TFA (10 molar equivalents) and kept at 20-22 $^\circ$C for 3 h. After addition of 100 mL CHCl$_3$ the solution was evaporated in vacuo leading to a yellow oil which was further purified by stirring with K$_2$CO$_3$ (aq) (10g/10ml) and EtOAc (20ml) and finally extracted. Combined organic phases were dried (MgSO$_4$), filtered and evaporated at 40 $^\circ$C in vacuo to a viscous yellow oil. This crude material was further purified by using column Silica gel (35 g) chromatography by using dichloromethane/methanol/ammonium hydroxide gradient elution. Clean product was collected in fraction 3-7 affording a pale yellow oil still containing 1 eq of ammonium trifluoroacetate which was removed after final washing of its AcOEt solution with NH$_4$OH (aq) saturated with NaCl followed by extraction with EtOAc and THF. Combined organic phases, dried (MgSO$_4$) and evaporated at 40 $^\circ$C in vacuo provides 6 a clear oil (1.206g, yield
83.7%)

NMR of 6 (DMSO-d6): δ 7.93 (brs, NHCO, 1H), 4.09 (d, 2.4, -OCH2-alkyne, 2H), 3.62 (t, 6.1 Hz, alkyneCH2-OCH2-), 3.54 (s, -OCH2CH2O-, 4H), 3.51 (t, 5.5 Hz, -OCH2CH2CONH, 2H), 3.42 (t, 2.4 Hz, alkyne-H, 1H), 3.40 (t, 5.1 Hz, -OCH2CH2NH2, 2H), 3.20 (q, 5.2 Hz, -CH2-NHCO, 2H), 2.87 (t, 5.2 Hz, -CH2-NH2, 2H), 2.33 (t, 6.4 Hz, -CH2-CONH, 2H).

Conversion 6 → 7 + 8 → 10 (Scheme 1 and Scheme 2)

1.206 grams of amine 6 was treated dropwise at r.t. (22 °C) for 2 h under stirring with an equimolar amount of CDI (0.757 g) in pyridine (15 mL) under N2 and kept under stirring at r.t. overnight.

LCMS of the crude reaction showed, besides the expected product 7, also formation of the product 8 due to internal cyclization of 7 itself. Puromycin was then added at different interval (overall added amount 2.546 g) keeping the bath at 100 °C for 7 h and left overnight a with stirring at r.t.. The crude was evaporated in vacuo at 50 °C affording a brown oil which was extracted by using different mixture of AcOEt/water and DCM/water partitioning system. The organic phase was purified by silica gel column chromatography (43 g) by using DCM/MeOH gradient elution obtaining at the end fractions containing almost pure 10 (LCMS >98%)

NMR of 10 (CDCl3): δ = 8.19 (s, Pur, 1H), 7.89 (s, Pur., 1H), 7.15 (d, 8.6 Hz, Pur., 2H), 7.02 (brd, 6.3 Hz, COCHNHCO, 1H), 6.83 (d, 8.6 Hz, Pur., 2H), 6.74 (brs, COCHNHCO, 1H), 6.12 (d, 7.7 Hz, PurCH-ribose, 1H), 5.73 (brs, NHCO, 1H), 5.60 (d, 4.5, 1H) 4.82 (t, J = 5.8 Hz, 1H), 4.58 – 4.47 (m, 2H), 4.14 (d, J = 2.4 Hz, 2H), 4.05 (t, J = 2.4 Hz, 1H), 3.90 (d, J = 12.7 Hz, 1H), 3.77 (t, J = 5.8 Hz, 2H), 3.76 (s, 3H), 3.68 (d, J = 11.0 Hz, 1H), 3.55 (d, J = 3.7 Hz, 8H), 3.49 (q, J = 5.9 Hz, 3H), 3.45 – 3.32 (m, 2H), 3.26 (dt, J = 14.4, 7.1 Hz, 1H), 2.820 (dd, 5.9,13.8 Hz, Ar-CH2-purom, 1H), 2.687 (dd, 8.3,13.8 Hz, Ar-CH2-purom, 1H)
Conversion 11 → 12 (Scheme 3)

