Development of an Immunotoxin Incorporating the Australian Jumper Ant Toxin, Pilosulin 1

BY

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ABSTRACT

Immunotoxins are therapeutic agents that directly target toxins to specific cells and are generally comprised of an antibody or antibody fragment (Fab or scFv) linked to a toxic moiety. The assessment of a number of immunotoxins in recent Phase I and II clinical trials has been very promising, particularly for the treatment of haematological malignancies. Since the majority of these incorporate large, potent bacterial or plant toxins, their therapeutic potential is limited by dose-limiting non-specific toxicity, immunogenicity and the need to be endocytosed. An alternative approach is to incorporate cytolytic toxins such as melittin and pilosulin that are smaller, less toxic molecules that act at the cell membrane. Both melittin- and pilosulin-based immunotoxins (mel-IT and pil-IT) have been developed by our research group. These cytolytic immunotoxins, which incorporate a scFv moiety specific for the human kappa myeloma antigen (KMA) expressed on human kappa myeloma cells and the human lymphoblastoid cell line, HMy2, display specific cytotoxic activity at micromolar concentrations against the target cell line. In contrast, immunotoxins in clinical and late stage pre-clinical studies are active at picomolar concentrations and thus it was deemed necessary to enhance the specific activity of mel-IT and pil-IT to ensure they could be effective at relevant clinical doses.

The pil-IT displayed greater cytotoxic potential as peptide studies indicated that pilosulin was four times more potent than melittin against white blood cells (WBCs) and additionally, the pil-IT was shown to be twice as toxic as the mel-IT on a molar basis. In order to identify the regions of pilosulin essential for cytolytic activity and thus develop a smaller immunotoxin, two recombinant constructs were generated that encoded truncated toxin domains; $P_{1-22}F$ (incorporating the N-terminal helix of pilosulin, amino acid residues 1 to 22) and $P_{23-56}F$ (incorporating the C-terminal helix, residues 23 to 56). Unexpectedly, both recombinant constructs displayed reduced cytolytic activity compared to the parent construct (pil-IT/ $P_{1-56}F$), due to reduced specific binding of the immunotoxins to the target cells, presumably as a result of incorrect tertiary folding of the expressed proteins. In a further attempt to increase the specific activity of the pilosulin-based immunotoxin, two additional constructs were generated; $P_{L21}F$ which had a longer linker arm between the full-length pilosulin peptide and the antigen-specific scFv moiety to enhance steric access of the toxin to the target

cell membrane, and $P_{A33K41}F$ which contained the C-terminal helical region of pilosulin in an enhanced helical conformation to aid membrane interaction and penetration. While preliminary studies indicated that both constructs had cytotoxicity comparable the parent, neither exhibited enhanced cytolytic activity.

Contaminant proteins were observed to be co-purifying with the immunotoxins raising a question as to whether these contaminants may have had the potential to affect the cytolytic activity of pil-IT. The most significant contaminant was identified as apoA-I, a 27-kDa hydrophobic serum protein that had previously been shown to inhibit the activity of cytolytic peptides and also to stabilise damaged membranes. As the FBS used to supplement the expression culture medium was identified as the source of apoA-I, the pil-IT immunotoxin was expressed in serum-free medium. The recombinant protein expressed under these conditions was extremely susceptible to proteolysis in the cell culture medium and attempts to block this proteolysis by supplementing the cultures with BSA or α2-macroglubulin were ineffective. However, supplementing the serum-free expression cultures with E-64, a specific cysteine protease inhibitor, blocked the majority of the proteolytic degradation of pil-IT and allowed affinity purification of a very pure immunotoxin preparation.

Unexpectedly, pil-IT expressed in this manner displayed significant non-specific toxicity compared to previous immunotoxin batches. It is possible that the presence of E-64 in the culture supernatant affected the tertiary fold of the immunotoxin so that it acted independently of the antigen binding specificity encoded by the scFv moiety, or that pil-IT in a very pure form is very toxic and non-specific (i.e. a true result of an 'apoA-I free'-immunotoxin preparation). Another issue requiring consideration was that the insect cell line used for expression of the non-specific toxic batches of immunotoxin in serum-free medium (High Five cells) was different to that used to express batches of specifically cytotoxic immunotoxin in serum-containing medium (Sf21 insect cells).

