Expert Opinion

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Vaccines & Antibodies

# TransMabs: cell-penetrating antibodies, the next generation

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Intracellular proteins are becoming attractive targets in diagnosis and for therapy such as in signal pathways, on enzymes, transcription factors and structural proteins. Antibodies have been used therapeutically for extracellular pathogens and for targeting cell-surface antigens. Antibodies normally do not pass easily through intact cellular or subcellular membranes in living cells. Methods to shuttle antibodies into living cells are either labour-intensive and/or compromise the structural and functional integrity of the cell or require the integration of genes for heavy and light chain production through gene therapy approaches. A new technology platform, 'SuperAntibody Technology', enables antibodies to be shuttled into living cells without harming them. Such cell-penetrating antibodies open new diagnostic and therapeutic windows. The term 'TransMabs' has been coined for these antibodies. Proof of principle has been achieved with a 17-amino acid peptide with membrane translocating properties, conjugated with anti-caspase-3 antibody. Such a TransMab inhibits significantly *in vitro* apoptosis-related events, such as caspase-3 activity, DNA fragmentation and spectrin cleavage. Anti-caspase-3 TransMab, therefore, could be utilised to inhibit apoptosis in a variety of diseases, such as Alzheimer's, Huntington's and Parkinson's. Unlike currently available peptide inhibitors, this TransMab is not expected to have *in vivo* toxic side effects and can only target activated forms of the enzyme. This paper discusses the advantages and limitations of cell-penetrating antibodies (TransMabs) to existing small molecule drug development approaches.

**Keywords:** antibody, apoptosis, cell-penetrating, SuperAntibody, TransMabs

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## 1. Introduction

Since the discovery by E von Behring and S Kitsato that sera from immunised animals contain neutralising antitoxin antibodies, antibodies began to dominate the science of immunology. Several Nobel prizes were awarded to immunologists who contributed to the structure, function, genetics and manufacturing of antibodies, emphasising the important role of humoral immunity for the well-being of humans and animals. Antibodies are the oldest and one of the most used tools in diagnostic work and research and development. Furthermore, antibodies have emerged as the largest market segment of immune-based therapeutics.

Due to of the success of monoclonal antibodies, one might question the need for any improvement of their activity and for expanding antibody usage. However, there are situations where monoclonal antibodies fail or underperform. This review discusses such situations and presents a platform technology that increases the potency of monoclonal antibodies to overcome their inherent deficiencies. This technology is designed to boost the potency of antibodies. To distinguish these kinds of engineered biologicals, the term SuperAntibody has been coined [1,2].

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Some monoclonal antibodies have a low affinity for a given target and it is not practical or economical to make a new and better monoclonal. The SuperAntibody technology (SAT) can overcome this deficit by increasing binding through crosslinking of target antigen (avidity).

Most antigen targets in signalling pathways transduce signals only when crosslinked, as in the case of a cellular receptor. Receptor density or spacing is critical for crosslinking, but is not typically achieved by bivalent antibodies. In some cases, creating a polyvalent antibody structure, such as an IgM, is able to induce crosslinking and signalling. Unfortunately, this class of antibody, even in humanised form, is not amenable to the scale-up and purification required for a clinical product. SAT represents a method of achieving dynamic crosslinking of target antigen with an IgG framework [3,4].

Antibodies, being large proteins, cannot pass through intact cellular or subcellular membranes in living cells. SAT can modify antibodies to penetrate into living cells without harming them. Such cell-penetrating antibodies open new diagnostic and therapeutic windows. A term has been coined for these modified antibodies, 'TransMabs'.

## 2. TransMabs: membrane-penetrating SuperAntibodies

Probes with low molecular weight have been developed that enter the cell without damage to the membrane and generate signals that can be monitored without harming the cell. However, their targeting specificity is limited and often highly crossreactive. Although surface antigens on pathogens or cells have been targeted, there are also intracellular proteins that can become attractive targets in diagnosis and for therapy, such as in signalling pathways, on enzymes, transcription factors and structural proteins. Standard methods to shuttle antibodies into living cells have been achieved by microinjection and vector-mediated antibody gene expression, as well as liposome-mediated transmembrane transport. These procedures are labour-intensive, may compromise the structural and functional integrity of the cell, and, as in the case of gene expression, require delivery and expression methods not commonly available in gene therapy.

It has been shown that naturally occurring polyreactive autoantibodies against DNA and nuclear ribonucleoproteins can penetrate the cellular and nuclear membrane of living cells despite their charge or hydrophilicity [5].

Therefore, the authors reasoned that it might be possible to endow ordinary antibodies with a membrane-penetrating property by linking them to a fusion protein containing a short membrane transport-facilitating peptide. Obviously, it was by no means expected that a small peptide could alter the properties of an antibody sufficiently to cause it to penetrate into cells.

Cellular import of proteins has been achieved by genetically engineering a fusion protein containing a short membrane facilitating peptide [6,7]. Cell entry is mandatory for viral replication and viruses have developed specialised protein sequences that facilitate their entry. Several peptides have been identified from such membrane transporting sequences (MTSs).

