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Short communication

Discovery of toad-derived peptide analogue targeting ARF6 to induce immunogenic cell death for immunotherapy of hepatocellular carcinoma

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Chemotherapeutic drugs such as doxorubicin (DOX) and oxaliplatin can induce immunogenic cell death (ICD) in tumor cells. Current studies have demonstrated that peptide-based drugs can also induce ICD in tumor cells. Unlike small molecule drugs, peptide drugs not only enhance the effectiveness of immunotherapy but also offer the advantage of low biotoxicity [1]. Adenosine diphosphate (ADP)-ribosylation factor 6 (ARF6) is closely related to drug resistance in promoting cancer malignancy and immune evasion, which therefore is considered a potential target for cancer therapeutics. However, the previously reported inhibitors of ARF6 exhibit poor bioavailability and high toxicity [2]. To date, no peptide drugs targeting ARF6 have been reported. Herein, the toad-derived peptide analogue buforin IIb was discovered to bind to ARF6. Our results demonstrated that buforin IIb was capable of inducing ICD and disrupting the transportation of the epidermal growth factor receptor (EGFR) in hepatocellular carcinoma (HCC) cell lines. Furthermore, the knockdown of ARF6 led to a decreased expression of ICD markers. In brief, we introduced a novel strategy for cancer immunotherapy using peptide drugs that target ARF6, providing a rationale for pursuing combination therapy for HCC.

We initially investigated the correlation between *ARF6* and numerous tumor drug resistance driver genes, and our findings revealed a positive correlation between *ARF6* and these genes, including Kirsten rat sarcoma viral oncogene homologue (*KRAS*), neuroblastoma rat sarcoma (RAS) viral oncogene homolog (*NRAS*), *EGFR*, fibroblast growth factor receptor 2 (*FGFR2*), platelet-derived growth receptor alpha (*PDGFRA*), among others (Figs. S1A and B). Additionally, *ARF6* was highly expressed in various types of tumors, and the survival rate of liver cancer patients with high *ARF6* expression was significantly lower (Figs. S1C and D). We then screened the database of anticancer peptides, and found 87 peptides with potential binding ability to ARF6 (Fig. 1A). The cell permeability was further evaluated and 65 peptides were found to have good cell permeability, among which the toad-derived peptide analogue buforin IIb (RAGLQFPVGRLLRRLLRRLLR) was the strongest (Fig. 1B). The binding capacity of buforin IIb to ARF6 was further confirmed through affinity ultrafiltration (Fig. 1C).

To identify the direct targets of buforin IIb in HepG2 cells, bio-buforin IIb was synthesized by reacting buforin IIb with N-hydroxysuccinimide (NHS)-biotin (Fig. S2). We identified the interacting proteins of buforin IIb in HepG2 cells through a pull-down experiment (Fig. 1D) [3]. After performing a differential screening (fold change > 1.5, P value < 0.05) and eliminating non-specific binding proteins, we finally identified a total of 149 potential binding proteins (Fig. S3), including ARF6, eukaryotic initiation factor 4A-III (EIF4A3), DEAD-Box Helicase 3 X-linked gene (DDX3X), staphylococcal nuclease domain-containing 1 (SND1), among others. Subsequently, we carried out Gene Ontology enrichment analysis on these proteins and discovered that they