4-hydroxy-2-butanone 11 (20g) was stirred for 5h in liquid ammonia at -76-71 °C under N₂; 240 mL of a methanol solution of hydroxylamine O-sulfonic acid (28 gr, 1.1 mol equivalent) in MeOH (200 ml) was then slowly added allowing the temperature to increase at 10-15 °C and kept under stirring for 4 days. The white precipitate was filtered off, washed with MeOH (2x 20mL), the volume of the solution was carefully reduced to 100 mL (care-volatile product!) in vacuo at 30 °C, the temperature was reduced to 3 °C and cooling kept at the same temperature during the addition in 30 minutes of 30 mL triethylamine (TEA, 1 molar equivalent). Then 28.8 gram of I₂ (0.5 molar equivalent) was slowly added until persistence of colour and the solution allowed to reach r.t. and then stirred again for 2h at 15 °C. The total volume of the solution was reduced in vacuo at 30 °C with great care (in order to avoid losses of volatile product) to 100 mL; this solution was finally diluted with saturated brine (200 mL) and extracted with ethyl ether (2x 200 mL), dried overnight on MgSO₄, its volume reduced first at 10 mL ad r.t. and finally evaporated in vacuum (100 mbar) affording a yellow liquid which was then distilled at 60-62 °C (9 mbar) to yield a clear colourless liquid 12 (81g, yield 34.4%).

NMR of 12 (CDCl₃): \( \delta \) 3.55 (q, 6.1 Hz, -CH₂OH, 2H), 1.64 (t, 6.1 Hz, -CH₂-CH₂OH, 2H.), 1.38 (brt, 6.1 Hz, -CH₂-OH, 1H), 1.07 (s, Me-azir, 3H).

Conversion 12 → 13 (Scheme 3)

A stirred solution of triphosgene (2.843 gr, 0.333 mol equivalent) and pyridine (2.275 gr, 1 molar equivalent) in THF (10ml) was kept at 18 °C under N₂ for 30 minutes followed by 5 minutes at 25 °C; then the obtained thick suspension was cooled to 0-5 °C and added dropwise with the diazirine 12 (2.880 g, 1 molar equivalent) in 9 mL of THF. Immediately began to form a sticky untr stirrable mixture, DCM (20mL) was added in order to aid solution mobility and allow the addition of the remaining part of diazirine 12. After 1h the suspension was rinsed with dry THF (2x1mL) to give a thinner suspension, stirred for 30 minutes at 0-5 °C, allowed to warm to r.t. filtered (GFF), evaporated in vacuo at 40 °C to yield a yellow oil 13 (4.26g, yield 91.1%), stored in dark under N₂.

NMR of 13 (CDCl₃): \( \delta \) 4.26 (q, 6.4 Hz, -CH₂OCOCl, 2H), 1.76 (t, 6.4 Hz, -CH₂-
CH$_2$OCOCl, 2H), 1.10 (s, Me-azir, 3H).

**Conversion 10 + 13 $\rightarrow$ 14 + 15 + 16 (Scheme 4)**

To a pyridine solution (7 mL) of puromycin derivative 10 (720 mg, 1 molar equivalent) stirred at r.t. under N$_2$ were added dropwise in 30 minutes 0.2 mL in DCM of diazirine 13 (155 mg, 1 molar equivalent) and the solution was kept at 0-5 C; after 2 further addition of 13 (2 molar equivalent) in 2 h, the reaction was quenched by addition of 10 mL of distilled water and 10 mL of AcOEt; organic phase obtained after extraction were dried over MgSO$_4$, evaporated in vacuo at 40 C to give a raw reaction products as an oil. The latter was then purified by Silica gel column chromatography (35g) by using EtOAc/MeOH gradient elution collecting 120 fractions of 25mL. By LC-MS and NMR analysis, fractions 13-30 contained almost pure the bis-aziridino derivative 16 (301mg, 3PC, purity 95%), fractions 57-90 the mono-aziridino derivative 15 (52 mg, 3PB, purity 96% ) and finally fractions 92-113 the expected mono-aziridino derivative 14 (275 mg, 3PA, purity 97%)

