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The ATTEMPTS delivery systems for macromolecular drugs

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Aiming at successful targeted drug delivery – a system that possesses both targeting and prodrug features that can be activated once the system reaches the target site upon systemic administration – would be desired to reduce systemic toxicity. Previously we proposed a heparin/protamine-based system for delivery of protease drugs such as tissue-specific plasminogen activator (t-PA). This approach, termed 'antibody targeted, triggered, electrically modified prodrug-type strategy' (ATTEMPTS), would permit antibody-directed administration of inactive t-PA and allow a subsequent triggered release of the active t-PA at the target site. This system can be adapted to target tumor tissues when protein transduction domain (PTD) peptide such as TAT is incorporated in the ATTEMPTS construct. Both *in vitro* and preliminary *in vivo* studies using TAT-gelonin (TAT-Gel) and TAT-asparaginase (TAT-ASNase) conjugates have demonstrated that the on/off regulation of the membrane translocation activity of PTD at tumor target, followed by intracellular delivery of cytotoxic macromolecular drug, can be accomplished. Hence, the PTD-mediated delivery system derived from our previous ATTEMPTS approach is a system that incorporates all of the targeting function, prodrug feature, release mechanism and cell entry mechanism and could become a generic system for delivery of macromolecular drugs.

Keywords: ATTEMPTS approach, prodrug, protein transduction domain (PTD) peptide, targeting

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1. Introduction: limitations of anticancer therapies

Anticancer drug therapies are beset by three bottleneck limitations. The first one is the absence of a preferential killing of tumor cells over normal cells for the drug. As known, most anticancer drugs interfere with cell replication and consequently exhibit similar effects towards proliferating tissues. While interaction with a desirable tumor target would result in therapeutic functions of the drug, exposure to inadvertent normal cells would lead to toxic side effects. The second limitation arises from the rapid clearance of water-soluble drugs with low molecular weights from the bloodstream, and the proteolytic degradation and/or immunogenicity of large nucleic acid or protein type drugs. The third limitation lies in the inability of most drugs to cross cell membranes. At present, efficient delivery of therapeutic compounds can only be achieved when the molecules are small (typically less than 1000 Daltons) and hydrophobic. Yet, low molecular weight drugs often localize more efficiently in normal tissues than in tumors, thereby rendering higher toxic effects [1], owing to the high interstitial pressure and unfavorable blood flow within rapidly growing tumors [2,3]. There is virtually no anticancer drug that by itself could overcome all these three limitations, and hence a variety of delivery systems were designed to accommodate such needs; all

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with the same milestone goal of achieving the maximum drug efficacy and minimum drug-induced toxicity.

The first limitation of lacking tumor selectivity can be circumvented by attaching the drug to a targeting component such as an antibody or a peptide ligand (e.g., RGD for targeting the endothelial cells). Yet, targeting alone would not completely abort the toxic effects because the drug could still exert activity on peripheral tissues along its traveling path to the target site. A combination of the targeting and prodrug features in a delivery system, where the drug remains inactive during the targeting and delivery process but is then converted at to the original active form at the targeted site, deems to be the only solution to this limitation. Indeed, based on this principle, an approach termed 'ADEPT' [4,5], which permits a specific enzymatic conversion of a prodrug at the target site to its active parent drug, has attained reasonable success in delivering only small therapeutic agents to the tumor without the drug-induced toxic effects.

The second limitation, which is related primarily to the pharmacokinetic properties of the drugs, can be managed with relative ease. In natural systems, the pharmacokinetic behaviors of many small drugs are regulated by a series of transport proteins [6,7]. Therefore, binding such drugs to a carrier or a macromolecule would normally prolong their circulation times. In addition, conjugation of a biomolecule with poly (ethylene glycol) (PEG) has also been extensively attempted as another means to prevent degradation and improve circulation time of the drugs [8]. The ability of PEG to discourage protein – protein interaction could also reduce the immunogenicity of the linked protein drugs. Alteration of the pharmacokinetic properties alone, however, would not advance the cell targeting or internalization ability of the delivered drugs. To this regard, these manipulations, at best, can serve only as a supplement in improving the functionalities of a carrier or a delivery system.

Of the abovementioned three limitations, the third one – inability of the drug to enter cells – is probably the most challenging to overcome. A wide variety of methods has been attempted to deliver proteins and other macromolecules into living cells, including microinjection [9], scrape loading [10], electroporation [11], liposomes [12], bacterial toxins [13,14], and receptor-mediated endocytosis [15-18]. Most of these methods, however, are inefficient or not for practical uses, cause appreciable cell deaths, or result in uptake into intracellular vehicles without effective cytoplasmic delivery. The most preferred and widely used method is the receptor-mediated endocytosis approach, which relies on binding of antibodies (or ligands) on the modified drug conjugate (or carrier) to the antigen determinants (or receptors) on the surface of the targeted cell. Internalization is then followed via the endocytic route [19]. Despite some success, this cell-entry method suffers a number of shortcomings on its application to delivering antitumor agents. One shortcoming is that not all antibodies are endocytosable, and therefore selection of an appropriate antibody for both targeting and cell internalization for a specific tumor could present a major challenge. Second, antibodies normally penetrate tumors quite slowly and display good tumor:blood ratios for only several hours after intravenous injection [20]. Indeed, biodistribution studies have shown that crossing the endothelial layer of the tumor blood vessel is such a slow process that it severely limits the absolute tumor uptake of the antibody-drug conjugates [21]. A third shortcoming is the presence of the 'antigen barrier' [22,23] that may sequester the antibody in perivascular regions, preventing a homogeneous distribution of the drug conjugates within the tumor mass. The most serious drawback of the receptor-mediated endocytosis, however, is that it requires the invagination and vasculation of the membrane lipid bilayer to form free cytoplasmic vesicles [24]. Since macromolecular drugs such as proteins that have entered cells via this pathway remain enclosed within the lipid vesicles, they do not have access to the cell cytoplasm. It seems reasonable to assume that the escape from endocytic vesicles is the rate-limiting step in achieving true cellular delivery, yet many applications of this cell-entry method fail to recognize this.