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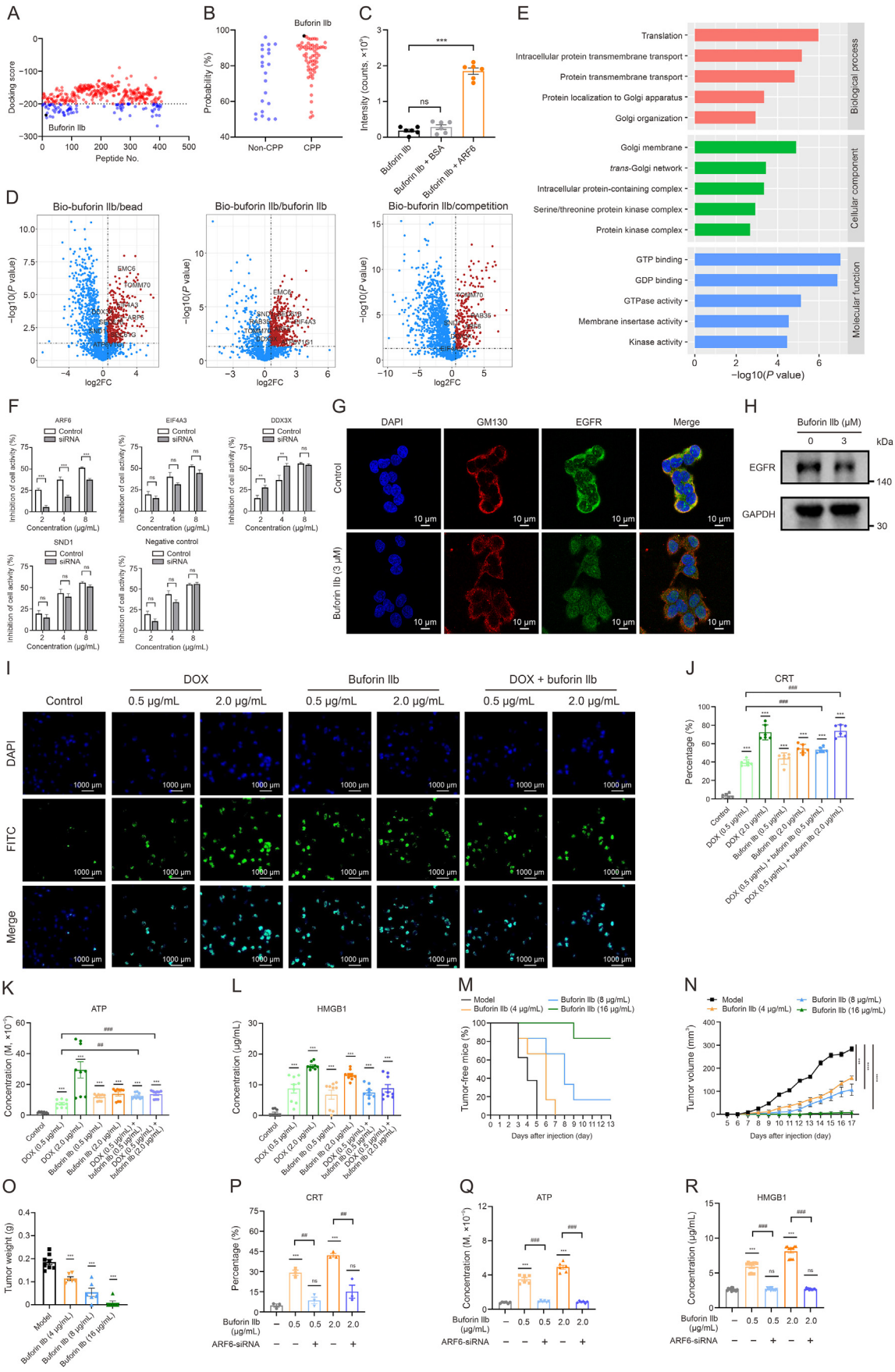
A
Docking score
Peptide No.
Buforin IIb
B
Buforin IIb
Probability (%)
Non-CPP
CPP
C
Intensity (counts, ×10⁶)
***
ns
Buforin IIb
Buforin IIb + BSA
Buforin IIb + ARF6
D
Bio-buforin IIb/bead
Bio-buforin IIb/buforin IIb
Bio-buforin IIb/competition
−log10(P value)
log2FC
E
Translation
Intracellular protein transmembrane transport
Protein transmembrane transport
Protein localization to Golgi apparatus
Golgi organization
Golgi membrane
trans-Golgi network
Intracellular protein-containing complex
Serine/threonine protein kinase complex
Protein kinase complex
GTP binding
GDP binding
GTPase activity
Membrane insertase activity
Kinase activity
Biological process
Cellular component
Molecular function
−log10(P value)
F
ARF6
EIF4A3
DDX3X
SND1
Negative control
Control
siRNA
Inhibition of cell activity (%)
Concentration (μg/mL)
G
DAPI
GM130
EGFR
Merge
Control
Buforin IIb (3 μM)
10 μm
H
Buforin IIb (μM)
0
3
kDa
EGFR
GAPDH
140
30
I
DOX
Buforin IIb
DOX + buforin IIb
Control
0.5 μg/mL
2.0 μg/mL
DAPI
FITC
Merge
1000 μm
J
CRT
Percentage (%)
Control
DOX (0.5 μg/mL)
DOX (2.0 μg/mL)
Buforin IIb (0.5 μg/mL)
Buforin IIb (2.0 μg/mL)
DOX (0.5 μg/mL) + buforin IIb (0.5 μg/mL)
DOX (0.5 μg/mL) + buforin IIb (2.0 μg/mL)
K
ATP
Concentration (M, ×10⁻⁶)
L
HMGB1
Concentration (μg/mL)
M
Model
Buforin IIb (4 μg/mL)
Buforin IIb (8 μg/mL)
Buforin IIb (16 μg/mL)
Tumor-free mice (%)
Days after injection (day)
N
Tumor volume (mm³)
Days after injection (day)
O
Tumor weight (g)
Model
Buforin IIb (4 μg/mL)
Buforin IIb (8 μg/mL)
Buforin IIb (16 μg/mL)
P
CRT
Percentage (%)
Buforin IIb (μg/mL)
ARF6-siRNA
Q
ATP
Concentration (M, ×10⁻⁶)
Buforin IIb (μg/mL)
ARF6-siRNA
R
HMGB1
Concentration (μg/mL)
Buforin IIb (μg/mL)
ARF6-siRNA

