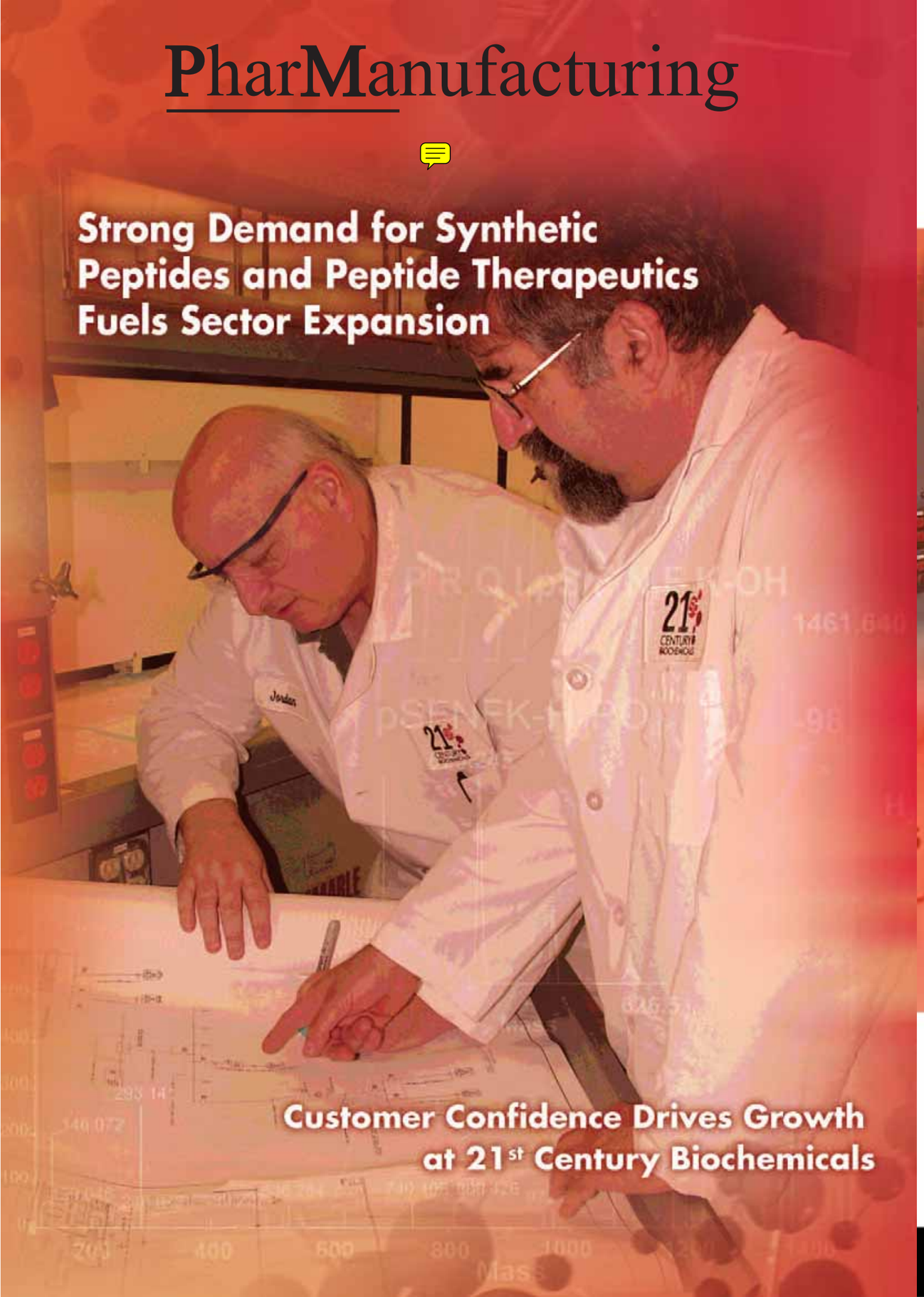
# PharManufacturing

## Strong Demand for Synthetic Peptides and Peptide Therapeutics Fuels Sector Expansion



**Customer Confidence Drives Growth at 21st Century Biochemicals**

# Cell Permeable Peptides: Precision Tools for Signal Transduction Research

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Since the middle 1990's, the intracellular delivery of small molecules, peptides, oligonucleotides and even proteins has been the subject the intense research and debate. A variety of peptides have been discovered that are capable of delivering cargo into cells, termed cell-penetrating or cell-permeable peptides (CPPs), also referred to as PTDs (protein transduction domains), MAPs (model amphipathic peptides) or MPS (membrane-permeable sequences). The three most common peptides used as CPPs are highly cationic peptides: the Antennapedia homeodomain peptide (penatratin),[1] amino acids 48-60 of the HIV-tat protein,[2] and arginine-rich/polyarginine peptides (ARPs). In addition, signal sequences from various proteins,[3-5] a human calcitonin (hCT)-derived carrier peptide[6] and lipids such as stearate and myristate have been used to successfully deliver cargo into cells.[7] Thus we will refer to cell-permeable delivery systems from this point on as cell-permeable vectors (CPVs) made up of the various peptides and lipids currently in use to transfer materials into cells.

A summary of some of the CPV-cargo combinations that have been used successfully to deliver cargo into cells are listed in the table below. Several things are readily apparent from the information in this table: there are a variety of CPVs that can deliver cargo into cells; the cargo can act to inhibit or activate various cellular systems; and not all CPVs work all the time, as evidenced by the inability of a signal sequence to deliver an ERK inhibitory peptide to its site of action when cationic CPVs were shown to deliver the same cargo successfully. Further it was observed that CPVs added to the N-terminal side of the cargo seems to be more active than when added to the C-terminal side.[7] It should be noted that the "sidedness" of the CPV-cargo molecule could be related to the free amine at the N-terminus of the cargo in this case. Being an internal peptide sequence, the free amine is not part of the native protein and thus the presence of this additional charge might be inhibitory, rather than the orientation of the CPV and cargo.