2. ATTEMPTS construct: targeted t-PA delivery

Previously, we have developed a system with both targeting and prodrug features that can be activated once the system reaches the target site upon systemic administration: an ATTEMPTS enzyme delivery system using thrombolytic agents (e.g., urokinase, tissue plasminogen activator (t-PA)) as an example, before applying this system to anticancer drug delivery. These enzyme drugs are plasminogen activators (PA) that convert an inactive pro-enzyme plasminogen to an active protease plasmin. Fibrinolysin in turn degrades fibrin, the principal component of the structural lattice of a thrombus. Plasmin, however, also degrades several circulating blood-clotting factors impairing the haemostatic system. Like any typical therapeutic agent without substrate specificity, conversion of the circulating plasminogen (i.e., the normal substrate) would lead to systemic generation of excess plasmin, whereas conversion of the fibrin-bound plasminogen (i.e., the target substrate) to the fibrin-bound fibrinolysin by the thrombolytic agent would result in dissolution of a thrombus. It is primarily because of this indiscriminate feature in attacking both the fibrin-bound and circulating plasminogen by the agent, that thrombolytic therapy also carries the risk of hemorrhage as its major side effect [25]. As depicted in **Figure 1**, the ATTEMPTS delivery system is termed based on the abbreviation of 'Antibody Targeted, Triggered, Electrically, Modified, Prodrug-Type Strategy [26]. It comprises a large protein complex made of two components: a targeting component that consists of an antibody (Ab) chemically linked with an anionic heparin molecule; and a drug component that consists of the enzyme drug modified with cationic species. In order to preserve binding domain of the antibody, heparin was chemically conjugated via carbohydrate moiety on the

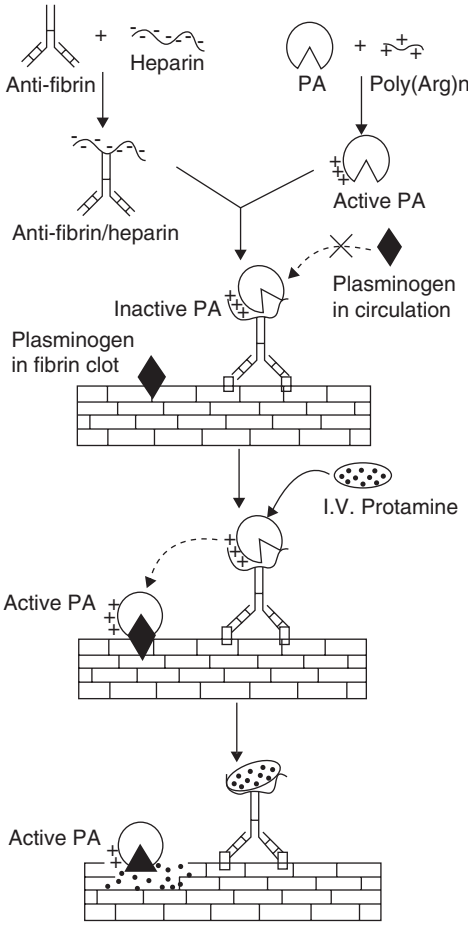

Figure 1. Schematic illustration of the ATTEMPTS approach.
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Table 1. Changes in levels of fibrigogen, antiplasmin and plasminogen after the plasma was treated with t-PA or heparin-bound mt-PA+ [28].

Plasma	Fibrinogen	Antiplasmin	Plasminogen
Untreated	100%	100%	100%
Treated with t-PA	61 ± 8	80 ± 4	62 ± 3
Treated with heparin-inhibited mt-PA+	89 ± 10	98 ± 2	95 ± 4

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Fc region of the antibody instead of random attachment via primary amines [26]. The two components are linked via a tight but reversible electrostatic interaction. Since the cationic species are relatively small, the modified enzyme would retain its catalytic activity. This activity, however, would be inhibited after binding with the Ab-heparin counterpart, primarily owing to the blockage of the active site of the enzyme by these appended macromolecules. Therefore, similar to a 'prodrug' type of approach, the antibody – enzyme complex will be without catalytic activity during administration and yet accumulate at the target site because of the function of the attached antibody. Since protamine is a USFDA-approved clinical drug with a much stronger affinity to heparin, it can be used safely and effectively as a competing agent to trigger the local release of the modified enzyme from the protein complex at the target site. The active enzyme would therefore be able to act selectively and discriminatively on substrates present only at the target site.

In vitro studies have demonstrated the feasibility of this approach. A cationic octa-peptide containing poly $(Arg)_7$ sequence with a cysteine residue at the C-terminus was successfully linked to t-PA by using SPDP coupling method. To obtain $(Arg)_7$-modified t-PA product (termed mt-PA$^+$) with the required heparin-binding strength, the reaction mixture was purified using a heparin affinity column. This purification method was based on the principle that, since ATIII eluted from this heparin column at 0.6 M NaCl, whereas protamine did so at 1.2 M NaCl, mt-PA$^+$ fractions eluted in between these two salt concentrations would possess the required heparin-binding strength. Quantitative fibrinolysis assay demonstrated on/off regulation by heparin and protamine on degradation of the fibrin gel by t-PA [27].