were mainly involved in protein transport processes and localized in the Golgi (Fig. 1E). We hypothesized that buforin IIb might affect the division and proliferation of cancer cells by interacting with these proteins. Interestingly, we observed a significant reduction in inhibition only after transfection of ARF6 small interfering RNA (siRNA) (Fig. 1F). Previous studies have shown that knocked down ARF6 disrupted the plasma membrane (PM) localization of EGFR in HeLa cells [4]. Notably, we found that buforin IIb disrupted the PM localization of EGFR in HepG2 cells (Figs. 1G and H).

Buforin IIb exhibits a significant inhibitory effect on HCC *in vitro* and possesses tumor cell targeting properties [5]. We demonstrated that it exerted significant anticancer effects in nude mice transplanted with HepG2 cells. Furthermore, the tumor inhibition rate *in vivo* was significantly enhanced by the combined administration of buforin IIb and DOX (Figs. S4A–D). Hematoxylin and eosin staining revealed that no significant pathological injuries were observed in either the buforin IIb or the combined administration groups (Fig. S4E). No significant changes in body weight were noted across all groups (Fig. S5A). The combined administration improved the organ coefficient of liver and spleen tissues (Figs. S5B–F). These results indicate that buforin IIb can inhibit the growth of liver cancer cells *in vivo*, and the combined administration of buforin IIb and DOX exhibits enhanced antitumor efficacy.

We further evaluated the induction effect of ICD in tumor cells by the cationic anticancer peptide buforin IIb. Our findings revealed that buforin IIb induced an increase in the levels of calreticulin (CRT), adenosine triphosphate (ATP) and high mobility group box 1 (HMGB1) in HepG2 cells, with effects similar to those of DOX (Figs. 1I–L). Moreover, the combined administration of DOX and buforin IIb increased the expression of CRT and the secretion of ATP (Figs. 1I–K). The induction effect of buforin IIb was further investigated in a tumor vaccination model, which demonstrated a reduction in tumor incidence in mice and a significant increase in the tumor inhibition rate (Figs. 1M–O). Buforin IIb improved the organ coefficient of liver, spleen, and lung tissues (Fig. S6). No relationship between ARF6 and ICD has been reported. Consequently, we evaluated the effect of ARF6 on ICD induction in HCC. We constructed HepG2 cells with stable expression of green fluorescent protein (GFP)-CRT (a fusion of CRT with GFP) through lentivirus transfection (Fig. S7). Following further transfection of ARF6-siRNA, the expression of CRT and secretion of ATP and HMGB1 induced by buforin IIb in HepG2 cells were significantly decreased (Figs. 1P–R and S8).

To optimize buforin IIb as an effective drug, we designed three buforin IIb analog peptides (B1, B2, and B3) and predicted their three-dimensional structures using AlphaFold (Figs. S9A and B). The results of the hemolysis assay suggested peptides B1 and B2 may offer enhanced biosafety (Fig. S9C). Moreover, the therapeutic index of all buforin IIb analogs increased compared to that of buforin IIb itself, with peptides B1 and B2 exhibiting the most significant improvements (Fig. S9D). All materials and methods used in this study are detailed in the Supplementary data.

In summary, we discovered that the toad-derived peptide buforin IIb disrupted EGFR transport and induced ICD to inhibit the growth of HCC by targeting ARF6. Additionally, the combined administration of buforin IIb and DOX could lead to more effective treatment outcomes. Our findings may extend beyond buforin IIb analog peptides, as other peptide drugs targeting ARF6 in future clinical applications may share a similar mechanism of action. This provides a rationale for pursuing combination therapies with chemotherapeutic drugs for HCC.

