EUROPEAN JOURNAL OF CANCER 47 (2011) 773–783

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A novel hybrid peptide targeting EGFR-expressing cancers

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ARTICLE INFO

Article history:
Received 29 July 2010
Received in revised form 22 October 2010
Accepted 27 October 2010
Available online 26 November 2010

Keywords:
Drug resistance
Drug targeting
EGFR
Hybrid peptide
Immunotoxins

ABSTRACT

Several potential molecular-targeted anticancer drugs focus on the inhibition of receptor tyrosine kinase and tumour growth, but these tyrosine kinase inhibitors (TKI) have been reported that the mutations of kinase-related signal molecule genes in cancer cells lead to the drug resistance. To overcome this issue, we have designed a novel targeting anticancer 'hybrid-peptide' EGFR-lytic peptide, in which epidermal growth factor receptor (EGFR) binding peptide is conjugated with a newly designed lytic-type peptide containing cationic-rich amino acids that disintegrates the cell membrane to kill cancer cells. In this report, cytotoxic activity of EGFR-lytic peptide was investigated in various human cancer and normal cell lines. It was found that the resulting conformational change in the novel lytic peptide enabled it to bind selectively to the membrane of cancer cells, and due to its acquired synergistic action, hybrid peptide demonstrated selective destruction of cancer cells as swiftly as 10 min after exposure. Treatment with EGFR-lytic peptide exerted a sufficient *in vitro* cytotoxic activity against TKI-resistant cancer cells with K-ras mutations. Moreover, *in vivo* analyses revealed that this peptide displayed significant antitumour activity in mouse xenograft models of both human K-ras mutation negative and positive cancers. Thus, hybrid peptide can be a unique and powerful tool for a new cancer-targeted therapy.

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1. Introduction

Several potential molecular-targeted anticancer drugs on the market inhibit receptor tyrosine kinase and tumour growth. In some cases, mutations of kinase-related signal molecule genes in cancer cells result in the resistance to these tyrosine kinase inhibitors (TKIs). Recently, it is revealed that K-ras mutations are significantly associated with a lack of response not only to epidermal growth factor receptor (EGFR) TKIs but also to EGFR antibody drugs like cetuximab in patients with non-small-cell lung cancer and advanced colorectal cancer.[1] To overcome this critical issue, we have designed a novel molecular-targeted anticancer drug named hybrid peptide that directly kills cancer cells superior to signal pathway blockers.

Immunotoxins, monoclonal antibodies or ligands against overexpressed proteins on the surface of cancer cells conjugated to plant or bacterial toxins, have been extensively investigated for their possible use as anticancer agents.[2] A number of immunotoxins have been tested in preclinical and clinical trials, and interleukin-2-diphteria toxin fusion protein (IL2-DT; Ontak™) has been approved for the treatment of cutaneous T-cell lymphoma.[3,4] In addition, *Pseudomonas* exotoxin-based immunotoxins including interleukin-4-*Pseudomonas*

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doi:10.1016/j.ejca.2010.10.021

exotoxin [IL4(38-37)-PE38KDEL] and interleukin-13-*Pseudomonas* exotoxin (IL13-PE38QQR) fusion proteins have been tested in clinical trials.[5,6] Both Diphtheria toxin and *Pseudomonas* exotoxin act by catalytically inactivating the elongation factor 2 in the ribosome complex, after taken into lysosomes, activated and translocated into the cytosol. This mechanism of action enables the immunotoxins to effectively destroy dormant, non-replicating tumour cells.

Although the targeting approach towards cancer utilising bacterial toxin-based immunotoxin is fascinating, its limitation lies in the liver toxicity due to the bacterial toxin and immunogenicity caused by the toxic proteins.[3,5,7] In addition, molecular size of immunotoxins is generally larger compared to chemical compounds or fragment antibody drugs, and this might prevent drugs from efficiently penetrating into tumour mass in the human body. To overcome these issues, new generation immunotoxins with evolutional approach are needed.

