Impact of Counter-ion in Peptide on Studies in Different Research Fields

Review 201907

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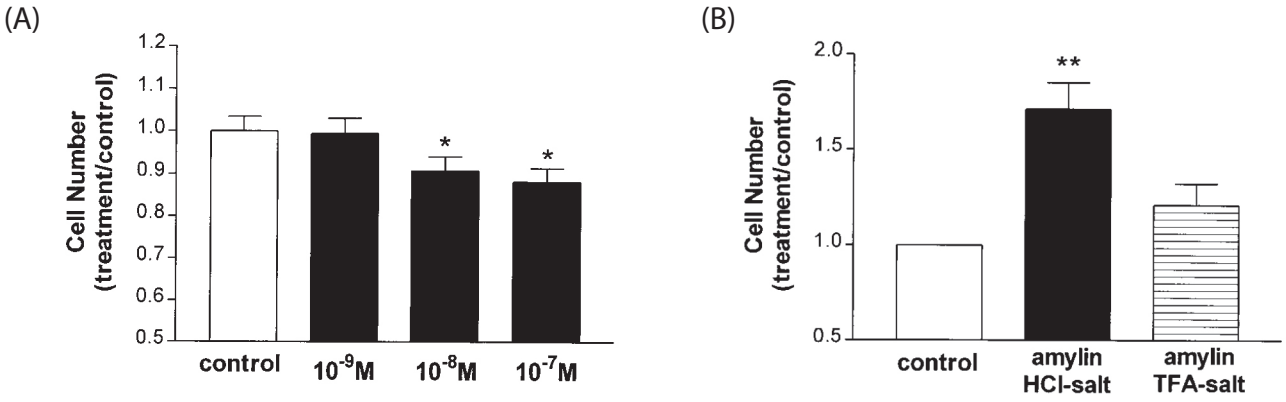
Impact of Counter-ion in Peptide on Studies in Different Research Fields

Current peptide synthesis is mainly based on the solid phase peptide syntheses (SPPS), which was pioneered by Merrifield. After the optimization from Boc-protected strategy into Fmoc-protected strategy, peptide can be achieved through milder reaction condition with improved quality. In Fmoc-protected strategy, trifluoroacetic acid (TFA) is used to cleave the peptide from the resin support, and is added in the mobile phase for a better separation in purification. During lyophilization of the purified peptide, free TFA is removed, but residual TFA remains in the peptide preparation in anionic form, which is also known as counter ions. In chemical terms, a counter ion is an ion of opposite charge to another ion in a solution or an electrochemical system. Specifically, peptides lacking basic amino acids, such as Arg, His and Lys or containing blocked N-termini, require TFA for protonation and hence, are obtained as TFA salts. Thus, it is inevitable for peptide to contain TFA salt in the final product, and peptides are usually delivered as TFA salts. TFA counter ions are able to affect both the biological and physico-chemical properties of peptides, and for this reason, peptides should be converted to a hydrochloride or biologically equivalent salt before assessment of their biological effects is undertaken.

TFA residues in the custom peptides may cause unpredictable fluctuations in the experimental data. For example, TFA at nM level can affect the results of cell experiments, in some cases inhibiting cell growth as low as 10 nM (Cornish *et al*.) and in some cases promoting cell growth at higher doses (0.5–7.0 mM) (Ma *et al*.). It has also been found in scientific studies that TFA may act as an allosteric regulator on GlyR of glycine receptor (Tipps *et al*.), increasing receptor activity at lower glycine concentrations. In the in vivo setting, TFA can trifluoroacetylate amino groups in proteins and phospholipids (Satoh *et al*. 1985), which can elicit antibody responses (Trudell *et al*. 1991; Furst *et al*. 1997). Besides, TFA hampers structure studies by affecting the spectrum absorption.