**NMR of 3PA (400 MHz, DMSO-d6)** $\delta$ 8.28 (s, purom,1H), 8.23 (s, purom, 1H), 8.01 (d, 7.8 Hz, NHCO, 1H), 7.90 (brt, 5.3 Hz, CH$_2$NHCO, 1H), 7.12 (d, 8.6 Hz, purom, 2H), 6.80 (d, 8.7 Hz, purom, 2H), 6.20 (d, 8.1 Hz, COCHNHCO, 1H), 6.17 (d, J = 4.8 Hz, NHCO, 1H), 6.12 (t, 5.8, NHCO,1H), 5.99 (d, 2.1 Hz, purom, 1H), 4.58 (m, riboseCH-NHCO, 1H), 4.55 (m, riboseCH-1H), 4.44 (q, 6.1 Hz, NHCOCHNHCO, 1H), 4.21 (dd, 2.7,12.0 Hz, -riboseCH2-OCO,1H), 4.17 (dd, 6.5,12.0 Hz, riboseCH2-OCO,1H), 4.07 (d, 2.4 Hz, -OCH2-alkyne, 2H), 4.01 (m, CH-ribose, 1H), 4.00 (t, 6.2 Hz, -CH2-OCO, 2H), 3.70 (s, CH3)2-N, 6H), 3.61 (t, 6.4 Hz, alkylneCH2-OCH2-, 2H), 3.47 (s, -OCH2CH2O, 4H), 3.31 (t, 5.1 Hz, -OCH2CH2NHCO, 2H), 3.39 (t, 5.1 Hz, -OCH2CH2NHCO, 2H), 3.30 (s, OCH3 purom, 3H), 3.18 (q, 5.8 Hz, OCH2CH2NHCO, 2H), 3.08 (q, 6.0 Hz, -CH2-NHCONH, 2H), 2.84 (dd, 5.9,13.8 Hz, Ar-CH2-purom, 1H), 2.69 (dd, 8.3,13.8 Hz, Ar-CH2-purom, 1H), 2.31 (t, 6.4 Hz, -CH2-CONH, 2H), 1.64 (t, 6.2 Hz, -CH2-aziridine, 2H), 1.00 (s, Me-azir, 3H).
NMR of 3PA 1H NMR (400 MHz, Chloroform-d) δ 8.24 (s, 1H), 7.94 (s, 1H), 7.18 – 7.10 (m, 2H), 6.94 (d, J = 6.8 Hz, 1H), 6.86 – 6.77 (m, 2H), 6.72 (s, 1H), 6.03 (d, J = 7.5 Hz, 1H), 5.86 (d, J = 2.3 Hz, 1H), 5.66 (s, 1H), 5.50 (t, J = 5.6 Hz, 1H), 4.62 (dd, J = 12.9, 6.2 Hz, 2H), 4.52 – 4.40 (m, 2H), 4.29 (dd, J = 12.0, 5.0 Hz, 1H), 4.21 – 4.10 (m, 3H), 4.06 (t, J = 6.6 Hz, 2H), 3.76 (d, J = 7.5 Hz, 5H), 3.61 – 3.53 (m, 6H), 3.53 – 3.31 (m, 4H), 3.31 – 3.20 (m, 1H), 3.01 (h, J = 6.9, 6.5 Hz, 2H), 2.56 – 2.39 (m, 3H), 1.82 (s, 2H), 1.65 (td, J = 6.6, 2.7 Hz, 2H), 1.04 (s, 3H).

13C NMR (100 MHz, DMSO-d6): 171.9 (s), 169.3, 157.2, 157.1 (d, CH-purom), 153.4, 151.5, 149.1, 137.2(d, CH-purom), 129.6 (d, CH-purom), 128.9, 118.9, 112.8 (d, CH-purom), 89.2 (d, CH-purom), 79.7 (d, CH-purom), 76.4, 72.2 (d, CH-purom), 69.3(t, ) 69.1(t, ), 68.5 (t, ) 66.8 (t, CH-purom),65.2 (t, CH-purom), 62.5 (t, ), 65.2 (t, t, ), 56.7 (t, ), 54.4 (q, ), 53.9 (d, CH-purom), 50.1 (d, CH-purom), 38.5 (t, ), 38.0 (t, ), 37.5 (t, ), 35.2 (t, ), 32.4 (t, ), 18.7 (q, ).

