Diabetologia (2019) 62:2158–2160
https://doi.org/10.1007/s00125-019-04996-6

LETTER

CD40-targeting $KGYY_{15}$ peptides do not efficiently block the CD40–CD40L interaction

Damir Bojadzic[1,2] · Peter Buchwald[1,2]

Received: 19 July 2019 / Accepted: 31 July 2019 / Published online: 9 September 2019

To the Editor: Co-signalling interactions, which include costimulatory and coinhibitory interactions and act as immune checkpoints, are important immunomodulatory therapeutic targets. Biological products that interfere with these interactions have achieved considerable clinical success in the treatment of cancer on the one hand, and autoimmune diseases and transplant recipients on the other. Alternatives to biological products are also being explored as they have the potential to lead to safer, less immunogenic, orally bioavailable agents; however, like all other protein–protein interactions, co-signalling interactions are challenging to target by smaller molecules [1]. Among these interactions, the one between CD40 and CD40 ligand (CD40L, also known as CD154) is of particular interest as a therapeutic target for the prevention of rejection in islet cell transplant recipients, as well as the prevention, or possibly even reversal, of type 1 diabetes. As published in *Diabetologia* [2], Wagner and co-workers designed a set of peptides to target CD40. They claimed that one of these peptides, the 15-mer $KGYY_{15}$, prevented hyperglycaemia in the NOD mouse model of type 1 diabetes, representing a potentially significant advancement. Recently, Coppieters and co-workers raised doubts regarding these claims in a letter [3]. Wagner and colleagues countered this in a response highlighting the complex nature of this interaction and the lack of in cellulo data [4].

In support of the concerns raised by Coppieters and co-workers, we would like to point out that, as part of our own work aimed at identifying small-molecule inhibitors of the CD40–CD40L interaction [5, 6], we have tested the $KGYY_{15}$ peptides, which were designed based on the CD40L domain that is critical for interaction with CD40, and found that in our hands they did not block the human CD40–CD40L interaction. In particular, in our human CD40–CD40L binding assay published in *J Med Chem* in 2017 [5], mouse $KGYY_{15}$ (VLQWAKKGYYTMKSN) showed no inhibitory activity whereas human $KGYY_{15}$ (VLQWAEKGYYTMSNN) showed some inhibition, but with an IC_{50} of only 154 μmol/l (see Supporting Information Figure S7 in our study [5]). As we have noted, there are two different $KGYY_{15}$ peptides, one designed to target the murine CD40–CD40L interaction, and another one with a slightly different sequence (see underlined amino acids above) designed to target the human one. Coppieters and co-workers have only reported testing the mouse 15-mer peptide and a corresponding scrambled 15-mer control. We originally looked at the inhibitory activity of both human and mouse peptides, but only in the human system.

Because of the issues raised recently, we retested them in both the human and the murine binding assay, as well as in our previously used CD40L sensor cell line. The $KGYY_{15}$ inhibitory peptides used here were acquired from the same manufacturer as those in the original publication by Wagner and colleagues (New England Peptide, Gardner, MA, USA) [2], and they both had molecular mass confirmed by mass spectral analysis and purity ≥95% as quantified based on HPLC (as determined by the manufacturer). As controls in these assays, we included the corresponding blocking antibodies (MAB617 from R&D Systems, Minneapolis, MN, USA, RRID:AB_2291414, and MR-1 from BioXCell, West Lebanon, NH, USA, RRID:AB_1107601, for the human and mouse system, respectively) as well as our DRI-C21095 small-molecule inhibitor [6], and a promiscuous protein–protein interaction inhibitor of relatively low potency that we have identified earlier (erythrosine; Sigma-Aldrich, St. Louis, MO, USA, cat. no. 198269) [7]. Assays and their quantification to obtain IC_{50} values were performed as described previously [5, 6]. The human assay (huCD40 and huCD40L, both from Enzo Life Sciences, San Diego, CA, USA, cat. nos ALX-522-016-C050 and ALX-522-015-6010, respectively)

✉ Peter Buchwald
pbuchwald@med.miami.edu

[1] Diabetes Research Institute, Miller School of Medicine, University of Miami, 1450 NW 10th Ave, Miami, FL, USA

[2] Department of Molecular and Cellular Pharmacology, Miller School of Medicine, University of Miami, Miami, FL, USA

essentially reconfirmed our previous results, showing a very low inhibitory activity for the human peptide, with an IC_{50} of 97 μmol/l (95% CI 70, 135 μmol/l) vs our previously obtained 154 μmol/l (95% CI 117, 201 μmol/l) [5], and essentially no inhibitory activity for the mouse one ($IC_{50} > 1$ mmol/l) (Fig. 1a). For comparison, in this assay, the blocking antibody is almost five orders of magnitude more potent, with a low nanomolar IC_{50} (3 nmol/l), our DRI-C21095 small molecule is about 1000 times more potent, and even the promiscuous inhibitor erythrosine is about 50-fold more potent (IC_{50} = 2 μmol/l) (Fig. 1a).