Impact of TFA on Cell Growth

For cell toxicity examination, Cornish reported the TFA suppression of proliferation to osteoblasts and chondrocytes in a dose-dependent manner. TFA reduced cell numbers into fetal rat osteoblast cultures after 24 hr. The impairing happens as low as 10 nM, and 100nM TFA would inhibit 10% of osteoblasts growth. Notably, same treatment by HCl didn't hazard to either osteoblasts or chondrocytes in same case. Moreover, when the activities of the TFA and hydrochloride salts of amylin, amylin-(1-8), and calcitonin were compared in osteoblasts, cell proliferation was consistently less with the TFA salts of these peptides, resulting in failure to detect a proliferative effect or wrongly attributing an anti-proliferative effect. This finding indicates purified peptides in concentrations above 10 nM in whatever cell or tissue type should take the salt into consideration.



TFA increasing cell viability (Ma *et al.*) can also be found in case of being metabolized for certain cell line or tissues. For example, TFA could induce the murine glioma cell growth at micromolar concentration. One day of treatment with TFA caused a slight concentration-dependent enhancement of cell growth. Exposure to TFA (0.5-7.0 mM) for 1 or 5 d elevated the [3H]leucine incorporation in a dose- and time-dependent manner. The results suggested that TFA stimulated cell growth and enhanced protein synthesis. Peptide-based assays in which TFA acts as a cell growth stimulator or inhibitor can introduce experimental variability, as well as false positive or reduced signals.

Impact of TFA on immunogen

TFA has been reported to exert inflammatory responses. Evaluation of the potential impact of counter-ion on immunogen has also been investigated. Mice were typically used in most studies, and showed very different clinical signs when they were treated with different counter-ions and salt levels.

Anne *et al.* reports that a 21-residue peptide (MOG35-55) derived from myelin oligodendrocyte glycoprotein (MOG) with varied TFA levels and counter-ion type result in induced experimental autoimmune encephalomyelitis (EAE) differently. They compared the development of clinical signs in female C57Bl6 mice immunized with two commercially available MOG_{35-55} peptides of similar purity but different peptide fraction (MOG-A being 45% peptide content and 55% TFA; MOG-B being 72% peptide content and 18% TFA). And they found that after a single immunization using MOG-A, containing a lower amount of peptide, mice exhibited a chronic disease with moderate severity, and with little recovery. In contrast, after a single immunization with MOG-B which contained about 60% more peptide, the mice showed less severe clinical signs, and by the end of the study four of the 15 mice recovered completely, reducing the incidence to 60%. The booster immunization with MOG-A resulted in a more rapid appearance of symptoms and more severe disease. A single immunization with MOG-A induced a chronic disease course with some recovery at later stages, whereas immunization with MOG-B induced a similar course of disease but with significantly lower averaged clinical scores despite a higher peptide content, suggesting that TFA influences an early stage of EAE leading to a more rapid disease onset.

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To determine if the counter ion could influence the disease, they compared MOG-B-containing TFA with MOG-B-containing acetate. Although disease incidence and severity were similar, the average day of disease onset occurred approximately 5 days earlier with the use of MOG-B-containing TFA, which that reducing the TFA content by exchanging it for acetate caused a delay in the average day of onset.

Table 1 Summary of disease parameters

	Peptide fraction	Counter ion	Group size	Day of onset	Inc max	Inc day	MACS	MACS day	AMCS	Score at end	Inc at end
Study 1 no booster											
MOG-A	45.4%	TFA	16	10.5 ± 0.7	100%	12	2.8 ± 0.2	17	3.1 ± 0.2	2.1 ± 0.2	100
MOG-B	72.0%	TFA	15	12.2 ± 0.5	87%	16	1.9 ± 0.3 [a]	16	2.0 ± 0.3 [g]	1.1 ± 0.3	60
Study 2 booster											
MOG-A	45.4%	TFA	15	10.1 ± 0.6	100%	12	3.9 ± 0.1 [b]	21	4.1 ± 0.1 [h]	3.7 ± 0.2	100
MOG-B	72.0%	TFA	17	13.4 ± 0.2 [e]	82%	21	2.6 ± 0.4 [c,d]	21	2.8 ± 0.4 [i]	2.3 ± 0.4	82
Study 3 TFA versus acetate with booster											
MOG-B	80.0%	TFA	9	11.6 ± 0.8	78%	16	2.2 ± 0.6	16	2.4 ± 0.4	1.9 ± 0.8	78
MOG-B	88.0%	Acetate	9	16.4 ± 1.7 [f]	78%	18	2.0 ± 0.5	18	2.8 ± 0.6	1.8 ± 0.8	78
					100%	28	2.1 ± 0.3	28			

Inc Max, maximum disease incidence during study; MACS, maximum average clinical score, the highest average daily score for all mice in the group; AMCS, average maximum clinical score, the average of the highest scores for all mice in group across entire study.