| Cell Permeable Vector | Cellular Localization Signal (optional) | Peptide Cargo |
|---|---|---|
| D/L polyarginine (4-10) | | src SH-2 domain[8] |
| HIV-tat | | JNK1 peptides[9] |
| HIV-tat | | rasGAP cleavage fragment[23] |
| HIV-tat | MLS | Fusion Protein[25] |
| HIV-tat | | PLC-gamma-1 SH2 domains[26] |
| Anntenapedia (penatratin) | | NEMO blocking peptide[24] |
| Anntenapedia (penatratin) | | P65 peptide[10] |
| Transportan (Tp10) | NLS | Plasmid[22] |
| Signal sequence | NLS | NF-κ[3] |
| Alternating aromatic & basic AA | NLS | Antioxidant[11] |





Research suggests that there are several modes of cell permeability by peptides, which can employ both classical endocytic and non-endocytic internalization pathways. Not only do some studies point to the mechanism of cellular entry of cationic CPPs being via an endocytic pathway,[12,13] but it also appears that the entry of the CPP-cargo may involve clathrin-coated vesicles and perhaps lipid raft microdomains.[14, 15] Foerg et al.[15] have shown that the sweet arrow peptide and a branched form of human calcitonin 9-32 do associate with lipid raft microdomains during their endocytic uptake. It also appears that the unbranched form of hCT(9-32) which is not highly cationic, may undergo endocytosis in a lipid raft-independent manner. Recent studies by Hawiger's group suggest that for signal sequence-derived hydrophobic regions (SSHRs),[4] cellular uptake may occur independently of endocytosis.[16] They found that cellular uptake of SSHR-cargo peptides was temperature dependent, occurred in ATP depleted cells, and enter cells with passing though the endosomal compartment. It is reasonable to assume therefore that two mechanisms of cellular entry are at play: uptake via an endocytic pathway for cationic CPVs and a non-endocytic pathway for SSHRs and most likely acylated peptides. Targeting CPVs and their cargo to various cellular compartments can also be achieved through the incorporation of nuclear[3] or mitochondrial localization sequences,[11,20] NLS and MLS respectively (see Table).