The authors have utilised the transport-peptide (MTS) technique to overcome the shortcomings of existing techniques in order to study intracellular structures and their biological functions.

This novel modification allows antibodies to penetrate the cellular membrane of living cells without affecting the cell, as the authors have demonstrated [8,101]. A 17-amino acid MTS that facilitates transport across membranes was site-specifically attached to monoclonal antibodies directed against intracellular components [8,101]. The antibodies conjugated to the MTS-peptide were transported into and bound to intracellular structures. This was visualised by means of confocal and fluorescence microscopy.

In this model, the authors have used a monoclonal antibody, 5D10, that does not react with any protein in the cytoplasm of the mouse fibroblast NIH 3T3 cells. MTS-5D10 cells can be detected intracellularly after 3 h of culture, but the unconjugated 5D10 could not. The translocation of non-antigen-specific MTS-5D10 follows kinetics, which reaches a maximum after 12 h of culture as long as high concentrations of conjugates are maintained in the culture. The distribution of internalised immunoglobulin appears uniform in the cytoplasm. The nucleus shows much less Ig-positive material. The cell morphology and viability after culture with MTS-5D10 remains normal. Sectional analysis reveals that the Ig-positive material is intracellular with very little attachment to the plasma membrane [8].

In contrast were results with antibodies to two intracellular cytoskeletal proteins, actin and paxillin. After converting these antibodies into TransMabs, the authors could demonstrate specific localisation on cytoskeletal structures using confocal microscopy. This new method enables the study of intracellular processes in living cells in a 'time-lapse' fashion [8]. For example, it will be possible using TransMabs to observe the movement of intracellular structures following signal induction and to detect molecular interactions in signal transduction circuits.

Antibodies against cytoplasmic and nuclear antigens involved in signalling and cell cycle control are also suitable for study with this approach. Certainly, these cell-penetrating TransMabs have potential as diagnostic reagents. However, the authors sought to demonstrate their utility to create therapeutic drugs able to modulate specific metabolic and proliferative functions in cells. Therefore, the authors studied the MTS-peptide linked to an antibody targeting caspase-3, an enzyme involved in triggering apoptosis [9] (**Table 1**).

Apoptosis, or programmed cell death, is essential in the process of development. For example, immature B cells are eliminated by apoptosis, which is essential for the maintenance of immune tolerance [10].

Transforming growth factor (TGF)-β induces apoptosis in a time-dependent manner in various cells, particularly hepatoma cells [11-13]. Coincident with the onset of DNA fragmentation is the cleavage of alpha-II-spectrin into 150-,

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**Table 1. Comparison of MTS-conjugated anti-caspase-3 antibody with naked antibody on inhibition of apoptosis (adapted from [9]).**

| Methods of detection | Treatment* | |
|---|---|---|
| | ***Anti-caspase-3*** | ***MTS-anti-caspase-3*** |
| Caspase-3 activity | 10.4‡ | 34.2 |
| Cleavage of spectrin | 30.1 | 60.5 |
| DNA fragmentation | 34.7 | 68.4 |
| DNA laddering | 19.8 | 57.2 |
| Counted cell death | 35.9 | 69.8 |

Jurkat cells were pretreated with different antibody (1 μg/ml) for 1 h following AD treatment for 4 h.
*Anti-caspase-3 = rabbit monoclonal anti-caspase-3 antibody. For each experiment, there was also an AD-treated group and a caspase-3 inhibitor (DEVD-fmk)-treated group. Cleavage of spectrin was detected using western blot, DNA fragmentation was measured using cell-death ELISA, and cell counting was performed by Trypan Blue exclusion assay. n ≥ 2 for each data.
‡The percentage inhibition was calculated compared with that of the caspase-3 inhibitor.
AD: Actinomycin D; ELISA: Enzyme-linked immunosorbent assay;
MTS: Membrane transporting sequence.

115-, and 110-kDa fragments [14]. Alpha-II is a cytoskeletal actin-binding protein (alpha-fodrin). Antibiotics such as cycloheximide or puromycin also rapidly induce apoptosis, which leads to the cleavage of alpha-II-spectrin via the known caspase-3 and calpain pathway [14].

It has been demonstrated by Brown *et al.* [14] that the broad spectrum caspase inhibitor, Boc-D-fmk (BD-fmk) completely blocks TGF-β-mediated apoptosis in mouse B cells, whereas specific caspase inhibitors are unable to prevent apoptosis. The cleavage of alpha-II-spectrin is, therefore, an indicator of apoptosis [14]. In addition, fluoromethyl ketone-conjugated peptides are strong broad spectrum caspase inhibitors [15].

The authors have used monoclonal and polyclonal antibodies to the active form of caspase-3 antibodies linked to the MTS-transmembrane peptide to target and modulate apoptosis in Jurkat T lymphoma cells. It could be demonstrated that these antibodies against the active form of caspase-3 (anti-active caspase-3) conjugated to the MTS peptide function as caspase inhibitors and can prevent apoptosis in T lymphoma cells [9].