LC/MS of 3PA Ascentis Express C18 (100 x 4.6mm, 2.7µm) Column Temp.: 25.0°C Mobile phase: 10-100% CH3CN+TFA (0.1 % v/v) : aq.TFA (0.1 % v/v) for 10 min with 5 min; Flow: 1.8 ml/min; Wavelength: 270 nm, retention time 4.74 minutes, purity higher than 97%. MS : m/z (ES+): 882 [M+H+], m/z (ESI-): 880 [M-H] –

NMR of 3PB (400 MHz, DMSO-d6) δ 8.48 (d, 7.8 Hz, NHCO, 1H), 8.26 (s, purom,1H), 7.93 (s, purom, 1H), , 7.93 (brt, 5.3 Hz, CH2NHCO, 1H), 7.12 (d, 8.6 Hz, purom, 2H), 6.85 (d, 8.7 Hz, purom, 2H), 6.29 (d, 4.1 Hz puromicin-H, 1H), 6.18 (d, 8.4 Hz, COCHNHCO, 1H), 6.11 (t, 5.8, NHCO,1H), 5.99 (d, 2.1 Hz, purom, 1H), 5.55 (dd, 4.2, 6.7
Hz, riboseCH-OCO, 1H), 5.25 (t, 5.5 Hz, 1H), 4.78 (q, 7.1 Hz, riboseCH-NHCO, 1H), 4.55 (m, riboseCH-1H), 4.45 (dt, 6.1, 8.2 Hz, NHCOCHNHCO, 1H), 4.10 (d, 2.4 Hz, -OCH2-alkyne, 2H), 4.02 (m, -riboseCH2-OCO, 2H), 4.01 (m, CH-ribose, 1H), 4.00 (t, 6.2 Hz, -CH2-OCO, 2H), 3.74 (s, CH32-N, 6H), 3.65 (t, 6.4 Hz, alkyneCH2-OCH2-, 2H), 3.52 (s, -OCH2CH2O, 4H), 3.31 (t, 5.1 Hz, -OCH2CH2NHCO, 2H), 3.39 (t, 5.1 Hz, -OCH2CH2NHCO, 2H), 3.34 (s, OCH3 purom, 3H), 3.21 (q, 5.8 Hz, OCH2CH2NHCO, 2H), 3.11 (q, 6.0 Hz, -CH2-NHCONH, 2H), 2.85 (dd, 5.9, 13.8 Hz, Ar-CH2-purom, 1H), 2.71 (dd, 8.3, 13.8 Hz, Ar-CH2-purom, 1H), 2.37 (t, 6.4 Hz, -CH2-CONH, 2H), 1.64 (t, 6.2 Hz, -CH2-aziridine, 2H), 1.00 (s, Me-azir, 3H).

1H NMR (400 MHz, Chloroform-d) δ 8.28 (s, 1H), 7.81 (s, 1H), 7.22 – 7.14 (m, 2H), 6.89 – 6.81 (m, 3H), 6.60 (s, 1H), 6.10 (s, 1H), 5.97 (d, J = 7.6 Hz, 1H), 5.89 (d, J = 5.8 Hz, 1H), 5.76 – 5.69 (m, 1H), 5.44 (t, J = 5.5 Hz, 1H), 4.85 (td, J = 6.8, 4.0 Hz, 1H), 4.50 (q, J = 7.4 Hz, 1H), 4.21 – 3.97 (m, 6H), 3.93 (d, J = 12.9 Hz, 1H), 3.78 (s, 3H), 3.77 (t, J = 5.8 Hz, 2H), 3.70 (s, 1H), 3.64 – 3.55 (m, 6H), 3.52 (t, J = 4.9 Hz, 2H), 3.43 (p, J = 5.8 Hz, 2H), 3.40 – 3.31 (m, 2H), 3.12 – 2.96 (m, 2H), 2.50 – 2.42 (m, 3H), 1.68 (td, J = 6.5, 2.8 Hz, 2H), 1.04 (s, 3H).

13C NMR (100 MHz, Chloroform-d): 152.0 (d, CH-purom), 137.9 (d, CH-purom), 130.3 (d, 2CH-purom), 114.0 (d, 2CH-purom), 88.6 (d, CH-purom), 85.2 (d, CH-purom), 70.6 (d, CH-purom), 70.5 (t), 70.1 (t), 69.8 (t), 66.1 (t, CH-purom), 63.9 (t, CH-purom), 62.2 (t), 58.3 (t), 55.7 (q), 55.2 (d, CH-purom), 50.4 (d, CH-purom), 40.1 (t), 39.5 (t), 37.8 (t), 36.7 (t), 33.5 (t), 19.7 (q).