[a] $p = 0.0012$ versus Study 1, MOG-A (time 9 group); $F[12,1] = 2.78$

[b] $p < 0.0001$ versus Study 1, MOG-A (time 9 group); $F[12,1] = 3.72$

[c] $p = 0.0014$ versus Study 1, MOG-B (time 9 group); $F[12,1] = 2.14$

[d] $p < 0.0001$ versus Study 2, MOG-A (time 9 group); $F[13,1] = 5.02$

[e] $p < 0.05$ versus Study 2, MOG-A (t-test)

[f] $p < 0.05$ versus Study 3, MOG-B TFA (t-test)

[g] $p < 0.005$ versus Study 1, MOG-A (t-test)

[h] $p < 0.005$ versus Study 1, MOG-A (t-test)

[i] $p < 0.005$ versus Study 2, MOG-A (t-test)

Notably, TFA itself would not arouse immongen response according to the reports by Anne, and this has also been observed by our client in Astrazeneca, that 500 nM TFA itself did not increase or reduce the response in the PHA wells, indicating that the TFA does not affect specific or nonspecific T cell activation. So the immogen response and diversity should be a synergetic effect of both peptide and its counter-ion.

Impact of TFA on animal models

In animal experiments, TFA is very stable during metabolism and affects metabolism of the liver, with slight elevation of ATP in the liver and selected muscles (Air-aksinen and Tammisto, 1968), inhibition of glycolysis in rats intraperitoneally injected with TFA (Rosenberg *et al.,* 1970), inhibition of creatine phosphokinase (Airaksinen *et al.,* 1970), and declined levels of carbohydrate metabolism intermediates such as the lactate, pyruvate, glycero-1-phosphate, glucose 6-phosphate, glucose, and glycogen (Stier *et al.,* 1972). TFA concentrations in serum, liver and kidney reached their peak between 5 and 16 hr (Eckes and Buch, 1985). Acute toxicity of this compound in experimental animals is low; LD50 in mice is in excess of 2000 mg/kg (Airaksinen and Tammisto, 1968). Nevertheless, lesions in fetal liver and kidney as well as skeletal malformations in rodents were observed in TFA-treated rodents (Wharton *et al.,* 1979). In human, TFA affects binding of drugs to albumin and induces changes in fat and carbohydrate metabolism (Dale, 1985). The peak plasma bromide concentration, another key metabolite, occurred between 48 and 72 hr following halothane anesthesia (Tinker *et al.,* 1976).

In vivo toxicity was tested by Alessandro Pini, *et al.*. Mice were treated by purified M33-acetate and M33-TFacetate peptides was tested by intraperitoneal (IP) and subcutaneous (SC) administrations. No difference in acute toxicity (not shown) was recorded for both peptide forms and the LD50 was in line with Pini *et al.* (2007). However, we noted that substantial differences in toxicity signs were shown by mice immediately after peptide administration.