In the corresponding mouse assay (muCD40 from R&D Systems, cat. no. 1215-CD-050, and muCD40L from Enzo Life Sciences, cat. no. ALX-522-070-2010), the peptides also show similar and only very weak inhibitory activity (Fig. 1b). The human $KGYY_{15}$ peptide shows a slightly weaker inhibitory potency, with an IC_{50} of 202 μmol/l (95% CI 144, 288 μmol/l), while, interestingly, the murine one shows essentially none again, with an $IC_{50} > 1$ mmol/l. The blocking antibody (MR-1, BioXCell) shows a strong sub-nanomolar activity ($IC_{50} < 0.1$ nmol/l). Considering these IC_{50} values, it is not surprising that no binding could be seen by Coppieters and co-workers in an assay that used concentrations of only up to 3 μmol/l (of the mouse $KGYY_{15}$ peptide).

Finally, we also performed an activity test using an in vitro cell assay, which was not performed by Coppieters and colleagues [3], and is important since the CD40–CD40L interaction is indeed complex, as argued by Wagner and co-workers [4], and simple interaction-blocking assays may not yield relevant characterisation. Using the same (human) HEK Blue CD40L sensor cells that quantify NF-κB activation following CD40 stimulation (InvivoGen, San Diego, CA, USA, cat. no. hkb-cd40) as we used before [5, 6], we again observed only very low activity for the two peptides, with only a little (~33%) inhibition at the highest concentration tested (100 μmol/l) (Fig. 1c). In contrast, both the antibody and our small-molecule inhibitor were able to achieve maximal inhibition. The IC_{50} of DRI-C21095 was estimated as 9 μmol/l, consistent with our previous results [6]. Because of the weak inhibition, the IC_{50}s calculated for the peptides can be considered only as first estimates, but the human one shows activity in the same range as before ($IC_{50} \approx 250$ μmol/l), while the