**LC/MS of 3PB** C18 Eclipse (100 x 4.6mm, 2.7µm) Column Temp.: 25.0°C Mobile phase: 10-100% CH3CN+Formic acid (0.1 % v/v) : aq.formic acid (0.1 % v/v) for 10 min with 5 min; Flow: 1.8 ml/min; Wavelength: 270 nm, retention time 5.02 minutes, purity higher than 94%. MS : m/z (ES+): 882 [M+H+], m/z (ES-): 926 [M+HCOO] -

**NMR of 3PC** (400 MHz, CDCl3) δ 8.24 (s, purom, 1H), 7.94 (s, purom, 1H), 7.14 (d, 8.6 Hz, purom, 2H), 6.94 (d, J = 6.8 Hz, NHCO, 1H), 6.82 (d, 8.7 Hz, purom, 2H), 6.71 (m,
NHCO, 1H), 6.03 (d, 7.5 Hz, COCHNHCO, 1H), 5.66 (brs, riboseCH-NHCO, 1H), 4.60 (m, riboseCH-1H), 4.46 (dt, 6.1, 8.2 Hz, NHCOCHNHCO, 1H), 4.46 (dd, 12.0, 8.0 Hz, -CH2-OCO, 1H), 4.29 (dd, 12.0, 5.0 Hz, -CH2-OCO, 1H), 4.17 (m, -riboseCH2-OCO, 2H), 4.15 (d, 2.4 Hz, -OCH2-alkyne, 2H), 4.06 (m, CH-ribose, 1H), 3.78 (t, 6.2 Hz, -CH2-OCO, 2H), 3.76 (s, CH32-N, 6H), 3.65 (t, 6.4 Hz, alkyneCH2-OCO, 2H), 3.52-3.30 (series of m, 8H), 3.46 (s, OCH3 purom, 3H), 3.21 (q, 5.8 Hz, OCH2CH2NHCO, 2H), 3.01 (q, 6.0 Hz, -CH2-NHCONH, 2H), 2.45 (dd, 5.9, 13.8 Hz, Ar-CH2-purom, 1H), 2.42 (t, 6.4 Hz, -CH2-CONH, 2H), 2.35(dd, 8.3, 13.8 Hz, Ar-CH2-purom, 1H), 1.65 (t, 6.2 Hz, -CH2-aziridine, 2H), 1.04 (s, Me-azir, 3H).

LC/MS C18 Eclipse (100 x 4.6mm, 2.7µm) Column Temp.: 25.0°C Mobile phase: 10-100% CH3CN+Formic acid (0.1 % v/v) : aq.formic acid (0.1 % v/v) for 10 min with 5 min; Flow: 1.8 ml/min; Wavelength: 270 nm, retention time 5.02 minutes, purity higher than 97% MS : m/z MS : m/z (ES+): 1008 [M+H+], m/z (ES-): 1052 [M+HCOO] -
**Pipeline MS-data analysis.** Raw files from gel bands were analyzed using Proteome Discoverer 2.2 (Thermo Fisher) against UniProt/SwissProt human and bovine database (2017_01, 26169 protein entries) as the cells were cultured with bovine serum, using Mascot 2.6.0. The mass tolerance was set at 10 ppm for the precursor ions and at 20mmu for fragment ions. Carboxyamidomethylation of cysteine was used as a fixed modification and oxidation of methionine as variable modifications. Two missed cleavages were allowed. The precursor peak area was used for protein quantification. Raw files from beads were analyzed using Proteome Discoverer 2.2 (Thermo Fisher) against UniProt/SwissProt human database (2017_01, 20171 protein entries) using Mascot 2.6.0. The mass tolerance was set at 10 ppm for the precursor ions and at 0.8 Da for fragment ions. Carboxyamidomethylation of cysteine was used as a fixed modification and oxidation of methionine as variable modifications. Two missed cleavages were allowed. The precursor peak area was used for protein quantification. For in-column digestion data: all the gene having at list a signal in one of the four replicates were included in the analysis. For in-gel digestion data: we removed all the proteins that were not detected in the input and we kept all genes having at list a signal in one of the four replicates. For in-beads digestion data: to account for low abundant protein we set a value of 25998.91992 (minimal detected value in all samples) for all the protein not detected in 3PB-beads or PEG-beads samples. Since we used two different types of commercial beads for pull-down (Magar – Immagina Biotech cat- no016-00-007-2-1 and Sepharose – GE healthcare cat. no 28985799), we calculate the ratio of (D1) 3PB Magar-beads/ (E2) PEG magar beads and (D2) 3PB sepharose beads / (E2) PEG sepharose beads. All the proteins with a mean FC enrichment ≥ 1.3 or ≥ 2.5.