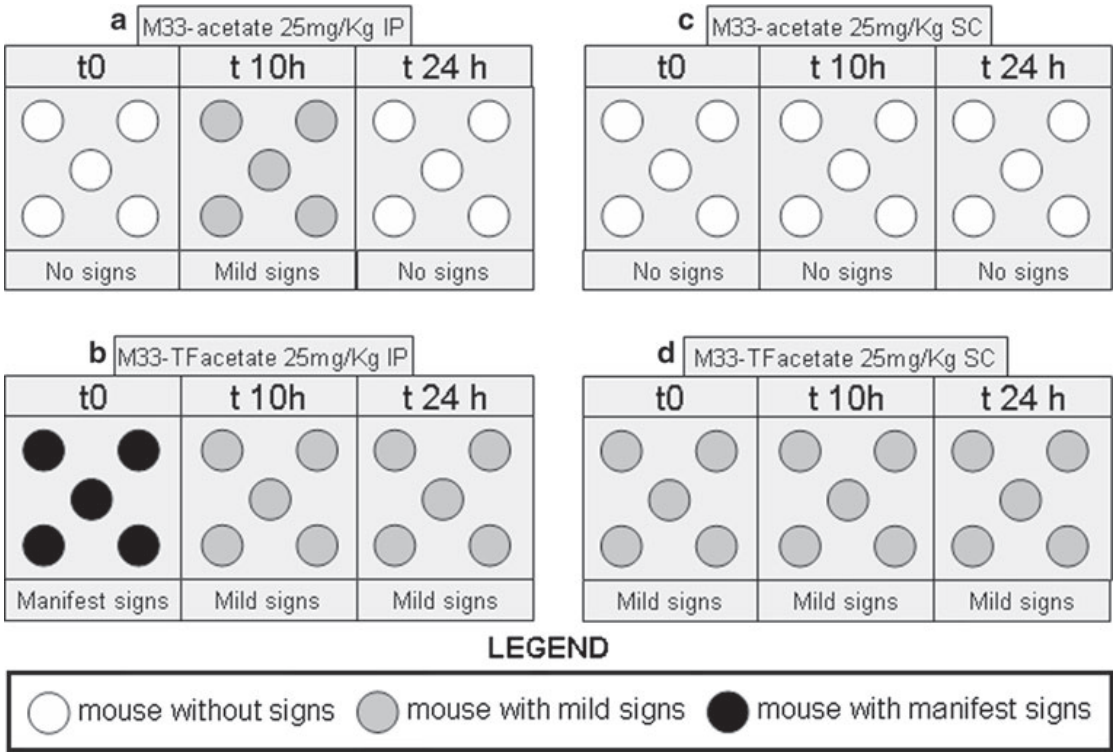

Figure 2. M33 toxicity *in vivo*. Five mice per group (each circle represents a mouse) were inoculated three times with M33-acetate (a, c) or M33-TFacetate (b, d) at the times indicated in the figure. In a and b mice were inoculated intraperitoneally (IP) with M33-acetate and M33-TFacetate, respectively. In c and d, mice were inoculated and subcutaneously (SC) with M33-acetate and M33-TFacetate, respectively. Different scales of grey indicate severity of signs shown by animals after injection. Mice inoculated with saline did not shown any signs of toxicity (not shown).

Impact of TFA on biological properties

To evaluate different counter-ion form and their impact on biological tests, Wojciech Kamysz and his coworkers selected 5 antimicrobial peptides (AMPs) and prepared into three counter-ion forms, namely, acetates, hydrochlorides, and trifluoroacetates. The five AMPs (CAMEL, citropin 1.1, LL-37, pexiganan, temporin A) were tested on to hemolytic activity against human red blood cells (hRBCs) and cytotoxicity (HaCaT). The results showed substantial difference between different salts, but the pattern is not consistent for the peptides. In general, the antistaphylococcal activity decreased in the order: CAMEL > temporin A > pexiganan > citropin 1.1 LL-37. The highest selectivity indexes were determined for CAMEL hydrochloride, pexiganan acetate, and temporin A trifluoroacetate. They observed that acetate counter-ions can substantially contribute to the hemolysis. For instance, the hemolysis of RBCs by pexiganan acetate (30.75% at 256 μg/mL) is substantially higher than that by both trifluoroacetate (7.04%) and chloride (8.51%). Similar conclusions can be applied to CAMEL.

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Cytotoxicity is also different, in the case of CAMEL and citropin 1.1, counter-ion type had a little impact on cytotoxicity. With temporin A, the trifluoroacetate proved to be the least toxic, while the acetate was the most. Interestingly, for three peptides, citropin 1.1, LL-37, and pexiganan, the lowest cytotoxicity was found for the acetate salts. These results are not consistent with the hemolysis pattern.

