Gene discovery and validation for neurodegenerative diseases

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Treatment of neurodegenerative diseases, such as Alzheimer's disease, multiple sclerosis, Parkinson's disease and amyotrophic lateral sclerosis (ALS), represents a major challenge for the pharmaceutical industry. These disorders have common and unique molecular pathological characteristics that result in serious reductions in nervous-system functionality. Key to developing novel and efficacious therapeutics is the discovery of new gene targets. Genomic, proteomics and bioinformatic analyses are identifying vast amounts of genes whose expression is associated with the pathology of a specific disease. Extensive validation studies performed in parallel with drug development are crucial for the selection of appropriate target genes. This review outlines some of the current progress in gene discovery for neurodegenerative disease.

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▼ Although difficult to group together, the diverse range of neurological disorders, such as Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), have a distinct commonality: a decrease in nervous system functionality owing to loss of neurons and/or glia through shared or discrete mechanisms. Severe clinical symptoms often include ataxia, dementia, amnesia, bradykinesia, blindness and paralysis. Some of these disorders only marginally affect the lifespan of the patient, thereby requiring expensive long-term care; however, current treatments rarely reduce disease progression. Therefore, there exists a major unmet medical need that demands the development of new and more-informed therapeutics for the efficacious treatment of neurodegenerative diseases.

Target discovery and validation

Essential to drug discovery and development is the identification and validation of new targets from the 30,000 genes currently estimated to be in the human genome. Gene discovery uses sophisticated genomic, proteomic and bioinformatic systems to analyze samples derived from animal models and diseased human tissue. Genomic and proteomic technologies increase the reproducibility and accuracy of gene expression analyses, and integration of bioinformatics techniques enables data to be analyzed, archived, disseminated, and cross-referenced with other databases [1,2]. Enhanced tissue-selection technologies mean that tissue repositories are also expanding, having a significant impact on gene-expression analyses [3].

Target validation is an important part of target selection, particularly in neurodegenerative diseases because of the abundance of complex signaling and regulatory pathways in the human nervous system. Characterization of the normal function of a gene, its role in disease pathogenesis, and contribution to other pathways, would increase the success rate of newly identified compounds. Therefore, new expression-analysis platforms, resources available in human tissue banks, data from the Human Genome Project and other animal genome databases, and data from animal models, are overlapping to create an exciting repertoire of technologies for target validation and subsequent pharmacology.

The common mechanism approach

Current gene discovery techniques identify gene-specific expression during disease progression as a means to circumvent side-effects that might result from targeting pathways involved in normal cellular functions. While many gene targets are selected because of their specific association with the pathology of a disease, identifying similarities in the pathogenesis of neurodegenerative diseases enables certain genes to be targeted for multiple disorders. General targets for neurodegeneration

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are Jun N-terminal kinases, p38 kinases, and glyceraldehyde-3-phosphate dehydrogenase [4,5]. The cyclin-dependent kinase, cdk5, is regulated by two non-cyclin activators, p35 and p39, and the Ca^{2+}-regulated protease, calpain, cleaves p35 into p25 to form the activate cdk5–p25 complex [6,7]. The activity of cdk5–p25 is largely restricted to post-mitotic cells of the nervous system and appears to be involved in cellular processes essential for neurodevelopment [8,9]. Other gene targets for neuroprotection have been identified in the cdk5–p25 pathway, for example, the phosphoprotein, DARP-32, which is involved in dopamine signaling [10]. The activation of cdk5 might play a role in the pathogenesis of both AD and ALS, therefore making it a possible target for both diseases.

This review aims to describe gene discovery and validation techniques for neurodegenerative diseases rather than gene discovery in trauma-related disorders, such as ischemia (stroke) and spinal cord injury. However, the common mechanism approach could identify signaling pathways that could also be used for targeting trauma.

Alzheimer's disease

Alzheimer's disease is the most common neurodegenerative disorder, affecting over four million people. Clinical characteristics include global dementia, and amnesia, with effects on attention and language. Neuronal loss is associated with characteristic extracellular senile plaques and intraneuronal neurofibrillary tangles (NFTs) [11]. Extracellular plaques are produced by the aggregated deposition of β-amyloid peptides (Aβ) derived from the proteolysis of amyloid precursor protein (APP) at discrete intramolecular sites (α, β and γ) [12]. Proteinases that might be responsible for APP formation have been identified [13,14]. The membrane-bound aspartic proteinase, β-secretase, cleaves at the β-site of APP to produce N-terminus Aβ peptides, and γ-secretase activity cleaves at the γ-site producing transmembrane-region Aβ peptides [15]. Transgenic mice expressing human APP and amyloid produced no amyloid when crossed with a β-secretase-knockout mouse [16].