As discussed earlier, bleeding produced by a PA drug is due to induction of systemic production of plasmin, which then depletes circulating fibrinogen, antiplasmin, and plasminogen. To examine if the ATTEMPTS approach could attenuate the bleeding risk, concentrations of fibrinogen, antiplasmin, and plasminogen were measured following incubation in plasma of an equivalent dose of either commercial t-PA or heparin-bound mt-PA$^+$ for 60 min. As shown in **Table 1** [28], whereas t-PA produced typical and significant depletion of these protein factors, heparin-bound mt-PA$^+$ did not elicit any statistically meaningful reduction in any of the parameters measured. These findings confirmed that bleeding risk of a PA drug could be eliminated or largely attenuated by using the ATTEMPTS approach.

Animal studies were also conducted using a rat thrombosis model to confirm the overall feasibility of the system. Sprague-Dawley rats (250 – 300 g; n = 40) were allocated to five groups (n = 8 for each group), and underwent ligation of the inferior vena cava (IVC) to initiate thrombus formation. Two days after thrombosis formation, the rats were treated with five test samples including: i) Group 1: saline buffer (negative control); ii) Group 2: mt-PA$^+$; iii) Group 3: mt-PA$^+$/Ab(59D8)-Hep; iv) Group 4: mt-PA$^+$/Ab-Hep (same as that in Group 3), followed by administration of 400 μg protamine 10 min later; and v) Group 5: 1 mg/kg commercial t-PA (positive control). The mt-PA$^+$ dose used in Groups 2, 3 and 4 studies was equivalent, in measured total chromogenic activity, to that of the commercial t-PA used in the Group 5 (control) study. Two hours after drug treatment, rats were killed and their IVCs harvested and examined for the thrombus size and histological

Table 2. Summary of *in vivo* results [28].

Group	Clot weight (mg)	Fibrinogen level (μg/ml)	Plasminogen level (μg/ml)
#1 Saline (control)	82.7 ± 13.2	1286 ± 182	179.5 ± 9.4
#2 mt-PA$^+$	48.3 ± 11.5	967 ±140	128.5 ± 8.9
#3 mt-PA$^+$/Ab-Hep	80.6 ± 12.2	1203 ± 296	185.0 ± 14.0
#4 mt-PA$^+$/Ab-Hep + Protamine (400 μg)	62.9 ± 9.9	1205 ± 34	175.0 ± 12.6
#5 t-PA (1 mg/kg) commercial (control)	55.9 ± 14.5	910 ± 162	121.5 ± 14.5

changes. Blood samples were also taken by using the cardiac puncture technique and then assayed for levels of fibrinogen and plasminogen using appropriate ELISA methods.

As shown in **Table 2**, the weight of the thrombus created by IVC ligation was relatively inconsistent, as reflected by the large variation observed in the control group (Group 1). The mt-PA$^+$ conjugate (Group 2) appeared to be slightly more potent than commercial t-PA (Group 5) in clot dissolution; however, because of the large variation in measuring the clot weight, this difference was statistically insignificant. When the mt-PA$^+$/Ab-Hep complex was administered (Group 3), no clot dissolution was observed, as the average weight of thrombi (80.6 ± 12.2 mg) observed in this animal group was statistically indistinguishable to that observed in Group 1 (82.7 ± 13.2 mg). These *in vivo* findings yielded a definite validation to one of our primary hypotheses, that is binding of the macromolecular Ab-Hep conjugate would render mt-PA$^+$ inactive, yielding the prodrug feature to this t-PA delivery system. When protamine was administered 10 min after the administration of the mt-PA$^+$/Ab-Hep complex, a substantial degree of clot dissolution was observed as the mean weight of the clot was reduced to a value (62.9 mg) comparable to that (55.9 mg) mediated by commercial t-PA. This finding provided a clear confirmation to our other hypothesis, that is protamine could be used *in vivo* to reverse heparin-induced inhibition on the fibrinolytic activity of mt-PA$^+$. It should be pointed out that, since the clot weight could not measured until after killing the animals, the large variation in the measured weight of clot dissolution could simply be due to the inability to measure the produced clot weight before the drug treatment [28].

Despite promise, the lack of a means of the ATTEMPTS system to facilitate an effective cellular drug uptake, however, restricts it to being applied solely to enzyme drugs that exert activities in the circulation or an extracellular environment.

3. Protein transduction domain (PTD) peptides and their integration into ATTEMPTS for the delivery of antitumor agents

3.1 Overcoming cell membrane barrier with PTD

In 1988, Green and Loewenstein [29] and Frankel and Pabo [30] independently reported that the full-length (86 amino acids) TAT protein derived from the Human Immunodeficiency Virus (HIV) could cross the cell membrane and transactivate a viral genome. Interest in applying this TAT protein to intracellular delivery of biological compounds, however, has dramatically heightened only since 1994, when Fawell *et al.* [31] demonstrated that heterogeneous proteins chemically crosslinked to a 36-amino acid domain of the TAT protein (TAT-(37-72)) were able to transduce into primary cells. Subsequent to the TAT discovery, several other proteins with a similar transducing capability were also identified, including the *Drosophila* homeotic transcription factor ANTP (encoded by the *antennapedia* gene) [32] and the herpes simplex virus type-1 (HSV-1) VP22 transcription factor [33]. To date, the domains responsible for protein transduction (termed PTD; protein transduction domains) have been successfully identified to be Residues #47-57 in TAT [34], Residues #267-300 in VP22 [29], and the third alpha helix (Residues #43-58) in ANTP [35]. Most recently, synthetic poly(arginine) peptides with more than six residues [36], either linear [37] or branch-chained [38], as well as the arginine-rich, non-toxic low molecular weight protamine (LMWP) peptides derived from protamine by our research group for clinical heparin reversal [39-41] have also displayed the ability to translocate macromolecules through cell membranes.