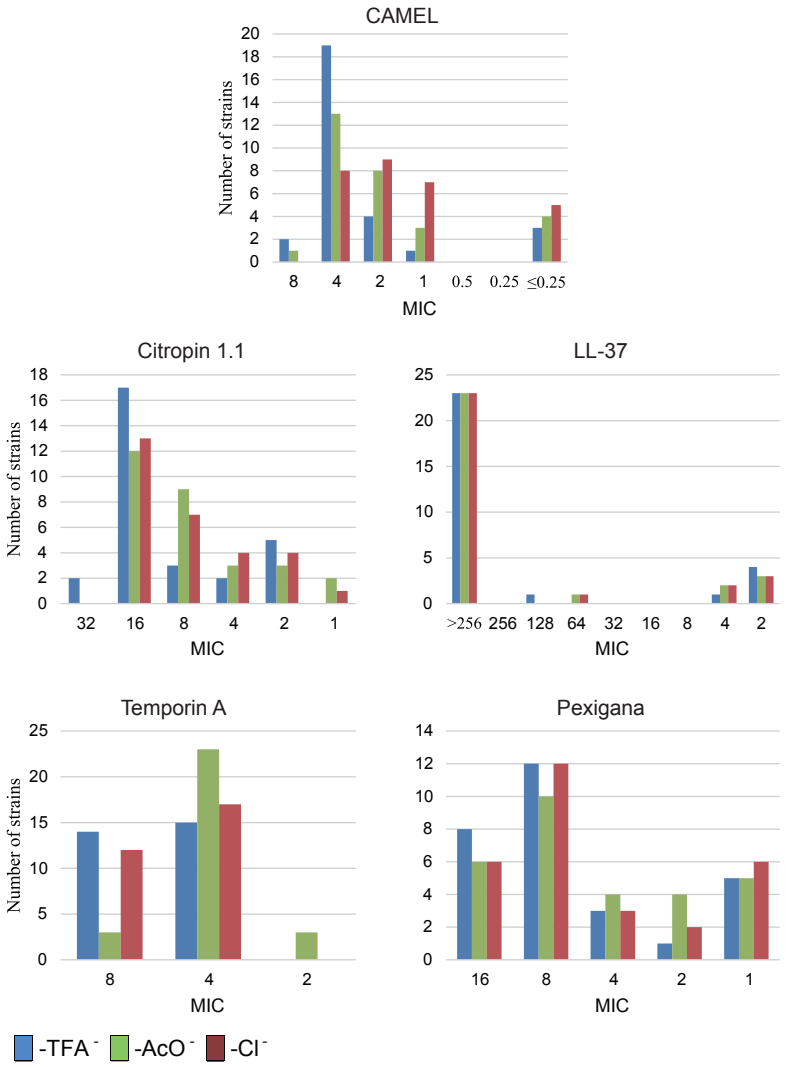


Figure 3. MIC of AMPs with counter-ion type.

Impact of TFA on spectrum study

In fact, counter-ions may also affect the secondary structure of peptides and proteins (Gaussier *et al.* 2002). The presence of different counter ions can affect hydrogen-bonding network and alter its structure (Blondelle *et al.* 1995) (Cinelli *et al.* 2001). Characterization of synthetic peptides, including conformational analysis, is routinely performed using IR and CD spectroscopy. Presence of TFA interferes with FTIR structural analysis by a strong IR band at around 1670 cm^{-1}, which overlaps the amide I band of peptides (1600-1700 cm^{-1}). This band overlaps or obscures the

amide I absorbance band of peptides, complicating peptide secondary structure determination experiments. And CD spectral data indicate that it induces a small increase in helical structures. Moreover, its capacity to decrease pH, in any buffer, induced difficulties in a rigorous study on pH dependence of structure-function relationships. But under same condition, HCl has not affect, so we recommend this counter-ion in structure studies.