The potential for moving cell permeable peptides from the research lab to the clinic is hampered in part by the nature of these molecules: peptides are meant to be degraded and as such there exists a myriad of proteases that act to rapidly degrade proteins and peptides. To combat this turnover, critical proteolytic sites can be modified, protease resistant d-amino acids can be employed, or peptidomimetics can replace peptides. Rennert et al. (2006)[17] have modified hCT 9-32 at amino acids 12 and/or 16 with N-methyl-phenylalanine or d-phenylalanine and have found that they are more stable metabolically and show no change in the rate or extent of cellular uptake. The retro-inverso, d-amino acid form of HIV tat (57-49) is even more efficiently translocated across cell membranes than the native form of the peptide.[18] Similarly, the d-amino acid form of penatratin was taken up more efficiently than the L-amino acid form[19].

The use of cell permeable peptides as a viable route to activate tumor suppressor genes such as p53 has been successful in both in vitro experiments and in mouse tumor models.
Snyder et al.[29] used this approach to introduce the retro-inverso (inverted, d-isomer) form of a transducible p53-activating peptide using HIV tat as the CPV. They demonstrated that this peptide could inhibit tumor cell growth *in vitro* as well as in a metastatic tumor mouse model. A review of the use of specific peptides, in some case cell permeable, as potential cancer therapies is reviewed in Privé and Melnick.[30] In particular, suppressor proteins, repressors and co-repressors are all convenient targets for cell permeable peptides, as they often depend upon protein-protein interactions to mediate their effects on cells. The ability of cell permeable peptides to disrupt protein-protein interactions at the micromolar or even submicromolar levels bodes well for their use as therapeutic agents (see Table 2, Prive and Melnick). Given the sensitivity of peptides to proteases, it is quite heartening to see the large number of successful studies that have been performed in vivo using cell permeable peptides.

"…the use of peptides, in some cases cell permeable, (could be used) as **potential cancer therapies**…"

Optimization of these peptide drug candidates through the use of peptidomimetics or retro-inverso peptides could play a major role in the development of new strategies to fight cancer.

The use of modified synthetic peptides as tools to understand the regulation of normal as well as pathological processes is also becoming quite popular. Two types of modifications that would be useful tools as cell-permeable peptides would be phosphorylated peptides and methylated peptides. Protein phosphorylation has been shown to play a key role in intracellular signal transduction (for review see Cohen[27]) and the methylation of histone proteins at numerous lysines/arginine has been shown to control chromatin remodeling and transcriptional regulation (for rev. see Jenuwein and Allis[28]). Given the importance of these protein modifications in many human disease states, one would expect that the introduction of modified peptides (or mimetics thereof) into cells will be used more often in the future to modulate key signaling pathways. These peptides can also be used in broken cell assays as a means to identify their protein binding partners (such as SH2 domains). Zero length, self-hydrolyzing crosslinkers such as EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride),[21] can be used to crosslink synthetic phosphopeptides to their protein targets. Once crosslinked, peptide fragmentation (CID MS/MS) can be used to identify the site(s) of interaction. The use of a cell-permeable, retro-inverso nonphosphopeptide corresponding to the target (binding) site of the phosphopeptide can then be used as a more stable cell-permeable tool.