A pro-domain of β-secretase is cleaved to produce the active protease [17,18]. Conserved sequences in the cytoplasmic domain of β-secretase suggest that it trafficks between the endosome and plasma membrane [19]. Targeting genes in protein-processing pathways might suppress active β-secretase formation and interrupt the plasma-membrane–endosome cycle to inhibit β-secretase extracellular localization. In addition, the extracellular domain of β-secretase is released into the blood through proteolytic digestion of membrane-bound β-secretase. As the released domain is inactive, promoting this release might reduce β-secretase cleavage of APP. Although the β-secretase-processing pathway has revealed targets that might decrease Aβ accumulation, inhibiting these pathways could affect the processing of other substrates crucial for normal cellular processes.

γ-Secretase activity is an intramembrane-located aspartyl protease. Though controversial, evidence suggests that this protease is presenilin 1 (PS1), and that PS1 auto-proteolysis is required for APP γ-cleavage [20]. Inhibitors of γ-secretase, and inhibitors that target the γ-secretase processing pathway, have been tested in human-APP transgenic mice, the result of which was reduced amyloid formation [21]. However, Notch receptors, which are involved in neurodevelopment, are also γ-secretase substrates [22,33]. Therefore, validation strategies will be fundamental in determining whether inhibiting Notch function has deleterious effects in animal models for AD.

Another approach to the removal of β-amyloid from the CNS is to enhance APP proteinases. Neprilysin is an endopeptidase that is downregulated during AD and might be involved in the degradation of APP [24]. Potential therapeutics could perhaps increase the activity of such peptidases with the aim of increasing APP degradation as it accumulates during AD.

Signal transduction through APP also appears to have pathological consequences in AD. Membrane-bound C-terminal fragments of APP form stable complexes with the adaptor proteins ShcA and Grb2 [25]. These adaptor proteins probably influence mitogen-activated protein kinase (MAPK) activity, which could be a useful signaling pathway to target during AD pathogenesis.

Recent reports have supported immunotherapy in AD [26]. After immunizing human-APP-expressing transgenic mice with a human Aβ peptide, there was a reduction of amyloid deposition, inhibition of Aβ production, removal of senile plaques, and restoration of cognitive functions [27–29]. Evidence indicates that there are at least two different mechanisms for Aβ removal. DeMattos *et al.* propose that anti-Aβ antibodies activate microglia to remove amyloid [30]. Other groups investigating the mediation of amyloid reduction have used an anti-Aβ antibody that does not cross the blood–brain barrier, and propose an anti-Aβ antibody 'sink' model that encourages migration of Aβ from the CNS [31]. Such antibody-based treatments might provide models for identifying new AD targets. The active transport mechanism could be targeted to transport Aβ transport from the CNS to blood plasma. Alternately, targeting proteins that regulate microglial activation might encourage increased phagocytosis and subsequent plaque digestion.

Complicating immune-based therapies further are data indicating that microglial activation could facilitate an inflammatory cascade and increase AD pathology. Activated microglia produce a neurotoxin that could enhance the microglial-mediated destruction of CNS tissue. In addition, glial cells appear to mediate neurodegeneration upon activation by processed amyloidogenic fragments present in AD brains, suggesting a role for aberrant protein processing in AD pathology [32]. Alternatively, microglial activation could prove to be beneficial by maintaining a steady-state level of Aβ deposition through digestion of β-amyloid.

The causative role of amyloid formation in AD has been controversial because Aβ plaque burden correlates poorly with neuronal loss [33]. However, intracellular NFTs, formed by aggregation of the microtubule-associated protein, tau, are associated with dementia during AD. Transgenic mice expressing mutant human tau proteins produced filamentous tau aggregations that induced neurodegeneration and altered the expression of genes associated with inhibition of apoptosis [34,35]. Mice expressing both mutant APP and tau genes have a more pronounced pathology, indicating a common pathway between APP and NFT deposition. Cdk5 might also play a role in NFT formation because p25 is elevated in AD brains along with hyperphosphorylated tau in NFTs [36,37], and transgenic mice overexpressing p25 develop AD-like lesions that might result from cdk5-mediated phosphorylation of tau [38].