Ethical statement

All methods used in this study were carried out in accordance with national animal care and use guidelines laws, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine, Nanjing, China (Approval No.: 202001A022).

CRediT author statement

Dihui Xu: Writing – original draft, Methodology, Formal analysis, Data curation, Investigation, Writing – review & editing. **Xiang Lv:** Investigation, Methodology, Writing – review & editing. **Meng Yu:** Methodology, Investigation, Writing – review & editing. **Ao Tan:** Investigation, Methodology, Writing – review & editing. **Jiaojiao Wang:** Methodology, Formal analysis. **Xinyi Tang:** Methodology, Investigation. **Mengyuan Li:** Investigation. **Wenyuan Wu:** Funding acquisition, Investigation. **Yuyu Zhu:** Funding acquisition, Investigation. **Jing Zhou:** Funding acquisition, Investigation. **Hongyue Ma:** Writing – review & editing, Funding acquisition, Conceptualization, Project administration, Resources, Supervision.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Fig. 1. Discovery of the toad-derived peptide analogue targeting adenosine diphosphate (ADP)-ribosylation factor 6 (ARF6) to induce immunogenic cell death (ICD) of hepatocellular carcinoma (HCC). (A) Docking score of anticancer peptides with ARF6 (Protein Data Bank (PDB) ID: 2A5D). (B) The cell permeability of peptides. (C) Affinity ultrafiltration of buforin IIb with vehicle, bovine serum albumin (BSA) or ARF6 ($n = 6$). (D) Identification of binding proteins with buforin IIb in HepG2 cells. Wild type HepG2 cell proteins were incubated with beads (Bead group), buforin IIb (Buforin IIb group), and bio-buforin IIb (Bio-buforin IIb group). HepG2 cell proteins, which were pretreated with buforin IIb, were incubated with bio-buforin IIb (Competition group). (E) Gene Ontology annotation analysis. (F) Inhibition of cell activity by buforin IIb against HepG2 cells after small interfering RNA (siRNA) transfection ($n = 12–18$). (G) Immunofluorescence staining of HepG2 cells, which were treated with vehicle or buforin IIb for 24 h. (H) Western blot analysis of expression levels of epidermal growth factor receptor (EGFR) in the membrane of HepG2 cells, which were treated with vehicle or buforin IIb for 24 h. (I) Immunofluorescence staining of HepG2 cells treated with vehicle, buforin IIb, doxorubicin (DOX) or combined administration (DOX + buforin IIb) for 4 h. (J) Percentage of cell membrane expression of calreticulin (CRT) ($n = 6$). (K) Secreted adenosine triphosphate (ATP) was detected by chemiluminescence assay ($n = 9$). (L) Secreted high mobility group box 1 (HMGB1) was detected by enzyme linked immunosorbent assay ($n = 9$). (J–L) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, the treated group versus the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, the combined administration group (DOX + buforin IIb) versus the model group. (M) The percentage of tumor-free mice in tumor vaccination model ($n = 6–8$). (N) Inhibitory effect of buforin IIb on the tumor vaccination model mice ($n = 6–8$). (O) Tumor weight ($n = 6–8$). (N, O) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, the treated group versus the model group. (P) Percentage of cell membrane expression of CRT ($n = 3$). (Q) Secreted ATP was detected by chemiluminescence assay ($n = 6$). (R) Secreted HMGB1 was detected by enzyme linked immunosorbent assay ($n = 12$). (P–R) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, the treated group versus the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, the ARF6-siRNA (+) group versus the ARF6-siRNA (−) group. Data were expressed as mean ± standard error of the mean (SEM). *P* values are determined by unpaired Student's *t*-test. CPP: cell-penetrating peptide; ns: no significance; GTP: guanosine triphosphate; GDP: guanosine diphosphate; GTPase: guanosine triphosphate hydrolase; EIF4A3: eukaryotic initiation factor 4A-III; DDX3X: DEAD-Box Helicase 3 X-linked gene; SND1: staphylococcal nuclease domain-containing 1; FC: fold change; DAPI: 4′,6-diamidino-2-phenylindole; GM130: 130 kDa cis-Golgi matrix protein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; FITC: fluorescein isothiocyanate.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2024.101038.

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