Translocation of biomolecules across the cell membrane mediated by TAT is so efficient that it can hardly be matched by any existing method including the most capable receptor-mediated endocytosis approach. Fawell and co-workers [31] demonstrated that biological signals could be readily detected even when the concentration of TAT in the cell culture medium was as low as 1 nm, and approximately 10^7 TAT molecules were able to enter one single cell. To improve the tumor-penetrating ability of scFv(19), an antibody fragment specific for targeting the ED-B domain of fibronectin in the extracellular matrix surrounding tumor neovasculature, Niesner and co-workers [42] attempted its conjugation with TAT. Results showed that the membrane translocation activity of TAT on the scFv(19)-TAT conjugate was so overwhelming that it completely masked the targeting function of scFv(19). As a consequence, a whole body distribution of the scFv(19)-TAT conjugates resembling to the pharmacokinetic profile of TAT when being administered alone was observed. Tumor targeting by the conjugates was almost non-existent, simply

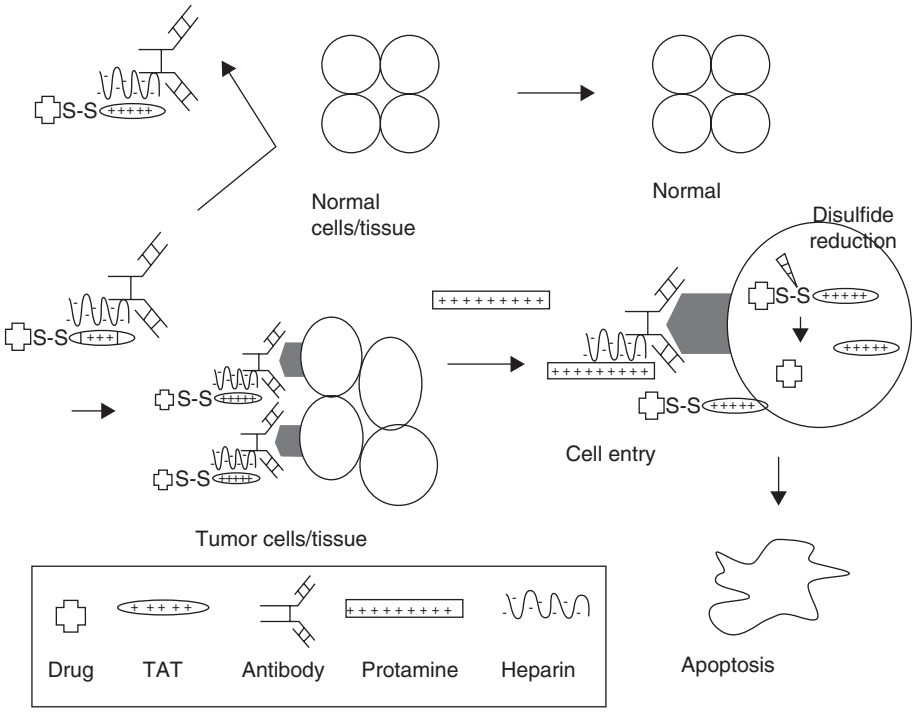

Figure 2. Schematic of the heparin/protamine delivery system in modulating TAT-mediated intracellular delivery of macromolecular drug.

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because most of the conjugates had already entered the cells due to TAT before scFv (19) could exert any targeting function. In a comparison study of the cell uptake efficiency between TAT and an antiestrogen receptor antibody using confocal microscopy, we also found that it took merely 30 min to transduce 90% of FITC-labeled TAT into MCF-7 breast cancer cells at 37°C, when it required more than 2 h to reach the same degree of transduction of the FITC-labeled antibody (unpublished data). Yet, the lack of selectivity of TAT-mediated cell entry renders this method an unacceptable practice in intracellular drug delivery, owing to the risk of causing drug-induced toxic side effects.

3.2 Incorporation of PTD peptide into ATTEMPTS

Based on the structural similarity between TAT and the poly(Arg)$_7$ peptide employed in our previous work of t-PA modification, the ATTEMPTS approach could actually be transformed, by replacing poly(Arg)$_7$ with a PTD peptide such as TAT, into a generic drug delivery system in achieving intracellular delivery of macromolecular drugs [43]. **Figure 2** describes this modified ATTEMPTS approach for tumor targeting and intracellular delivery of a generic macromolecular drug. As an example, gelonin [44], a membrane-impermeable, 30 kDa protein toxin that belonged to the type I ribosome-inactivating protein (RIP) family in cancer treatment, was linked to TAT using the SPDP activation method.

Figure 3 [45] showed both the confocal and FACS results using FITC-labeled gelonin. As seen in **Figure 3A**, there was virtually no cellular uptake of free gelonin. This finding was expected, as gelonin alone could not enter the cells. On the other hand, > 95% of the total FITC labels were recovered and found inside of almost the entire population of cells (see **Figure 3B**) within 1-h incubation of these cells with the TAT-Gel conjugates. **Figure 3C** shows that adding heparin to TAT-Gel completely inhibited the cellular uptake of these conjugates; presumably due to binding of heparin to TAT of the conjugate that prohibits TAT-mediated cell adsorption and subsequent internalization. This heparin-induced inhibition of TAT activity, however, was completely abolished when protamine was added to the heparin-inhibited TAT-Gel. As displayed in **Figure 3D**, both confocal and FACS results of the TAT-Gel conjugates, after being consecutively treated with heparin and protamine, resembled those observed in **Figure 3B** when the TAT-Gel conjugates were neither treated with heparin nor with protamine. These findings are of great significance because they provide a validation of the *in vitro* plausibility of our approach that heparin could be used to abort PTD-mediated cellular drug uptake, whereas protamine could be effectively used to resume this PTD-mediated cell translocation by reversing the heparin-induced inhibition.