Besides using animal models, microarray and proteomic studies of post-mortem AD brains have identified proteins involved in synaptic activity [39,40]. The sodium–potassium-coupled excitatory amino acid transporter 2 (EAAT2) was highly expressed in pyramidal neurons in the cortex of AD patients, and co-expressed with tau, supporting a role for glutamate toxicity in AD [41]. Other genes that might protect neurons during AD have also been identified (e.g. humanin [42]).

Multiple sclerosis

Multiple sclerosis is a CNS demyelinating disease and the most common neurological disorder in young adults. Symptoms include paralysis, blindness, chronic pain, and cognitive changes, which occur from 20 to 30 years-of-age and can continue for up to 30 years. Multiple sclerosis typically follows a relapsing–remitting cycle and can develop into a secondary progressive pattern. Although MS is thought to be an inflammatory disease, its pathology involves the death of CNS myelinating cells (oligodendrocytes) and neurons. Therefore, MS can be viewed as a neurodegenerative disease resulting from an inflammatory autoimmune attack of the CNS.

T cells and antigen-presenting cells (APCs) are crucial for disease progression and have long been cellular targets for MS. During epitope spreading, endogenous priming with new self-antigens produces new populations of autoreactive T-cells. These new self-antigen-specific T-cells appear to control the acquisition and progression of clinically defined MS [43]. Consequently, therapeutics are being developed to inhibit the activation of T-cells, the presentation of antigen by APCs, or inflammatory cell migration across the blood–brain barrier. Indeed, one current MS therapy uses β-interferon to immunomodulate myelin-specific T-cells by affecting the interleukin (IL)-10–IL-12 cytokine circuit during antigen presentation [44].

In the experimental autoimmune encephalomyelitis (EAE) mouse model, animals are immunized with peptides from myelin proteins to produce Th1 T-cell-mediated relapsing–remitting or progressive diseases, which are characterized by epitope spreading, demyelination, and axonal loss. Transgenics and knockouts in EAE mice have been useful in gene discovery, target validation and preclinical evaluation of new therapeutics.

Proinflammatory cytokines and their receptors and signaling pathways have been targeted for treatment of MS. Tumor necrosis factor (TNF) function has been inhibited with TNF-soluble receptors and anti-TNF antibodies [45]. Chemokines and their cognate G-protein-coupled receptors have been exploited because of their role in recruiting inflammatory cells to the CNS, and their applicability to HTS. Chemokines (e.g. CCL2, CCL5 and CXCL10) and receptors (e.g. CCR1, CCR5 and CXCR3) have differential expression in post-mortem MS brain tissue, cerebral spinal fluid (CSF) and during EAE [46].

Members of the integrin family have been targeted to reduce cell migration into the CNS. Antibodies against α4-integrin (VLA-4) and a modified VLA-4 peptide inhibited clinical characteristics and T-cell migration in EAE [47]. In addition, VLA-4 expression is downregulated in T cells from MS patients treated with interferon-β [48]. However, while anti-VLA-4 antibodies can inhibit EAE onset and reduce disease severity when administered preclinically, they exacerbate disease relapses when given at the peak of EAE or during remission [49].

Although voltage-gated potassium channels (Kv) are expressed by CNS neurons, Kv1.3 might be a useful target for MS because of its T-cell expression and involvement in T-cell activation. Kaliotoxin, a selective blocker of both Kv1.1 and Kv1.3, inhibits antigen-dependent T-cell activation and reduces clinical signs in EAE [50]. Specific pharmacological inhibitors of calcium-activated K-channels have also suppressed T-cell proliferation [51].

T-cell activation marker, dipeptidyl peptidase IV, or CD26 cleave the N-terminal dipeptide from proteins with penultimate prolines, hydroxyprolines, or alanines (a class of proteins that includes growth factors and chemokines). Dipeptidyl peptidase IV proteinase inhibitors treat EAE through mechanisms that suppress T-cell activation and induce transforming growth factor-β1 (TGF-β1) production [52,53].