**Cy3 cell labeling.** MCF7 cells were grown to 80% of confluence and treated with cycloheximide (CHX, 10 µg/mL, 5 min, 37 ºC) and the 3PC/3PB probe (10 min, 37 ºC). For Torin 2 and pp242 treatment, MCF7 cells were incubated with pp242 (250 nM) and Torin 2 (500 nM) at 37 ºC for 60 min before adding CHX. After that, cells were washed thrice with cold PBS (containing CHX 10 µg/mL), placed on ice and irradiated under a UV lamp at 365 nm for 5 min (0.75 J/cm2) followed by washing twice with PBS. Subsequently, cells were fixed and permeabilized with ice cold 100% methanol for 5 min at -20 ºC. Post permeabilization, cells were washed twice with PBS and incubated with 1 ml “click” buffer (PBS containing 2.5 µM picoly1-azide Cy3, 0.1 mM Cu++, 0.5 mM THPTA and 5 mM ascorbic acid) for 1 hour at room temperature. Cells were blocked with blocking buffer (5% BSA, 0.3% triton x 100 in PBS) for 1
hour and incubated with (i) the indicated primary antibody (1:200) and then with (ii) the secondary antibody (1:300) in 3% BSA, 0.2% saponin, 2.5% vol/vol polyvinyl sulfonic acid. Finally, cells were washed twice in PBS with a final overnight washing at +4°C in PBS + 0.1% tx100. The day after the slides were washed thrice with PBS, mounted with ProLong Gold Antifade (Invitrogen, Catalog no P36962) and viewed using a confocal microscope.

**Confocal Microscopy.** Laser scanning confocal microscopy (LSCM) analysis was carried out using a Leica DM6000CD microscope equipped with an argon laser source. MCF7 cells were immunostained with RPL26, calnexin or HSC-70 FITC (Ex 492 nm, Em.: 519 nm) and 3PB/3PC-Cy3 (Ex. 552 nm, Em 578 nm).

**3Px pull-down assays of ribosome complex.** After PP242 (250 nM, for 1h ) and Torin2 (500 nM for 1h) treatment, MCF-7 cells were incubated with cycloheximide (CHX, 10 µg/mL, 5 min, 37 °C) and 3PB (10 min, 37 °C), then, placed on ice and irradiated under a UV lamp at 365 nm for 5 min (0.75 J/cm2). Cells were harvested with hypotonic cytoplasmic lysis buffer and lysates (0.3 mL) were incubated with picoly-PEG4-biotin (1.6 mM), CuSO4 (80 µM), THPTA (400 µM) and sodium ascorbate (4 mM), on a wheel for 1h at 4 °C. After click reaction, the cell lysates were incubated with 100 µL of Dynabeads MyOne™ Streptavidin C1 (ThermoFisher), on a wheel for 30 min at 4 °C.