Preliminary *in vivo* studies were conducted using CT-26 colon carcinoma-bearing BALB/c mice. About 10^6 CT-26 cells

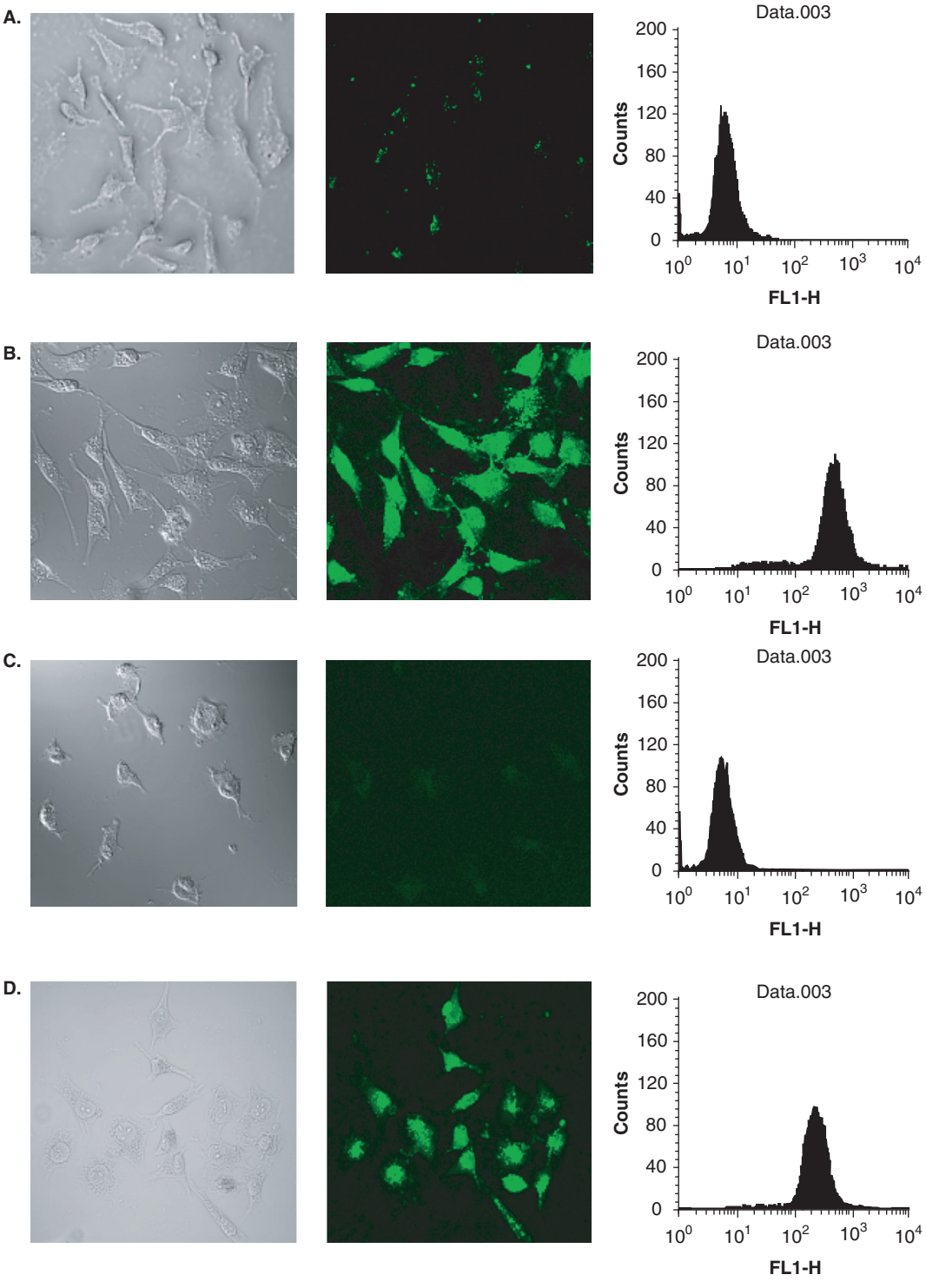

Figure 3. Cellular uptake of TAT and TAT-Gel into MCF-7 cells. The left two panels show confocal microphotograph section of DIC image and correspoinding fluorescent image, whereas the right panel shows the FACS results (ordinate, number of cells; abscissa, fluorescent intensity). Cells were treated with FITC-labeled: (**A**) Gelonin; (**B**) TAT-Gel; (**C**) TAT-Gel + heparin; and (**D**) TAT-Gel + heparin and then + protamine. The protein concentration was adjusted to 10 nm, and the incubation time was 1 h at 37°C.

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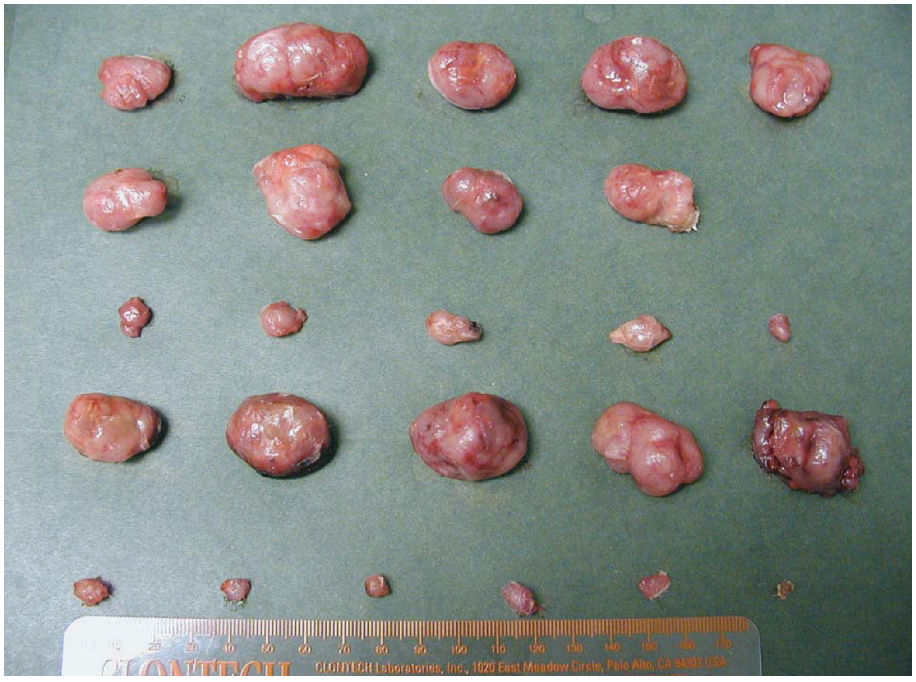

