The Synthesis of Conjugated Peptides Containing Triazole and Quinolone-3-Carboxamide Moieties Designed as Anticancer Agents

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Background: Cancer is a major health concern in human populations worldwide, and due to its causes being multifactorial, it is not easily curable. Many attempts have been made to tackle this disease in hopes of finding effective anticancer agents which are not harmful to healthy tissues. Peptides with several medicinal activities have been shown to be good candidates as anticancer agents to replace common classic anticancer drugs. Peptides in conjugation with either biologically active heterocyclic compounds or anticancer drugs may result in new molecules compiling the biological benefits of both individual compounds within a unit structure.

Objective: In this study some triazole-peptide conjugates as well as ciprofloxacin-peptide conjugates were designed, synthesized, and their anticancer activities evaluated. A normal skin cell line, NIH3, was also employed to determine the safety profiles of these conjugates.

Materials and Methods: Two peptides, YIGSR and LSGNK were synthesized by the solid phase peptide synthesis (SPPS) method using Wang resin. Cell viability was examined by employing the MTT assay. To determine the cytotoxicity of the triazole and ciprofloxacin conjugates, two human cancer cell lines were employed; HepG2 (human liver cancer cell line) and LNCaP (human prostatic carcinoma cell line). A human skin fibroblast cell line was also included for comparison.

Results: MTT results showed that all the compounds could inhibit the viability of cancerous cells in a concentration-dependent manner.

Conclusions: The results showed that these peptide conjugates are toxic against the aforementioned cancerous cells and thus may raise a hope for finding new anticancer agents made by such strategy in the near future.

Keywords: Anticancer activity, Cancer cells, Conjugated peptides, MTT assay, Solid phase peptide synthesis

1. Background
Cancer is one of the major leading causes of death in human populations globally. The death toll from cancer is estimated to rise and involve 70% more cases by 2030 (1). So far, various treatments have been practiced to combat cancer, among them radiation, surgery, and hormonal, biological and chemotherapy. Unfortunately, high financial costs and adverse effects can cause a heavy treatment burden on patients (2). Moreover, the appearance of drug resistance as a consequence of long-time use of conventional anticancer agents leads patients to face even worse situation (3, 4). Therefore, compounds with new identities which give better activity and few side effects are in high demand. Small peptides with anticancer activity have been shown to fulfill this demand, as they have desirable pharmacokinetic properties such as good solubility, fast blood distribution, high uptake by target tissues, and rapid elimination from the body (5). Peptides also produce low toxic effects on the body and its immune system.
Moreover, these molecules are amenable to different chemical modifications, producing compounds with various physico-chemical properties (6). Changes in properties may make peptides into molecules with desired pharmacokinetic and, perhaps, better pharmacodynamic activity (7). Anticancer peptides may affect cancerous cells through various mechanisms, including cell necrosis by cell membrane lysis and cell apoptosis through mitochondrial dysfunction and angiogenesis inhibition (5, 8). Peptides can also be employed for cell-membrane penetrating and/or cell targeting of known anticancer agents (9, 10). However, peptides have shown some undesirable properties such as low biological stability and short half-life (2). To tackle these drawbacks, many efforts have been implemented to improve peptides through different structural modifications (6, 11, 12). Also, hybridization and conjugation of peptides with other chemicals and active compounds have promised to produce molecules with favorable biological activities while containing fewer undesirable properties of peptides (1, 13, 14).

2. Objectives
The current study aimed to design and synthesize two kinds of peptide conjugates and evaluate their anticancer activities against cancerous cell lines. It is hoped that such peptide conjugates can potentiate anticancer activities of individual agents comparably when the agents are used alone. Meanwhile, by employing a normal cell line, the safety profiles of these peptide conjugates will be determined.

3. Materials and Methods
All the chemicals including protected amino acids, Wang resin, and reagents for peptide synthesis were provided by Bachem AG (Switzerland) or Santa Cruz Biotechnology Inc. (USA). Solvents were purchased from Sigma-Aldrich. Mass spectra of the samples were recorded on (Agilent 6410 QQQ) LC Mass spectrometer (USA). Plate reader instrument (Infinite® M200, TECAN, Switzerland) was employed to read absorption in the MTT assay.

3.1. Peptide Synthesis on Resin
Two peptides with anticancer activities, YIGSR and LSGNK, were synthesized by the solid phase peptide synthesis (SPPS) method using Wang resin, as previously reported (15).