**Immunoblotting.** List of antibodies used:

| Antibody                        | Company Supplier | Dilution/amount (IP, immunoprecipitation; WB, immunoblotting) | Catalog no. |
|---------------------------------|------------------|----------------------------------------------------------------|-------------|
| Mouse monoclonal anti calnexin | EMD Millipore    | IP – 6 µg WB – 1:3000                                            | MAB3126     |
| Mouse monoclonal anti puromycin| EMD Millipore    | IP – 6 µg WB - 1:5000                                            | MAB3126     |
| Rat monoclonal anti puromycin  | EMD Millipore    | WB - 1:5000                                                      | MABE341     |
| Rabbit monoclonal anti GRP78   | Abcam            | WB – 1:1000                                                      | ab108613    |
| Sheep polyclonal anti serum albumin | Abcam      | WB - 1:1000                                                      | ab8940      |
| Rabbit polyclonal anti RPL18   | Abcam            | WB -1:1000                                                       | ab207555    |
| Rabbit IgG                      | Pierce – Thermo Scientific | IP - 6 µg WB - 1:1000                                          | 31235       |
| Mouse IgG                       | Pierce – Thermo Scientific | IP6- µg WB - 1:1000                                             | 31903       |
| Antibody                          | Company Supplier | Dilution/amount (IP, immunoprecipitation; WB, immunoblotting) | Catalog no. |
|----------------------------------|------------------|---------------------------------------------------------------|-------------|
| Mouse monoclonal anti HSP90     | Abcam            | WB - 1:2000                                                   | ab13492     |
| Rabbit monoclonal Anti ENO1     | Abcam            | WB - 1:5000                                                   | ab155102    |
| Rabbit monoclonal GRP78         | Abcam            | WB - 1:2000                                                   | Ab108615    |
| Mouse monoclonal anti HSP70     | Abcam            | WB - 1:2000                                                   | ab2787      |
| Rabbit monoclonal anti RPL26    | Abcam            | WB - 1:2000                                                   | ab18110     |
| mouse monoclonal anti eEF1A     | Millipore        | WB - 1:1000                                                   | 2787685     |
| Ribosomal protein S6             | Cell Signaling Technology | WB - 1:1000                                               | 4858S       |
| Ribosomal protein pS6 (235/236)  | Cell Signaling Technology | WB - 1:1000                                               | 2211S       |
| Actin Beta                       | Sant Cruz        | WB - 1:1000                                                   | Sc-69879    |
| h-VEGF                           | RD systems       | WB - 1:1000                                                   | BAF293      |

HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (used at 1:10,000 dilution, catalog no. sc-2004, catalog no. sc-2005), while Streptavidin-HRP (Trascendent kit, catalog no. L5080, used at 1:1,000 dilution) was purchased from Promega. The chemiluminescence was acquired by ChemDoc-It (Bio-Rad) and analyzed with ImageJ software (v 1.45s). Precision Plus Protein Standard Kaleidoscope standard (Biorad catalog no. 161-0375) or PageRuler Prestained Protein Ladder (Termo Fisher, catalog no. 26617) was used as ladder protein marker.
Supplementary Figures

Targeting translation activity at the ribosome interface with UV-active small molecules