Gene discovery studies have used freshly isolated monocyte-depleted peripheral-blood mononuclear cells from MS patients, consequently avoiding RNA degradation, which occurs in post-mortem brain tissue [54]. The IL-7 receptor – which enhances pro-inflammatory cytokine production – and metalloproteinase-19 – which might function in blood–brain barrier breakdown during leukocyte migration – were both elevated in patients with MS. EAE mice and control mice were included in another analysis of MS demyelinating lesions [55]. 5-Lipoxygenase (5-LO), which catalyzes the last synthesis step of leukotrienes, was upregulated in MS and EAE. Inhibitors of 5-LO might reduce leukocyte migration in a mechanism independent of adhesion molecules (e.g. VLA-4) [56].

Chabas and colleagues used parallel microarray analysis of EAE brains, and EST sequencing from cDNA libraries of MS brain to identify the upregulation of osteopontin [57]. Gene knockouts of osteopontin showed reduction of EAE severity by inhibition of T-cell activation and induction of IL-10. Osteopontin also inhibits cell lysis and, consequently, might protect neurons during inflammation; therefore, it is necessary to examine osteopontin function during the stages of disease where neuronal loss is more pronounced.

The histopathology observed in MS and EAE enables comparisons to be made between different types of demyelinating lesions and might identify targets involved in lesion development. Lock *et al.* used microarray analysis of acute lesions, chronic active lesions, and chronic silent lesions to produce a molecular 'portfolio' of the genes that might be involved in lesion development during disease [58]. However, the altered expression of candidate target genes could have physiological and developmental effects independent of their role in generating autoimmunity [59].

Gene expression analyses of discrete lesions from MS patients and tissue from specific stages of EAE are producing a collection of targets, some of which might be useful in promoting regeneration during MS. Oligodendrocyte progenitor cells have been identified in chronic lesions of MS patients, supporting a therapy directed at inducing remyelination [60]. Although inhibition of apoptosis might appear an effective therapy for protecting degenerating oligodendrocytes and neurons, such a therapy might also prevent apoptosis of autoreactive T-cells, thereby perpetuating CNS inflammation [61]. Careful target selection and validation is necessary to develop an MS therapy that not only inhibits inflammation but also protects the CNS.

Parkinson's disease

Parkinson's disease is the most common neurodegenerative disease after AD, and is caused by the death of neurons in the substantia nigra that use dopamine as a neurotransmitter. Symptoms are rigidity, bradykinesia, akinesia, resting tremor, and progressive symptoms, including dementia. However, at the point of diagnosis, 70–80% of the dopaminergic neurons have already been lost. Therefore, the most efficacious therapies would aim to protect the remaining neurons.

Levodopa (L-dopa), which is converted to dopamine *in vivo*, is the current treatment for PD. However, L-dopa therapy is only effective for about five years, after which the patient must use dopamine agonists to stimulate the dopamine receptor. Compounds that inhibit dopamine-degrading enzymes are also being tested in PD. Disappointingly, many PD patients respond poorly to these drugs, despite studies suggesting they might offer neuroprotection when used before L-dopa [62]. Most compounds for PD are directed at increasing or sustaining the available dopamine pool rather than inhibiting the continued neuronal loss. More sustainable therapies need to couple neuroprotective therapies with dopamine replacement therapy.

Caspase proteases are essential for apoptosis and are currently being targeted as a treatment of PD. In human post-mortem PD brains, caspase-3 expression correlates with the loss of dopaminergic neurons [63]. A similar correlation was observed using a mouse model of PD, where the selective degeneration of nigrostriatal dopamine neurons is induced by injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Inhibition of caspases-2, -3, and -9 also produced neuroprotection in dopaminergic neurons cultured with methyl phenylpyridinium, the active form of MPTP. Using dopaminergic cells from MPTP-treated mice, and post-mortem PD brain tissue, Viswanath and colleagues showed that inhibition of caspase-9 deterred caspase-3 and -8 activation, implicating caspase-9 as an upstream regulator [64]. However, rat dopaminergic neurons treated *in vitro* with caspase inhibitors were not protected upon serum removal or in a 6-hydroxydopamine-grafting model. Careful validation in well-characterized PD models and in human tissue is crucial for evaluating caspase as a PD target, especially when other systematic side-effects of apoptosis inhibition might include autoimmunity and neoplasia.