To address this question, the pil-IT was expressed in High Five insect cells in the presence of FBS and then affinity purified with a Tween-20 wash step to dissociate the apoA-I from the immunotoxin. While this produced a pure preparation, non-specific cytolytic activity was again observed, although to a lesser degree than that observed for

the batches expressed with E-64 supplementation. Tween-20 was found to contribute to some of this non-specific activity but was not the sole factor, as pil-IT expressed in High Five cells in the presence of FBS and purified without Tween also exhibited non-specific cytotoxicity. Thus it was likely to be a result of either (i) expressing the immunotoxin in High Five insect cells which, in contrast to Sf21 cells, may generate a tertiary fold in the immunotoxin that allows it to act non-specifically, or (ii) the absence (or low levels) of apoA-I, which when present in the immunotoxin preparation, may have inhibited its non-specific cytolytic activity and/or repaired toxin–induced cell membrane damage, with the strength of this effect varying for different cell membranes.

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ABBREVIATIONS

3'	three prime
5'	five prime
A	amps
aa	amino acid(s)
AcNPV	*Autographa californica* nuclear polyhedrosis virus
ADCC	antibody-dependent cellular cytotoxicity
apoA-I	apolipoprotein A-I precursor
approx	approximately
AP	alkaline phosphatase
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indopyranoside
BJP	Bence Jones protein
bp	base pair
BP9	BacPAK9 vector
BSA	bovine serum albumin
CM	complete media
CMP	complete media with 1% Pluronic acid
CNBr	cyanogen bromide
C-terminus	carboxyl terminal end
D	Dalton
dAbs	domain antibodies
dpi	days post infection
dH_20	deionised water
DNA	deoxyribonucleic acid
dNTP	deoxynucleic triphosphate
dsFv	disulfide stabilised single chain variable fragment
DT	Diphtheria toxin
E.coli	*Escherichia coli*
EtOH	ethanol
Fab	fragment antigen binding
FBS	Foetal bovine serum
h	hours
hpi	hours post infection
HPLC	high performance liquid chromatography
IT	immunotoxin
κ	kappa
Kb	kilobasepairs
kDa	kilodalton
KMA	kappa myeloma antigen
LDH	lactate dehydrogenase
M	molar (moles/litre)
Mab	monoclonal antibody
mel	melittin
mel-IT	melittin-based immunotoxin
MeOH	methanol
MM	multiple myeloma
μg	micrograms

μl	microlitres
ml	millilitres
min	minutes
mM	millimolar
MOI	multiplicity of infection (number of virus particles per host cell)
MWCO	molecular weight cut-off
NBT	nitro blue tetrazolium
nM	nanomolar
nmol	nanomoles
NT	No tween wash step during purification
N-terminus	amino terminal end
p0	viral passage 0 (transfection)
p1	viral passage 1
p2	viral passage 2
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	Pseudomonas exotoxin
pfu	plaque forming unit/s
pi	postinfection
pil	pilosulin
pil-IT	pilosulin-based immunotoxin
pil-IT H5	pilosulin-based immunotoxin expressed in High Five insect cells
pil-IT Sf	pilosulin-based immunotoxin expressed in Sf21 insect cells
pmol	picomoles
PVDF	polyvinylidinedifluoride
rpm	rotations per minute
RT	room temperature
RTA	ricin toxin A chain
s	seconds
scFv	single chain variable fragment
SDS	sodium dodecyl sulphate
SFM	serum free media/medium
SUPAMAC	Sydney University Prince Alfred Macromolecular Analysis Centre
TAE	tris-acetic EDTA
3-D	three dimensional
TBS	tris buffered saline
Tris	tris(hydroxymethyl)aminomethane
TW	Tween wash step during purification
U	unit
UV	ultraviolet
VH	variable heavy
VL	variable light
w/v	weight by volume

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