3.2. General Procedure for the Synthesis of Ciprofloxacin –Peptide Conjugate
Peptide -ciprofloxacin conjugates were synthesized according to the published procedure (16). Briefly, Fmoc-ciprofloxacin (Fluorenyl methoxycarbonyl-ciprofloxacin) was synthesized first from a ciprofloxacin base, and in parallel, peptide sequences were constructed on Wang resin using a SPPS method with the Fmoc strategy (17, 18). Then, Fmoc-ciprofloxacin was attached to N-terminal unprotected resin-linked peptides through amide bond formation. Finally, the Fmoc group was removed from ciprofloxacin, and the conjugate ciprofloxacin-peptide was cleaved from the resin (16).

3.3. General Procedure for the Synthesis of Triazole -Peptide Conjugates
The triazole - peptide conjugate was synthesized according to the published procedure (15). Briefly, the triazole compound, 4-(4-phenyl-1H-1, 2, 3-triazol-1-yl) benzoic acid, was synthesized through several steps, and then, the triazole compound was connected to the previously synthesized resin-bound peptides unprotected with a similar procedure as mentioned above. Finally, triazole-peptide conjugate was cleaved from the resin (15).

3.4. Cell Toxicity Study
To determine the cytotoxicity of the triazole-peptide and ciprofloxacin-peptide conjugates, two human cancer cell lines were employed: HepG2 (human liver cancer cell line) and LNCaP (human prostatic carcinoma cell line). A human skin fibroblast cell line was also included in the study for comparison. Cell toxicity experiments were carried out in accordance with previously reported methods (19, 20), with some modifications. The cells were cultured in RPMI1640 medium enriched with fetal bovine serum (FBS, 10%), penicillin (100 µg.mL⁻¹), and streptomycin (100 µg.mL⁻¹) at 37 °C under CO₂/air (5:95%) condition. Cell viability was examined by MTT assay based on the reduction of the yellow tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide or MTT) and its conversion to purple formazan crystals through the action of metabolically active cells. The cells were seeded in 96-well plates at the
concentration of $10^4$ cells/well, incubated for 24 h, and then treated with various concentrations (10, 100, and 1000 µM) of the synthesized compounds for 48 h. The MTT reagent (10 µL, 5 mg.mL$^{-1}$ in PBS) was added to each well, and the plates were incubated for 4 hours at 37 °C. The medium solution containing MTT was removed, and dimethyl sulfoxide (100 µL) was added to each well to dissolve the formazan crystals. The plates were then maintained at 37 °C for 20 min. Finally, the plates were transferred into the plate reader instrument, and the absorbance was measured at 570 nm.

3.5. Statistical Analysis
GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. Multiple comparisons were considered among the groups using one way ANOVA followed by Tukey’s post hoc test. All data was shown as arithmetic mean ± S.E.M of triplicate (at least) determinations. Significance was accepted at $p < 0.05$.

4. Results

4.1. Peptide and Peptide Conjugates Synthesis
Two peptides, YIGSR (C1) and LSGNK (C2), were synthesized by the solid phase peptide synthesis (SPPS) method using Wang resin which gave 80% and 85% yields, respectively. One part of each peptide, before cleavage from the resin, was connected N-terminally to Fmoc-ciprofloxacin as well as the triazole compound, 4-(4-phenyl-1H-1,2,3-triazol-1-yl)benzoic acid through amide bond formation. After Fmoc removal, the peptide conjugates were cleaved from the resin. The chemical structures of the ciprofloxacin-peptide conjugates and triazole-peptide conjugates are depicted in Table 1. Mass spectra results gave the appropriate molecular weights, i.e., m/z 830.7 (M+1) for ciprofloxacin-LSGNK conjugate (Fig. 1, Cipro-C1), m/z 907.7

### Table 1. Chemical Structures of peptides and peptide conjugates. The letters and words in the table stand for as follows; R: alkyl group including hydrogen, H: hydrogen, A: the chemical structure (A), B: the chemical structure (B), C1: the peptide LSGNK, C2: the peptide YIGSR, Cipro: ciprofloxacin.

| R   | A               | B               |
|-----|-----------------|-----------------|
| H   | LSGNK (C1)      | YIGSR (C2)      |
|     | Cipro-C1        | Cipro-C2        |
|     | Triazole-C1     | Triazole-C2     |
The triazole-peptide conjugates displayed molecular ion peaks at the appropriate, i.e., m/z 764.8 (M+1) for triazole-LSGNK (Fig. 1, Triazole-C1) and 841.7 (M+1) for triazole-YIGSR (Fig. 1, Triazole-C2).