Mitochondrial-associated pathways have also been targeted for PD, because MPTP toxicity is mediated by inhibition of mitochondrial complex I, resulting in decreased ATP production and increased oxygen radicals. Some PD patients have deficiencies in mitochondrial respiratory function, and such oxidative stress can induce apoptosis [65]. In female PD patients, increased levels of 8-hydroxy-2′-deoxyguanosine or 8-hydroxyguanosine, a marker for DNA, correlates with RNA oxidative damage [76]. A clinical trail using rasagiline might have slowed PD progression by targeting the mitochondrial membrane [67].

Genes expressed by cells other than dopaminergic neurons have also been targeted for PD. Minocycline, a second-generation tetracycline, prevents dopaminergic neuron loss by an apparent inhibition of nitric oxide production by glia, and studies using a dopaminergic cell-line indicated that microglial activation induces dopaminergic cell death [68,69]. Although these data support a possible treatment of PD with anti-inflammatory therapies, anti-inflammatory drugs that produce neuroprotection in animal models for PD have not been protective in clinical trials [70].

Human genetic studies of PD have identified mutations in α-synuclein, a component of Lewy bodies [71]. Interestingly, both wild-type and mutant α-synuclein caused fibril formation and a reduction in cell viability that appears to be related to oxidative stress [72]. Other genetic studies have identified genes in ubiquitin-mediated proteolysis pathways (e.g. Parkin and ubiquitin C-terminal hydrolase L1), indicating that proteolytic pathways could be useful targets for PD [73,74]. Microarray analysis of PD brains has identified elevated expression of genes involved in oxidative stress [75]. With the loss of tolerance to oxidative stress as a possible major contributor to PD pathology, other targets derived from pertinent signaling pathways could be investigated for therapeutic potential.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis results from the selective death of motor neurons in the spinal cord, brain stem and cerebral cortex, and affects ~30,000 people, with ~5,000 new cases in the USA each year. Symptoms generally start with weakness in the legs, arms and bulbar muscles, and often progresses to include all the voluntary muscles, producing leg and arm paralysis, loss of speech, muscle wasting, and difficulty in swallowing and breathing.

Significant progress in ALS etiology was made with the discovery that 15–20% of familial ALS is caused by mutations in a gene encoding the detoxification enzyme, copper–zinc superoxide dismutase (SOD-1) [76]. As most SOD-1 mutations are gain-of-function, and SOD-1 knockout mice have no motor neuron pathology, neuronal loss might not be caused by a reduction in SOD-1 activity [77]. Indeed, transgenic mice overexpressing human mutant SOD-1 display paralysis despite wild-type SOD-1 expression [78].

Mutant SOD-1 might induce motor neuron loss by alternate pathways that elevate oxidative damage in neurons through the accumulation of toxic free-radicals or glutamate excitotoxicity [78,79,80]. Concentrations of glutamate were elevated in the CSF of ALS patients, and riluzole – a drug that reduces glutamate-mediated transmission – slows ALS progression [81,82]. However, the large number of ion channels, receptors and related signaling pathways that are regulated by glutamate complicates target selection for glutamate excitotoxicity.

Excitatory amino acid transporter 1 regulates glutamate re-uptake at the synaptic cleft and has been implicated in ALS [83]. Excitatory amino acid transporter 2 -knockout and -knockdown mice have motor neuron degeneration, and EAAT2 protein was reduced in brains from sporadic ALS patients [84,85]. As EAAT2 expression is decreased in transgenic mutant SOD-1 mice, oxidative damage to EAAT2 could lead to glutamate accumulation and induce excitotoxic death in neurons that are already susceptible to excitotoxicity [86].

Reduced mitochondrial function might contribute to motor neuron loss by decreasing the chemical energy required to maintain membrane potential. Motor neurons might not be able to regulate glutamate receptors and transporters, thereby increasing cellular vulnerability to glutamate excitotoxicity. Mitochondrial pathology has been detected in ALS patients and SOD-1 mice along with decreased activity of the mitochondrial enzyme, cytochrome *c* oxidase [87,88].

The histopathology of ALS and SOD-1 transgenic mice is characterized by motor neuron inclusions of neurofilament aggregations (composed of subunits NF_L, NF_M, and NF_H) [89]. Uncoordinated expression of neurofilament subunit proteins leads to aggregates of unassociated NF proteins in NF-knockout mice. However, NF aggregates could be neuroprotective by supplying Ca^{2+}-binding sites that act as sinks to bind increased cellular concentrations of calcium, thereby protecting against excitotoxicity. Although NF aggregates themselves have not yielded targets, they have contributed important validation data for the role of excitotoxicity in ALS.