Figure 4. Excisd tumor from treated mice. From top to bottom represent tumors excised from mice treated with: Lane #1: PBS solution (average tumor mass: 3.16 ± 0.65 g; n = 5); Lane #2: 100 μg gelonin (2.86 ± 0.58 g; n = 4); Lane #3: 110 μg TAT-Gel (0.21 ± 0.15 g; n = 5); Lane #4: 110 μg TAT-Gel + 20 μg heparin (2.86 ± 0.57 g; n = 5); and Lane #5: 110 μg TAT-Gel + 20 μg heparin + 60 μg protamine (0.12 ± 0.09; n = 6).
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were implanted subcutaneously into each mouse, and drug treatments were started 3 weeks after tumor implantation, when the tumors reached a size of ~ 100 mm^3. Since no appropriate antibodies were commercially available at the time of experiments, the targeting feature of the proposed delivery system was alternatively managed by intratumoral injection of the test compounds. Five test compounds were included in the initial tumor regression studies. They were: i) PBS solution (control); ii) gelonin (100 μg); iii) TAT-Gel (100 μg gelonin equivalent); iv) TAT-Gel + heparin (20 μg); and v) TAT-Gel + heparin (20 μg) + protamine (60 μg). Each CT-26-bearing mouse was given a total of nine treatments; once every 2 days. Thirty days after initial treatment, mice were sacrificed and their tumors were excised and analyzed. As shown in **Figure 4** [10], tumor growth in the control group (i.e., with injection of the PBS solution; Lane #1; Top) was very significant and continued gradually over the 4-week span. Four weeks after PBS injection, the average tumor mass in this control group was 3.16 ± 0.65 g. As expected, mice treated with gelonin alone did not display regression in tumor growth, since gelonin itself could not penetrate the tumor. An average tumor weight of 2.86 ± 0.58 g (Lane #2) was observed 4 weeks after treatment. This average tumor mass was indistinguishable ($p < 0.05$) statistically from that of the control group (i.e., 3.16 ± 0.65 g). Co-administration of free TAT with gelonin in the treatment revealed no difference on tumor regression, as an average tumor mass of 2.92 ± 0.58 g (n = 4) was observed 4 weeks after treatment (results not included). On the other hand, mice treated with TAT-Gel displayed a significant regression in tumor growth, as the tumor weight was reduced to a relatively insignificant value (0.21 ± 0.15 g; Lane #3). Adding heparin to the TAT-Gel solution prior to its injection revealed a complete inhibition on TAT-mediated gelonin uptake, as no statistically meaningful regression on tumor mass was observed. Four weeks after this treatment, the tumor grew to a mass of 2.86 ± 0.57 g (Lane #4); which is comparable to that seen in the control group (3.16 ± 0.65 g; Lane #1). Addition of protamine to the heparin-inhibited TAT-Gel completely reversed this inhibition and resumed the cytotoxic activity of TAT-Gel, as tumor growth was reduced to an insignificant mass value of 0.12 ± 0.09 g (Lane #5; Bottom) [45].

The modified ATTEMPTS approach was further applied to treat diffuse tumors as we selected L-asparaginase (ASNase) as a model macromolecular drug to demonstrate the *in vitro* [44] and *in vivo* [46] feasibility of this system. ASNase is an enzyme drug that has been approved by the USFDA for induction of remission in patients with acute lymphoblastic leukemia (ALL) [47]. The mechanism of ASNase action is attributed to a systemic depletion of the non-essential amino acid asparagine (ASN). Unlike normal cells, which are able to synthesize asparagine, some leukemic cells lack this ability and

Table 3. Hematological parameters of various treatment groups.

Parameters	Negative control	Positive control	ASNase	TAT-ASNase
wbc (K/μl)	6.21	9.57	8.44	7.22
rbc (M/μl)	10.53	3.52	6.71	8.63
Hemoglobin (g/dl)	15.3	5	9.5	11.6
% Hematocrit (%)	49.5	17.7	31.6	36.2

Approximately 150 μl of blood was removed retro-orbitally from three mice in each treatment group using heparinized capillary tubes according to the procedures described in the materials and methods. The blood from each treatment group was pooled together and analyzed at the hematology laboratory at the University of Michigan Hospital. The 'negative control' represented that mice were injected with RPMI 1640 solution only whereas the 'positive control' represented that mice were injected with L5178Y cells pre-incubated with PBS buffer.

thus rely on the extracellular supply of asparagine for survival. A shortage of asparagine leads to inhibition of DNA and RNA syntheses in leukemic cells, thereby impairing cellular functions, and their subsequent death.