## Proteomics and a 21st Century Approach to Peptide Manufacturing

Modern biomedical research has moved rapidly from the era of cloning and gene sequencing to the era to proteomics and systems biology. As such, the direction of research and development has moved from requiring simple, linear peptides to more complex, highly modified peptides such as cell permeable peptides and their cargo. It is incumbent upon peptide manufacturers to build the infrastructure necessary to manufacture these modified peptides and to put into place rigorous QC methods to insure the fidelity of the peptides being manufactured. It is common nowadays for peptide requests to include one or more of the following modifications: phosphorylation (tyr, thr and/or ser), methylated lysine (mono, di, tri), lysine-ε-acetyl, dimethyl arginine (symmetrical and/or asymmetrical), N-α-acylation, O-methylation, to name just a few. To meet the demands of manufacturing such complex peptides, we have put into place state of the art instrumentation and highly educated manpower to not only manufacture peptides with unusual and/or multiple modifications, but through the use of high end instrumentation, such as the ABI QSTAR XL Pro, a Q-o-TOF nanospray mass spectrometer, to provide sequencing for each peptide we manufacture as part of our standard QC procedures. In order to accurately analyze





peptides with unusual side-chain modifications, it is imperative that high quality peptide suppliers move away from low accuracy mass spectrometers towards instrumentation with higher accuracies and sensitivities. For example, the QSTAR is accurate to 10ppm (translating to an error of only +/-0.02 Da for a 2,000 Da peptide) whereas a typical MALDI-TOF, the workhorse of the peptide industry, is accurate to only 250 ppm, or +/-0.5 Da! Given the mass differences between Lys and Gln of 0.036Da, between Asp/Asn or Glu/Gln of <1.0Da, and with numerous modifications adding to the potential ambiguity, the QC of peptides utilizing simple mass analysis and low accuracy instrumentation should be avoided.

In summary, CPVs are rapidly being a key research tool to probe specific protein-protein interactions as well as signal transduction in general.[21] As a complement to siRNA, CPV delivery of cargo peptides to selectively disrupt specific pathways may result in the development of novel and specific drugs to combat a large variety of human diseases. In particular CPV's can selectively disable individual functions present on multifunctional signaling proteins; this selective functional disruption is impossible using siRNA technology.

### About the Authors:

**Jordan B. Fishman**, President/CEO of 21st Century Biochemicals, received his PhD in Biochemistry and Carcinogenesis/Toxicology in 1984 from the Univ. of TN-Oak Ridge and joined the faculty at BU School of Medicine (BUSM) in 1985 and the faculty at the Univ. of Mass. Medical School in 1988. The author of over 30 scientific papers and book chapters, Dr. Fishman has been a founder of several peptide and reagent companies since 1992. **Michael A. Shia**, Director of Cell Biology/Immunology, received his PhD in 1984 from BUSM, and joined the faculty at BUSM in 1990. He became a principal investigator at US Genomics and then founded Cambridgeport Consultants, assisting emerging biotech companies to develop scientific plans and funding strategies. **Eric A. Berg**, VP of Operations, received his PhD in Neuroscience from BUSM in 2001. Dr. Berg was a postdoctoral fellow and research faculty member at the National Mass Spectrometry Research Resource at BUSM, joining 21st Century Biochemicals in 2004. Dr. Berg's has extensive expertise in complex mixture separations, post-translational/synthetic modification analysis and identification.

### About 21st Century Biochemicals, Inc (www.21stcenturybio.com):

21st Century Biochemicals, Inc., located in Marlborough, MA, (in the USA), was founded in March 2003 by Dr. Jordan Fishman. Occupying 15,000 SF of state of the art laboratory and administrative space, 21st Century Biochemicals utilizes high-end instrumentation and has assembled a staff comprised of scientists from many different scientific disciplines to manufacture high quality custom peptides and antibodies for its customers worldwide. Nanospray mass spectrometry using a QSTAR XL Pro and CID MS/MS provide sequence confirmation of every purified peptide resulting in 100% peptide fidelity and tremendous customer satisfaction. The use of LCMS provides unparalleled QC of difficult synthetic peptides. 21st Century Biochemicals has a capacity of over 700 peptides per month, can handle projects from milligrams to >100 grams and peptides up to 100 amino acids in length. Modified peptides, in particular phosphorylated and methylated peptides, and their corresponding modification state-specific antibodies, are a specialty.

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