4.2. Evaluating Anticancer Activity
Anticancer activities of the two synthesized peptides, their ciprofloxacin/triazole conjugates, and ciprofloxacin as well as the triazole compound were examined separately on LNCaP and HepG2 cell lines. The overall results of all compounds showed more than 50% cell viability inhibition against the two cell lines, LNCaP and HepG2. Figure 2 shows the results for cell toxicity of C1 and C2 peptides and their conjugation with ciprofloxacin in LNCaP cells at different concentrations compared with ciprofloxacin. Figure 3 shows the results regarding viable inhibition of C1 and C2 peptides and their conjugation with ciprofloxacin in HepG2 cells compared with ciprofloxacin. In Figure 4, the results for cell toxicity are shown for C1 and C2 conjugated with triazole against LNCaP cells compared with the triazole compound. Figure 5 shows the findings regarding the cell toxicity of C1 and C2 conjugated with triazole achieved by employing HepG2 cells compared with the triazole compound.

5. Discussion
Hybridization is an important strategy for the modification of anticancer peptides (ACPs) which would improve therapeutic efficacy, reduce toxicity, etc. (21, 22). From the medicinal chemistry approach, it is a common practice to employ amide linkage (a stable covalent bond) for connecting two biologically active molecules (hybridization) in order to enhance/improve the pharmaceutical properties of the resultant molecule. Such linkage is quite stable in the environment (in vitro), but it is disconnected when the hybrid molecule enters a biological system and faces esterase or amidase enzymes. In the present study, this strategy was used to prepare peptide conjugates. In each peptide conjugate, one anticancer peptide was connected to a known chemical agent as an anticancer compound, so that both components of each peptide conjugate could participate and increase the bioactivity of the final molecule. It is noteworthy that the both peptides (7, 23) as well as the other anticancer agents, i.e. ciprofloxacin and the triazole compound used in the experiments, were known molecules with good stability.

5.1. Peptide and Peptide Conjugate Synthesis
Two peptides LSGNK (C1) and YIGSR (C2) were synthesized on the basis of the solid phase peptide synthesis (SPPS) method using the Fmoc strategy (17, 18). With this strategy, the peptide sequence was constructed on Wang resin as a solid support through the direction of the carboxyl end (C-terminal) of the

Figure 1. Mass spectra of the peptide conjugates: Ciprofloxacin –LSGNK (Cipro-C1), Ciprofloxacin-YIGSR (Cipro-C2), Triazole-LSGNK (Triazole-C1), Triazole-YIGSR (Triazole-C2).
Figure 2. Viability inhibition of C1 and C2 peptide conjugated with ciprofloxacin on LNCaP cells. Values are presented as mean ± SD of three independent experiments (n = 3). The star values are significantly different from the corresponding control (ciprofloxacin) (*p < 0.05, **p < 0.01). ns: not significant.

Figure 3. Viability inhibition of C1 and C2 peptide conjugated with ciprofloxacin on HepG2 cells. Values are presented as mean ± SD of three independent experiments (n = 3). The star values are significantly different from the corresponding control (ciprofloxacin) (*p < 0.05, **p < 0.01). ns: not significant.
Figure 4. Viability inhibition of C1 and C2 peptides conjugated with triazole on LNCaP cells. Values are presented as mean ± SD of three independent experiments (n = 3). The star values are significantly different from the corresponding control (triazole) (*p < 0.05, **p <0.01)). ns: not significant.

Figure 5. Viability inhibition of C1 and C2 peptides conjugated with the triazole on HepG2 cells. Values are presented as mean ± SD of three independent experiments (n = 3). The star values are significantly different from the corresponding control (triazole) (*p < 0.05, **p <0.01)). ns: not significant.
peptide. By deprotecting the other end of the peptide sequence (amino end), it was possible to connect N-terminally any chemical agent containing a free carboxyl group to the peptide by forming an amide (peptide) linkage. Peptide C1 has already been reported as an anti-proliferative agent against human colorectal cells (23), and peptide C2 has demonstrated an inhibitory effect on tumor growth and metastasis of human fibrosarcoma cells by inducing apoptosis (7). Furthermore, ciprofloxacin as an antimicrobial drug has been proven to present anticancer activity on human prostate (24) and other carcinoma cells, such as cancerous colorectal (25), lung (26), and bladder cells (27). For the peptide conjugation, a triazole compound was used as an anticancer agent. In the literature, the triazole moiety has shown various biological activities and attracted the attention of many scientists for use in their studies (28, 29). The anticancer activity of compounds containing a triazole moiety has also been documented in several publications (30-34).