Despite its efficacy in enhancing disease remission rate and prolonging complete remission duration in ALL patients, ASNase therapy is nevertheless confounded by a number of serious toxic effects, particularly to organs associated with high protein production (e.g., liver, pancreas), owing to the systemic depletion of asparagine. The underlying principle of using the modified ATTEMPTS approach in intracellular ASNase delivery was that elimination of nutrient asparagine (followed by apoptosis) would presumably occur only within the tumor cells, thereby lowering therapeutic dose as well as aborting the toxic side effects on the high protein production organs (e.g., liver, pancreas) resulting from systemic depletion of asparagine. Using the PTD-mediated ATTEMPTS approach, the chemically constructed TAT-ASNase conjugates would be able to translocate into the MOLT-4 cells and elicit the cytotoxic effects, but this PTD-mediated intracellular ASNase uptake could also be regulated (with on/off control) by the addition of heparin and protamine [44].

For the animal model of ALL, we chose a well-established mouse lymphoma model – DBA/2 mice with L5178Y cells that are ASNase-sensitive. Preliminary survival studies were carried out with animals (n = 6 each group) inoculated with L5178Y cells that were given saline or 0.5 U of either ASNase or TAT-ASNase. The mean survival times of the positive control mice (saline), free ASNase group and TAT-ASNase-injected mice were found to be 9.8, 10.0 and 11.2 days, respectively [46]. Mice treated with TAT-ASNase exhibited an extended survival rate of approximately 13% over those treated with the same dose of free ASNase and control.

More importantly, TAT-ASNase treated mice exhibited a significantly improved hematological and liver histological status than the control groups [46]. Blood samples were drawn from the mice 8 days after the initial tumor implantation and were measured for white blood cell count (WBC), red blood cell count (RBC) and hematocrit. As shown in **Table 3**, the positive control animals exhibited a much greater increase in WBC and decrease in hematocrit compared with the results of the TAT-ASNase-treated mice. It is known that an increase in WBC would reflect the elevation of the animal's immune system in combating tumor progression. In addition, tumor growth was also reported to cause significant decrease in RBC and hematocrit [48]. Based on these hematological results, it was concluded that the TAT-ASNase tumor-injected animals encountered the slowest tumor progression; as reflected by the much smaller decrease in hematocrit and increase in WBC compared with results of the positive control animal group. Furthermore, metastases of L5178 tumor cells in the livers of the treated animals were also examined. Livers were removed from the mice 8 days after initial tumor implantation, fixed in 10% formalin, and later sectioned and stained by hematoxylin and eosin. As seen in **Figure 5**, both the positive control and free ASNase-treated mice displayed a diffused infiltration of tumor cells in the liver (**Figure 5B** and **C**, respectively). In sharp contrast, infiltration of L5178Y cells in the TAT-ASNase-treated mice was mostly focal (**Figure 5D**); similar to the results seen in the negative control group where no tumor cells were injected (**Figure 5A**). Based on analysis of these histological results of the liver sections, it was concluded that tumor growth in the TAT-ASNase-treated mice was significantly slower than the positive control group at day 8 after tumor implantation.

In summary, both *in vitro* and *in vivo* results from studies on ATTEMPTS were extremely exciting, promising and convincing, and demonstrated the overall plausibility of the proposed drug delivery approach on four key fronts: i) covalent conjugation of PTD (e.g., TAT) to a protein toxin could render this originally cell-impermeable macromolecular drug highly cytotoxic towards various cancer cell lines; ii) heparin can be used successfully to inhibit the cell transduction activity and, consequently, regulate the cytotoxic effects of the PTD-linked protein towards non-targeted tissues; iii) masking the transmembrane activity of PTD with heparin could completely preserve the functions of the targeting moiety of the delivery system and prevent PTD from degradation by circulating proteases; and iv) protamine, the universal clinical heparin antidote, can be used successfully to reverse heparin-induced inhibition on PTD and resume the cytotoxic effects of the TAT-protein conjugate towards targeted tumor cells.

4. Expert opinion

Only a few approaches show promise in subduing the three limitations, as mentioned in the Introduction, and thus deserve further discussion. One such approach is termed 'TAP' (tumor-activated prodrug) therapy [49], in which the small cytotoxic drug is conjugated to a tumor-specific antibody