These results indicate that MTS-conjugated antibodies can be used as vehicles to target apoptosis-related enzymes in the cell. The scheme in **Figure 1** depicts how TransMab anti-caspase-3 enters the cell and blocks activated caspase-3.

The advantages of using TransMabs in apoptosis are their specific target recognition in the cell and their expected lower toxicity in patients compared with conventional apoptosis inhibitors. By comparison, the active peptide inhibitor Boc-fmk is toxic to mice [16-18] and could not be considered a candidate for clinical development. The therapeutic utility of MTS-linked antibodies should be pursued further in preclinical studies to modulate apoptosis in a variety of diseases.

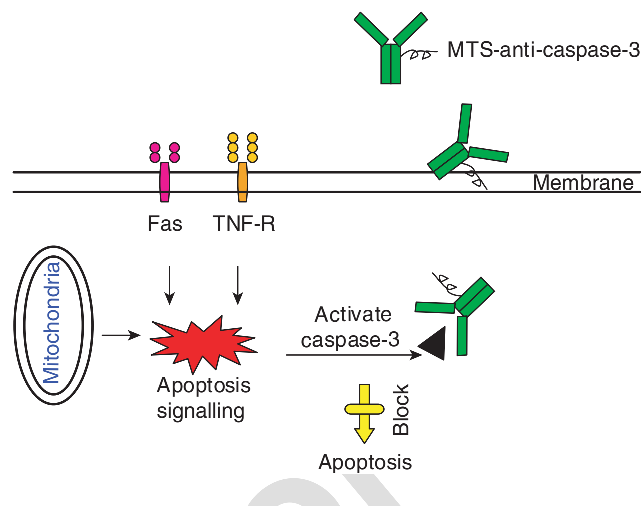


**Figure 1. Scheme of TransMab pathway.** The action of MTS-conjugated antiactive-caspase-3 antibody. After cells receive apoptotic signalling initiated from Fas, TNF-R or mitochondria, pro-caspase-3 will be cleaved and become active caspase-3, which functions as an executor of apoptosis. Attached with an MTS peptide, which enhances cell membrane penetration, anti-active caspase-3 antibodies could rapidly accumulate and target active caspase-3, leading to the inhibition of apoptotic cell death.
MTS: Membrane transporting sequence; TNF-R: Tumour necrosis factor receptor.

**Box 1. Advantages of TransMabs over small molecule drugs.**

- Specificity – reactivity to single, conformational-dependent epitopes can distinguish isoforms and activated forms
- Potential for toxicity – no inherent toxicity or target organ toxicity
- Pharmacokinetic advantage – up to one millionfold
- Rapid feasibility assessment – many antibodies to intracellular proteins already exist as diagnostic tools

## 3. Creating drugs where small molecule development is infeasible

From the previous example, detailing the creation of an anti-caspase-3 TransMab, the authors have demonstrated for the first time a method for using antibodies to target intracellular proteins that can be deemed a drug development platform. From this work, as well as with other intracellular targets, a number of general advantages of this approach over traditional or facilitated (computer-assisted drug design) small molecule drug development can be extrapolated (see **Box 1**).

The key asset of antibodies over any other drug development approach is their specificity. Antibodies can selectively recognise small modifications of proteins, peptides and

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organic molecules, as well as conformational changes that may be associated with changes in activities of enzymes, receptors or other biologically active components of a cell. As with the authors' studies with caspase-3, the specificity or inhibitory activity of existing peptides were insufficient to create a product candidate. The antibody in contrast, when enabled to enter cells, was not only an active inhibitor, but would recognise only an activated form of the enzyme. This means that *in vivo* it would target only enzyme in cells undergoing apoptosis. Even more contrasting was the difference in toxicity. The only active peptide inhibitor in cell culture was highly toxic in animals; the antibody conjugate, in contrast, had no inherent toxicity (not related to targeting of the enzyme). It is well known that many small molecule drugs fail in early animal or clinical testing due to unexpected toxicity. Perhaps what is underappreciated about today's human or humanised forms of antibodies is the period of time in which they circulate in the body. Typically, such antibodies display circulating half-lifes of 7 – 14 days when not targeting circulating lymphocytes [19,20]. When compared to small molecule drugs with half-lifes of minutes to hours, antibodies have a large advantage in the period of time in which sufficient quantities of the inhibitor are in the body to maintain blockade. Another advantage is that, in many cases, antibodies to intracellular targets may already exist or at least exist for a closely related target that allows for a rapid feasibility assessment.

## 4. Expert opinion

Despite all these advantages, we don't expect TransMabs to replace existing small molecule drugs for most applications. Antibodies cannot be delivered orally and at present cannot be manufactured with the same costs of goods as most small molecule drugs. However, we believe there are many drug development opportunities where this novel form of antibody can overcome problems that make it infeasible to develop a traditional drug.

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