Another neuronal inter-filament protein, peripherin, is upregulated in ALS inclusions, and peripherin transgenic mice develop a mild motor neuron disease [90]. Peripherin can assemble with the other neurofilament proteins to disrupt the intermediate filament cytoskeleton and inhibit

intracellular transport crucial for neuronal survival. One possible mechanism to reduce peripherin-mediated aggregate formation is to target the inflammatory cytokine, IL-6, and leukemia inhibitor factor (LIF), because both factors upregulate peripherin expression and are elevated in some ALS patients [91,92]. Interleukin-6 and LIF function through the JAK–STAT (Janus-family kinases–signal transducers and activators of transcription) signaling cascade, thereby offering a potentially large selection of targets. The involvement of IL-6 in ALS also suggests the possible use of inflammation targets for treating ALS.

The administration of neurotrophic growth factors to protect motor neurons has long been expounded. Ciliary neurotrophic factor, brain-derived neurotrophic factor and insulin-like growth factor slow motor neuron degeneration [93,94]. However, protein-based therapeutics are problematic and might require gene therapy or cell transplantation to deliver biologically active factors to diseased motor neurons.

An alternative approach uses small molecules to target components of the signaling pathways downstream of neurotrophic factor receptors. For example, increased cdk5–p25 activity was detected in transgenic SOD-1 mice, and cdk5 inhibitors increased neuronal survival [95]. Activity of cdk5–p25 might be linked to glutamate excitotoxicity because over-activity of the glutamate receptor leads to elevated concentrations of intracellular Ca^{2+}, which would produce p25 and activate cdk5–p25.

A 'phosphorylation sink' mechanism was proposed to explain the neuroprotective properties of NTs, where cdk5–p25 is trapped by NTs, inhibiting its activity [96]. However, the NF_H subunit might be a substrate of cdk5–p25, and the hyperphosphorylation of NF_H might induce formation of NT accumulations, which then sequester cdk5–p25 and induce neuroprotection. Furthermore, knockout experiments indicated a requirement for cdk5–p25 in normal neurodevelopment; a lack of cdk5–p25 activity causes neurodegeneration [6]. Therefore, although partial inhibition of cdk5–p25 could be neuroprotective, a more complete inhibition might interfere with the functions required for correct neuronal function.

Concluding remarks

With the almost surfeit collection of data from differential gene expression, protein sequence and pharmacogenomics analyses, the requirement for target validation in complex and multifactorial disorders such as neurodegeneration is obvious. Such is the growth of data that the Stanford Microarray Database (http://genome-www5.stanford.edu/microarray/smd/) is assembling a collection of microarray data and bioinformatics tools. Linking such microarray databases to gene replacement, transgenic, and genome databases, and data from clinical and pharmacogenomics studies, provides a vast resource that can be used for gene discovery and target- and compound-validation. This will facilitate the identification and timely progression of lead therapeutic molecules to provide effective amelioration of diseases that have the most profound unmet need.

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Erratum

Please note a correction to the article *Structural pharmacogenomics, drug resistance and the design of anti-infective super-drugs* by Edward T. Maggio, Mark Shenderovich, Ron Kagan, Dean Goddette and Kal Ramnarayan in the print version of *Drug Discovery Today*, 15th December 2002, Volume 7, No. 24, 1214–1220.

Figure 7 on p. 1217 should have been as below:

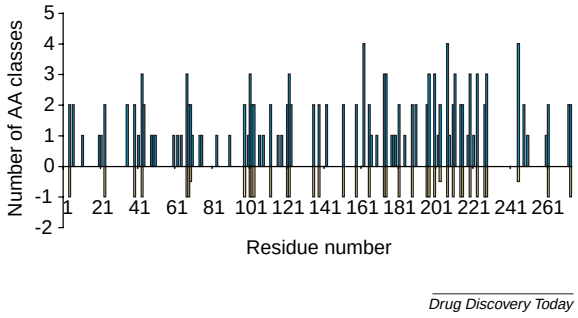

Fig. 7. The number of amino acid classes allowed at each residue position for HIV-1 reverse transcriptase. 38 positions (flagged below the X-axis) frequently exhibit mutation to an amino acid class different from the original class.

We would like to apologize for this inaccuracy and for any confusion that this might have caused.

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