In our experiments, a specific triazole molecule, previously designed and synthesized to be amenable for making amide linkage with peptides, and ciprofloxacin as an anticancer agent were attached to the two peptides, LSGNK (C1) and YIGSR (C2). The peptide conjugates, thus made, were then used to examine whether the anticancer activity of the resultant molecules is enhanced compared to triazole and ciprofloxacin molecules.

5.2. Evaluation of Anticancer Activity

This study assessed the effects of the synthesized anticancer peptides and their conjugates on LNCaP (human prostatic carcinoma cell line) and HepG2 (human liver cancer cell line) as well as NIH3 (human skin fibroblast cell line). The MTT results showed that all the peptide conjugate compounds demonstrated inhibitory action on the viability of cancerous cells with varying degrees in a concentration- dependent manner (Fig. 2- 5). Generally, C1 and C2 peptide conjugates demonstrated higher activity against the LNCaP cells’ viability than the HepG2 cells’ survival, considering ciprofloxacin and the triazole compound as control agents (see Fig. 2 and 4 compared with Fig. 3 and 5). In addition, all the peptide conjugates gave a similar pattern of inhibitory action against the viability of the LNCaP cell line (Fig. 2 and 4). This inhibitory pattern was repeated by the conjugated Cipro-C1 and Cipro-C2 in HepG2 cells but with less pronounced behavior compared with ciprofloxacin (Fig. 3). The both peptide conjugates showed more inhibitory activity only at the highest concentration (1000 µM). Compared with the triazole compound (Fig. 5), the both peptide conjugates, triazole-C1 and triazole-C2, showed noticeable viable inhibition in HepG2 cells only at the highest concentration (1000 µM). Moreover, the C2 conjugate was more effective than the C1 conjugate at this concentration.

It should be mentioned that C1 conjugates and C2 conjugates were more toxic (p<0.05) than their congeners, C1 and C2, against LNCaP cells in all different concentrations used in the experiments. Moreover, the toxicity of C2 conjugates, i.e. Cipro-C2 and triazole-C2, at 1000 µM concentration on LNCaP cells was more significant (p<0.01). Considering cell toxicity against HepG2 cell line, C1 conjugates and C2 conjugates were more effective than C1 and C2 congeners at 10µM concentration (p<0.05), but at higher concentrations (100 µM and 1000 µM) this activity was quite significant (p<0.01).

Overall, the current results showed that the conjugated peptides are more effective against the two employed cancerous cell lines compared with the cell toxicity of ciprofloxacin, the triazole compound, C1, and C2 used alone. These results are comparable with the previous results obtained in a similar experiment that employed MCF-7 cells (16). In another study in which peptide-drug conjugates were used against prostate cancer cell lines, the results showed that at low concentrations (10 µM or less), a significant degree of cell toxicity was not achieved by using the doxorurubicin-peptide conjugate (35). In fact, several factors may influence the cytotoxicity of peptide-drug conjugates on cancer cells, including the drug and peptide types, concentrations, and duration of incubation employed for such experiments (35). All the compounds were shown to be safe with low toxic activity (in the range of 2-6.5%) on the human skin fibroblast cell line (NIH3) used as normal cells to control the safety profile of these conjugated peptides.

6. Conclusion

These results demonstrated that the strategy of combining two anticancer agents into a unit identity (hybridization) could be effective and thus promise the development of anticancer agents with better properties. In such strategy, in addition to choosing the appropriate
peptide for joining to an anticancer agent, attention should be given to selecting proper cancer cells as the drug target. In the current study, the two peptides LSGNK and YIGSR conjugated with ciprofloxacin and a triazole molecule, showed cell toxicity more specifically against LNCaP cells than HepG2 cells, comparing ciprofloxacin and the triazole compound as control cell toxic agents. If peptides, in addition to being anticancer agents by themselves, play a role in combination with anticancer drugs as carriers for targeting specific tissues or organs, the benefits of the conjugated peptides may be doubled. The conjugated peptides made as such will be more favorable candidates for application in the clinical settings.

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Conflict of interest
The authors declare that there is no potential conflict of interest.

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