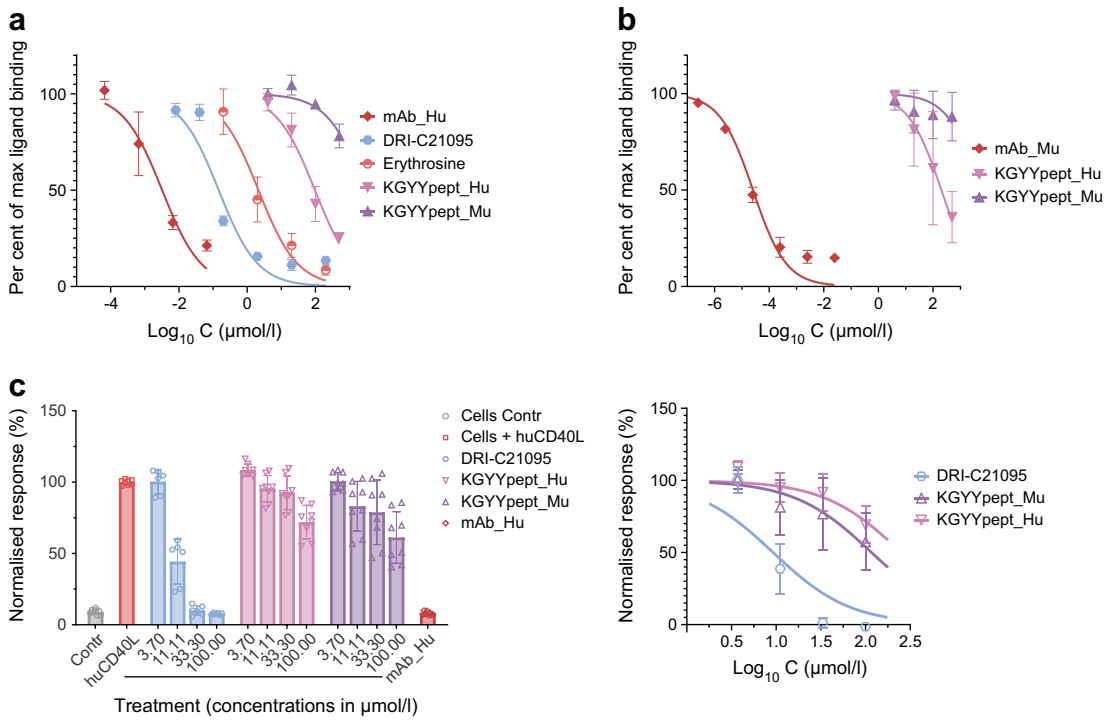

Fig. 1 CD40–CD40L interaction inhibitory activity of the human and murine $KGYY_{15}$ peptides. (**a**) Concentration-dependent inhibition of the human CD40–CD40L interaction quantified using a cell-free ELISA-type assay and fitted with standard binding curves using GraphPad Prism (GraphPad, La Jolla, CA, USA, RRID: SCR_002798) as described previously [5, 6]. In addition to the $KGYY_{15}$ peptides, a blocking antibody (mAb_Hu [MAB617]), our small-molecule inhibitor DRI-C21095, and a promiscuous inhibitor (erythrosine) have also been included. (**b**) Concentration-dependent inhibition by the $KGYY_{15}$ peptides and a corresponding antibody (mAb_Mu [MR-1]) using murine CD40–CD40L. Data in (**a**, **b**) are mean ± SD shown on semi-logarithmic plot with log-scaled concentrations (log_{10} C) on the horizontal axis. (**c**) Concentration-dependent inhibition of CD40L-induced NF-κB activation in HEK Blue CD40 sensor cells (red bar) by the $KGYY_{15}$ peptides and DRI-C21095 with an anti-CD40L antibody (mAb_Hu [MAB617]; 100 nmol/l) as positive control; the concentration response is also shown as a classic semi-logarithmic plot. Data in (**c**) are mean ± SD (normalised to CD40L-activated cells alone) for $n = 3$ independent experiments with duplicates for each condition. Contr, control; KGYYpept_Hu, human $KGYY_{15}$ peptide; KGYYpept_Mu, murine $KGYY_{15}$ peptide; max, maximum

mouse one seems to show some weak activity this time. In light of these IC_{50} estimates obtained in cell-free and cell-based assays, it seems unlikely that weekly injections at a dose of 1 mg/kg (≈0.5 μmol/l) [2] would produce observable therapeutic benefits, regardless of the elimination half-life. Overall, our earlier results [5] and those presented here (Fig. 1) are in line with those of Coppieters and co-workers [3] and support their doubts regarding the CD40 inhibitory activity and therapeutic utility of these $KGYY_{15}$ peptides [2].

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Funding This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Duality of interest The author(s) declare that they have no competing interests.

Contribution statement PB conceived the study, analysed the data and wrote the manuscript; DB designed and performed the assays and contributed to the writing of the manuscript. Both authors contributed to drafting the article or revising it critically for important intellectual content and approved the version to be published. PB is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Bojadzic D, Buchwald P (2018) Toward small-molecule inhibition of protein-protein interactions: general aspects and recent progress in targeting costimulatory and coinhibitory (immune checkpoint) interactions. Curr Top Med Chem 18(8):674–699. https://doi.org/10.2174/1568026618666180531092503
2. Vaitaitis GM, Olmstead MH, Waid DM, Carter JR, Wagner DH Jr (2014) A CD40-targeted peptide controls and reverses type 1 diabetes in NOD mice. Diabetologia 57(11):2366–2373. https://doi.org/10.1007/s00125-014-3342-5
3. Pagni PP, Wolf A, Lo Conte M et al (2019) CD40-targeted peptide proposed for type 1 diabetes therapy lacks relevant binding affinity to its cognate receptor. Diabetologia 62(9):1727–1729. https://doi.org/10.1007/s00125-019-4893-2
4. Vaitaitis GM, Olmstead MH, Waid DM, Carter JR, Wagner DH Jr (2019) CD40-targeted peptide proposed for type 1 diabetes therapy lacks relevant binding affinity to its cognate receptor. Reply to Pagni PP, Wolf A, Lo Conte M et al [letter]. Diabetologia 62(9):1730–1731. https://doi.org/10.1007/s00125-019-4945-7
5. Chen J, Song Y, Bojadzic D et al (2017) Small-molecule inhibitors of the CD40-CD40L costimulatory protein-protein interaction. J Med Chem 60(21):8906–8922. https://doi.org/10.1021/acs.jmedchem.7b01154
6. Bojadzic D, Chen J, Alcazar O, Buchwald P (2018) Design, synthesis, and evaluation of novel immunomodulatory small molecules targeting the CD40-CD154 costimulatory protein-protein interaction. Molecules 23(5):1153. https://doi.org/10.3390/molecules23051153
7. Ganesan L, Margolles-Clark E, Song Y, Buchwald P (2011) The food colorant erythrosine is a promiscuous protein-protein interaction inhibitor. Biochem Pharmacol 81(6):810–818. https://doi.org/10.1016/j.bcp.2010.12.020