EGFR has been an important tumour-specific target for drug therapies for many years.[8,9] EGFR plays important roles in cell growth, differentiation and migration. Its positive signalling is found to cause increased proliferation, decreased apoptosis and enhanced tumour cell motility and angiogenesis.[10] EGFR overexpression is frequently found in a wide spectrum of human tumours of epithelial origin, including breast, lung, gastric, colorectal, prostate, pancreatic and ovarian cancers.[11] All these findings have brought EGFR as an important target for receptor-mediated delivery system of drugs. Recently, several studies reported successful identification of peptide ligands of EGFR by screening phage display libraries, implicating possible drug delivery by targeting to EGFR.[12,13]

Therapeutic peptides are increasingly gaining popularity as therapeutic agents for a variety of applications[14], including tumour vaccine,[15] antimicrobial therapy[16] and nucleic acid delivery.[17] In addition, research and development of new cancer therapeutics involving peptide-based drug has been widely undertaken.[18,19] It is also known that peptide drugs are relatively easily synthesised using either recombinant or solid-phase chemical synthesis techniques and the production costs are generally affordable when compared to antibody-based therapeutics. Recently, Papo and Shai reported that a new lytic-type peptide (D-K_6L_9) composed of 15-amino acids diastereomeric sequence containing D- and L-forms of leucine and lysine disrupts the plasma membrane.[20,21] This peptide kills tumour cells better than normal cells, and disintegrates the cell membrane in a detergent-like manner. In addition, the peptide's diastereomeric sequence preserves its anti-tumour activity in serum and in the presence of proteolytic enzymes. On the other hand, it is suggested that the peptide's selectivity to the cancer cells is probably determined predominantly by an increase in the level of acidic components or phosphatidylserine on the cancer cell membrane.[20] Even though this lytic-type peptide has selective cytotoxicity between normal and cancer cells, this peptide still kills normal cells in lower concentration, and thus, it is not considered suitable for the combination with targeting moiety.

Using aforementioned and recent identification of peptide sequences binding to EGFR and lytic-type peptide sequence, we have developed a new hybrid peptide targeting EGFR-overexpressed cancer cells. This hybrid peptide, termed EGFR-lytic peptide, is composed of an EGFR-binding moiety and a novel designed lytic moiety that is stable when combined with targeting peptide with less toxic effect to normal cell lines compared to D-K_6L_9 peptide, with three glycine spacer. In this study, we demonstrated both *in vitro* cytotoxic activity and selectivity of cell death induced by EGFR-lytic peptide in seven human cancer cell lines derived from breast, pancreas, lung, prostate and brain cancer. In addition, we investigated the interaction of EGFR-lytic peptide with the cancer cell surface and the mode of action of peptide-induced cancer cell death. *In vivo* experiments also revealed that this novel hybrid peptide displayed significant antitumour activity.

2. Materials and methods

2.1. Materials

Gefinitib and Erlotinib were purchased from Toronto Research Chemicals (Ontario, Canada). Anti-EGFR mouse monoclonal antibody (Clone 225) and PD153035 were purchased from Calbiochem (La Jolla, CA).

2.2. Cell culture

Human breast cancer (BT-20 and MDA-MB-231), lung cancer (H322 and H460), pancreatic cancer (SU.86.86), prostate cancer (LNCaP), glioma (U251) and lung fibroblast (MRC-5 and WI-38) cell lines were purchased from the American Type Culture Collection (Manassas, VA). Human pancreatic cancer (BXPC-3) and colon cancer (HCT116 and DLD-1) cell lines were purchased from the European Collection of Cell Cultures (ECACC; Salisbury, Wiltshire, UK). Human embryonic kidney cell line (HEK293) was purchased from RIKEN Cell Bank (Tsukuba, Japan). Human colon cancer cell line (SW837) was purchased from HSRRB (Osaka, Japan). Cells were cultured in RPMI1640 (BT-20, MDA-MB-231, H322, H460, SU.86.86, LNCap, U251, BXPC-3, DLD-1 and SW837), MEM (MRC-5 and WI-38), McCoy's 5a (HCT116) or D-MEM (HEK293) containing 10% FBS v/v (BioWest, Miami, FL), 100 μg/ml penicillin and 100 μg/ml streptomycin (Nacalai Tesque, Kyoto, Japan).

2.3. Peptides

The following peptides were purchased from Invitrogen (Carlsbad, CA):

1. Designed lytic-peptide: K**L**LLK**L**L**KK**LLK**L**LKKK-OH (bold letters are D-amino acids.)
2. EGFR binding + designed lytic hybrid peptide (EGFR-lytic peptide): YHWYGYTPQNVIGGGK**L**LLK**L**L**KK**LLK**L**LKKK-OH
3. D-K_6L_9 peptide: LK**L**LK**K**L**L**K**K**LL**K**LL-NH_2
4. EGFR binding + D-K_6L_9 peptide: YHWYGYTPQNVIGGG LK**L**LK**K**L**L**K**K**LL**K**LL-NH_2

Nos. 3 and 4 peptides were used for Supplementary Figs. S1 and S2 only.

All peptides were synthesised by use of solid-phase chemistry, purified to homogeneity (i.e. >90% purity) by reversed-phase high-pressure liquid chromatography and assessed by mass spectrometry. Peptides were dissolved in water and

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