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**Figure S1.** (a) Nucleus-cytoplasm fractionation and immunoblotting of MCF7 cells treated with 3PB (100 μM, 10 min). Ribosomal protein S6 and Histone 3 (H3) are used as cytoplasmic and nuclear marker respectively. (b) Labelling activity is UV and diazirino dependent. Immunoblotting of puromycin (top) and actin (bottom) on cytoplasmatic protein extract from growing HEK-293 cell in complete DMEM media, treated with the probes reported in the table on the left (all probes are used at 50 μM, with 10 min incubation time). UV-treatment: 365 nm, 0.75 J/cm². Lane 7 reports cells treated with 3PC without UV treatment. (c) Immunoblotting with an anti-puromycin antibody of cytoplasmic protein extracts from HEK-293 cells incubated with 3PA, 3PB or 3PC (50 μM, 10 min) and exposed to UV (365 nm) irradiation. (d) Chemical structure of the probes used in (b). (e) Increasing concentration of the 3PC probe UV activated in the cell lysate. (Top) Puromycin immunoblotting. (Bottom) Ponceau staining. (right) Best fit of the data set obtained from the total lane intensity (immunoblot) for each 3PC concentration. The concentration causing 50% of total binding (EC50) is reported.
Figure S2. (a.) Immunoblot with anti-puromycin antibody on total protein extracts from MCF7 cell treated with increasing concentrations of puromycin (incubation time: 2 hours) and 3PC (50 µM, 10 min). CHX + Puro: 350 µM CHX before puromycin treatment (20, 50, 100, 200 µM). 3PC treatment: (10 µM, 10 min); -, not treatment. (b) On the left, immunoblot with anti-puromycin antibody on total protein extracts from MCF7 cells treat with the indicated drugs. CHX, 350 µM, 5 min; Puromycin, 100 µM, 2 hours; 3PB, 50 µM, 10 min. On the right, CuAAC coupling with Biotin-azide was performed on the cell lysate before total protein extract and SDS-PAGE. The staining of the membrane was performed with Streptavidin-HRP. (c) Sketch that summarize the effect of puromycin treatment on the 3PB-3PBis labelling. (c) In-gel fluorescent image of total protein extracts from MCF7 cells treated with 3PB (100 µM), with or without 365-nm UV irradiation, and with or without CHX and puromycin at the indicated concentrations. Cell lysates were conjugated to a Cy3-azide reporter by CuAAC and the analysis performed by SDS-PAGE and fluorescence scanning. Ponceau membrane staining of the total protein content is reported below. Immunoblots were scanned with Typhoon Trio (Ex. max:530/Em. max :550) and analysed with ImageJ v1.45s.
Figure S3. (a) Overview of the experimental procedure for the proteomic LC-MS/MS analysis. (b) Table reporting accession number, gene name, molecular weight (MW) and PMSs peptide-spectrums match score for each of the top-6 genes. (c) Silencing of GRP78 and its effect on 3PC signals. MCF7 cells were transiently transfected with GRP78-siRNA for 24 h, followed by 3PC treatment (100 μM, 10 min) (siGRP78); not transfected 3PC-treated cells served as a control (Ctrl). A representative immunoblot is reported the right silencing of GRP78 and its effect on the 3PC signal. MCF7 cells transiently transfected with GRP78-siRNA for 24 h, followed by 3PC treatment (100 μM, 10 min) in a transfected (siGRP78) and a not transfected (Ctrl) condition. Black arrows: two main isoforms of GRP78 detected. Black arrows, two main isoforms of GRP78 that were detected. (d) Quantification of the immunoblot was performed by measuring the relative intensities of the bands compared to those of the internal controls (Actin). #, independent replicates (e) Immunoblot developed with the indicated antibodies using total protein from cells treated or not treated with VER-155008 (100 μM, 1 hour), followed by treatment with 3PC (100 μM, 10 min). The fold-change enrichment for duplicate experiments is reported. (f) Immunoblotting of heat shock protein HSPA1/HSP70, puromycin and GRP78 from MCF7 cells under normal conditions or during serum starvation (0.5% FBS, 18 hours) with 3PC (50 μM, 10 min). #, independent biological replicates; black arrows, two main isoforms of GRP78 that were detected.
Figure S4. (a) On the left, immunoblotting of eEF2, pS6 (235,236) and total pS6 after 3PB pull-down assays of ribosome complexes. On the right, pixel quantification of a representative immunoblotting. (b) Sucrose gradient absorbance profile of MCF7 cells at ~ 80% of confluence treated with DMSO or 3PB. (b, bottom) 3PB protein targets co-sediments with the translational machinery. Co-sedimentation profiles of 3PB/αPuro and RPS6 by immunoblotting. Each line corresponds to a sucrose fraction of the profile. Fractions related to the 80S pick or to the polysome region of the profile are indicated. Black arrows, GRP78 main bands.
Figure S5. Representative images of (a) 3PC and (b) 3PB-treated MCF7 (for RPL26) cells immunoassayed with RPL26 (up) and conjugated with Cy3 by Cu+ catalyzed click reaction. z-stack, single plane images. FITC Ex. Max: 492, Em. Max: 519. Cy3 Ex. Max 552, Em. Max 578. Images captured with Leica DM6000CD. Software LAS AF Version 2.7.3.9723. Scale bars, 25 µm (a) and 10 µm (M)
Figure S6. (a) 3PC main protein targets are EDTA sensitive. MCF7 cells were treated with 3PC and 8 mM EDTA (+ EDTA, red dotter line) and directly fractionated on a 15–50% sucrose gradient (-EDTA, black dotter line). Fractions were collected and analysed by western blot with antibodies against Puromycin and the ribosomal L26 protein (RPL26). (b) Co-immunoprecipitation analysis. 3PC-tagged proteins were immunoprecipitated with anti-puromycin or anti Hsc-70 antibody. Cell lysates before immunoprecipitation (Input) and immunoprecipitated (co-IP) were separated by SDS-PAGE. The immunoblot was performed with the indicated antibodies. M, mouse anti-puromycin antibody; R, rabbit anti-puromycin antibody. (c) Electropherogram profile obtained using a Bioanalyzer (Agilent total RNA kit) with total RNA isolated from 3PB beads and control beads incubated with HEK-293 cell lysate.