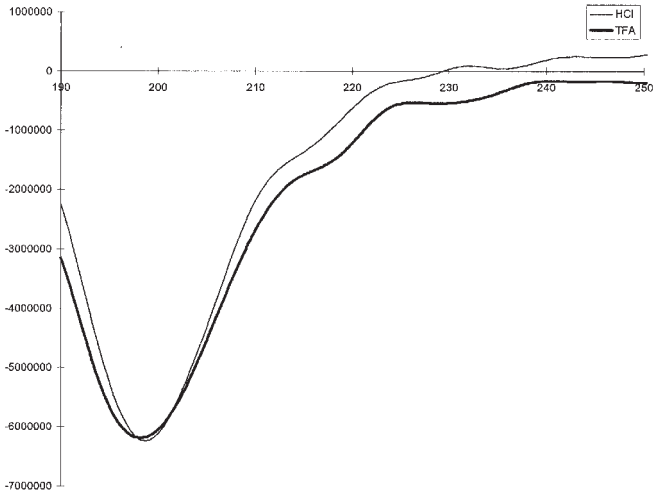
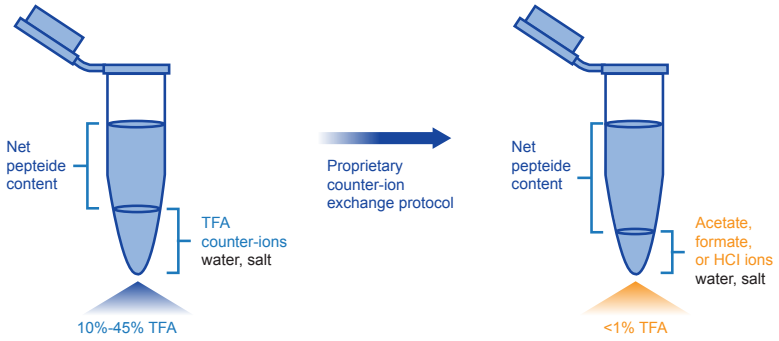


Figure 4. Effect of TFA and HCl on the CD spectra of pediocin PA-1 protein in aqueous solution. The spectra were recorded in the wavelength range of 190 to 300 nm.

Finally, TFA can reduce the pH of a peptide preparation, and thus alter the pH of subsequent assays.

How Does GenScript Accelerate to Achieve Better Result?

To make research easy, GenScript offers two kinds of TFA removal services, and could reduce TFA content to be less than <1% in peptide. Check TFA Removal Service to learn more which service is most suitable for your research.



Service Type	Final TFA Counterion %			
	HCl	Formate	Acetate	Phosphate
Guaranteed TFA removal	<1%	<1%	<1%	N/A
Standard TFA removal	~~10%-45%~~ <10%			

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References

1. Cornish J, Callon K E, Lin C Q X, *et al*. Trifluoroacetate, a contaminant in purified proteins, inhibits proliferation of osteoblasts and chondrocytes[J]. American Journal of Physiology-Endocrinology And Metabolism, 1999, 277(5): E779-E783.
2. Boullerne A I, Polak P E, Braun D, *et al*. Effects of peptide fraction and counter ion on the development of clinical signs in experimental autoimmune encephalomyelitis[J]. Journal of neurochemistry, 2014, 129(4): 696-703.
3. Ghanbari-Niaki A, Bergeron R, Latour M G, *et al*. Effects of physical exercise on liver ATP levels in fasted and phosphate-injected rats[J]. Archives of physiology and biochemistry, 1999, 107(5): 393-402.
4. Sikora K, Jaśkiewicz M, Neubauer D, *et al*. Counter-ion effect on antistaphylococcal activity and cytotoxicity of selected antimicrobial peptides[J]. Amino acids, 2018, 50(5): 609-619.
5. Gaussier H, Morency H, Lavoie M C, *et al*. Replacement of trifluoroacetic acid with HCl in the hydrophobic purification steps of pediocin PA-1: a structural effect[J]. Appl. Environ. Microbiol., 2002, 68(10): 4803-4808.
6. Blondelle S E, Ostresh J M, Houghten R A, *et al*. Induced conformational states of amphipathic peptides in aqueous/lipid environments[J]. Biophysical journal, 1995, 68(1): 351-359.
7. Cinelli S, Spinozzi F, Itri R, *et al*. Structural characterization of the pH-denatured states of ferricytochrome-c by synchrotron small angle X-ray scattering[J]. Biophysical journal, 2001, 81(6): 3522-3533.
8. Pini A, Lozzi L, Bernini A, *et al*. Efficacy and toxicity of the antimicrobial peptide M33 produced with different counter-ions[J]. Amino Acids, 2012, 43(1): 467-473.

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