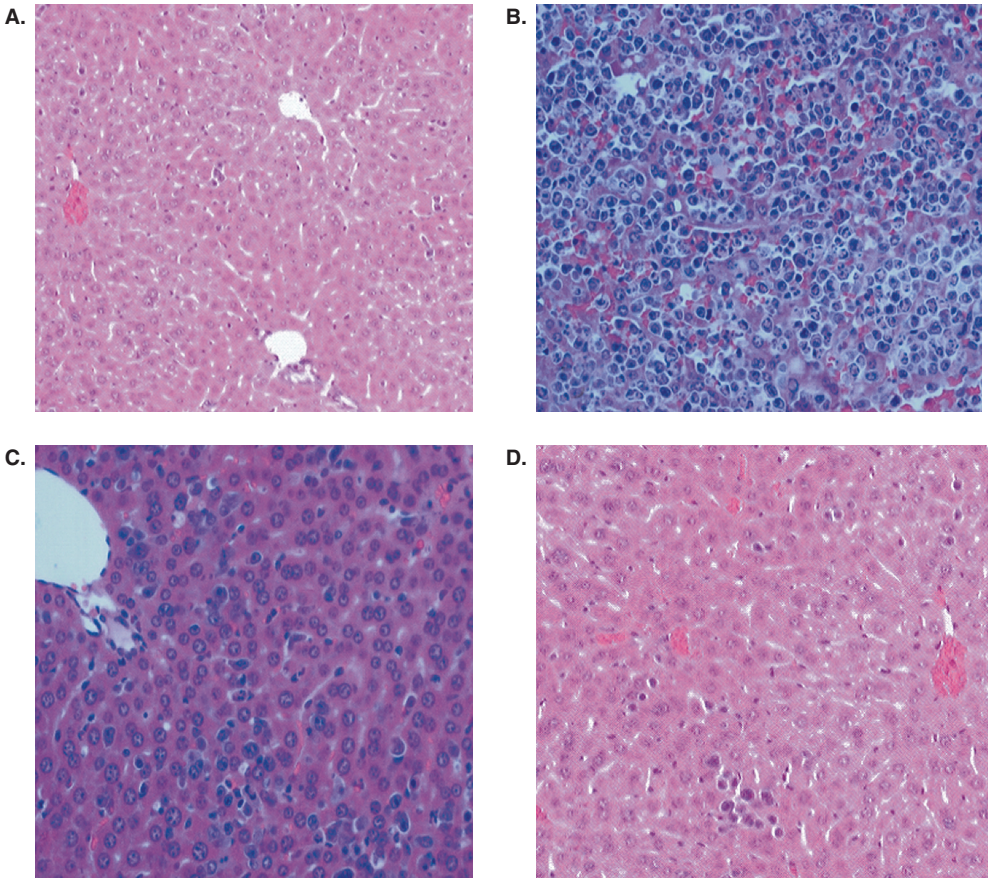

Figure 5. Liver metastases of L5178Y. (**A**) Negative control (i.e., mice were injected with RPMI 1640 solution only); (**B**) Positive control (i.e., mice were injected with L5178Y cells pre-incubated with PBS buffer); (**C**) ASNase-treated group (i.e., mice were injected with L5178Y cells pre-incubated with ASNase; and (**D**) TAT-ASNase-treated group (i.e., mice were injected with L5178Y cells pre-incubated with TAT-ASNase). Liver cells were fixed in 10% formalin and stained with hematoxylin-eosin. A 400X magnification was used.
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via either a hydrolysable linkage (e.g., hydrazone [50]) or a peptide linker [51] that is cleavable by lysosomal peptidases. Conjugation to the macromolecular antibody renders the small drug inactive during its travel to the target site. Once bound to the tumor cell surface, the drug-linked antibody internalizes the cell via endocytosis, and the drug is then released from the antibody carrier by hydrolysis or enzymatic breakdown of the linker, restoring the drug's original therapeutic potency. Early clinical trials of these antibody-drug conjugates were disappointing, as no major responses were observed in cancer patients after treatment [52,53]. This failure was later recognized as the inability of achieving a high concentration of the drug in the tumor cells; primarily owing to both the ineffectiveness of the endocytosis-mediated cell entry and the low drug loading that could be attained on the antibody (a loading of up to 8 drug molecules per antibody was obtained [54]). Later studies using antitumor agents (e.g., methyldithio-maytasinoid) that were 100 – 1000-fold more potent than the previous drug received some improvement, as a measurable tumor regression was observed in the tested animals [55]. A general review of prodrug strategies in cancer therapy appeared elsewhere [56], including two-step prodrug strategies involving carbohydrate moiety [57].

Another type of approach involves conjugation of drug molecules to nano-carriers such as water soluble polymers. This concept of using water-soluble polymers as drug carriers was proposed by Ringsdorf [58] and Kopecek [59] more than two decades ago and then validated by Duncan [60], Maeda [61] and others. Several drugs based on this type of delivery system are currently in clinical trials. Generally speaking, this approach uses the so-called EPR (enhanced permeation and retention) [62] effect for passive targeting and accumulation of the polymer carriers in tumor tissues. During angiogenesis, the nascent capillaries supplying nutrients to the tumor tissues poses large gaps between the vascular endothelial cells relative to healthy tissue types. These gaps render the blood vessels permeable to macromolecules (> 30 kDa), whereas the capillaries in normal tissues typically do not allow these

large molecules to traverse. Furthermore, the macromolecules tend to collect in the interstitial space of tumor because of a lack of a developed lymphatic drainage system. As these drug carriers accumulate, they can enter tumor cells via pinocytosis; a process that is also accelerated in rapidly growing tumor cells. This phenomenon is known as the EPR effect and has been documented for a variety of polymers [62,63] or other types of carrier (e.g., liposomes [64]) as a passive means for tumor targeting. To facilitate further the tumoral uptake, certain types of targeting moieties have been attached to these nano-carriers [65]. In addition, conjugation of PEG to such carriers has been explored to prolong the circulation time and enhance tumor accumulation; such as those long-lasting stealth liposomes [64].

Both the antibody- and polymer-based delivery systems have shown promise in subduing the aforementioned limitations in drug therapy. However, some of those systems endure deficiencies. As for the antibody-drug conjugate system, the macromolecular (e.g., enzyme) drug must be able to dissociate itself from the antibody counterpart once the conjugate is inside the cell cytosol, or otherwise the activity of the enzyme drug would be inhibited as a result of steric hindrance by the appended antibody, a principle widely employed in the so-called homogeneous enzyme immunoassay [66]. Yet, conjugation between both the macromolecular antibody and protein drug can hardly be achieved without yielding multiple linkages. It is unlikely that a macromolecular drug linked randomly with multiple bonds can be completely dissociated from the antibody counterpart by either hydrolysis or enzymatic degradation. The most outstanding deficiency of both types of delivery system, however, lies in the use of the receptor-medicated endocytosis (for immunotoxins) or phagocytosis (for particulates) for cell internalization; thereby inheriting the drawbacks of this method such as low efficiency and necessity for endosome escape of the drug. To this end, the need for a highly effective delivery system with a universal applicability to all drug types including hydrophobic, hydrophilic, small, or large compounds is acute and imperative, and the quest continues. A system that incorporates all of the targeting function, prodrug feature, release mechanism and cell entry mechanism would still be desired.

To that end, the PTD-mediated delivery system derived from our previous ATTEMPTS approach could become a generic system for delivery of macromolecular drugs of all types, including proteins, enzymes and nucleic acid-based products such as siRNA. Further expansion of this delivery system is currently underway in our laboratory.

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Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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