HHS Public Access

Author manuscript

J Mol Biol. Author manuscript; available in PMC 2016 May 31.

Published in final edited form as:

J Mol Biol. 2016 February 13; 428(3): 533–537. doi:10.1016/j.jmb.2015.12.019.

The Path of Least Resistance: Mechanisms to Reduce Influenza's Sensitivity to Oseltamivir

Angela M. Phillips and **Matthew D. Shoulders***

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139

The impact of a potential global influenza pandemic calls for the development and delivery of effective antiviral therapeutics [1]. Unfortunately, for all influenza therapeutics widely used in the clinic to date, the virus' high evolutionary capacity has led to the rapid appearance of drug-resistant variants in currently circulating strains [2–4]. Such resistance poses a serious threat to public health, demanding a global effort to generate improved treatment options [5]. The intelligent design of anti-influenza drugs that circumvent resistance requires a molecular-level understanding of the mechanisms involved, such that we can both predict and rationally sidestep such processes.

Current small molecule-based anti-influenza drugs consist of the neuraminidase inhibitors (the focus of this commentary) and the adamantanes, which block the M2 protein ion channel [6]. Neuraminidase is an influenza membrane glycoprotein responsible for cleaving sialic acid from host cell membranes and thereby potentiating viral release [7,8]. Phylogenetic analyses and high-resolution crystal structures of influenza neuraminidase in complex with the enzyme's natural substrate, sialic acid, revealed that residues in direct contact with the substrate are highly conserved among influenza strains (Figure 1A) [9,10]. Information from these high-resolution structures thus provided insight towards the rational design of neuraminidase inhibitors with nanomolar potency and high oral bioactivity [11]. Oseltamivir (Figure 1B) is an optimized compound derived from these studies that is currently a leading anti-influenza drug treatment [5,12,13]. However, oseltamivir displays a *C*6-pentyloxy group that interacts with a hydrophobic site in neuraminidase, whereas the native substrate sialic acid contains a glycerol moiety at *C*6 that does not interact significantly with the hydrophobic site [10,14,15]. This distinction has assisted the acquisition of drug-resistant mutations, by enabling neuraminidase variants to exclude oseltamivir from the active site while continuing to process sialic acid with high efficiency in the presence of the drug [14,15]. Alternatively, oseltamivir resistance-conferring mutations have also been observed in hemagglutinin that weaken binding to sialic acid receptors, alleviating the pressure on neuraminidase to cleave sialic acid for virion budding [16].

A commonly observed amino acid substitution in neuraminidase that confers oseltamivir resistance, H275Y, also results in decreased neuraminidase stability and surface expression

*To whom correspondence should be addressed: Matthew D. Shoulders, Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Building 16, Room 573A, Cambridge, MA 02139, Phone: (617) 452-3525, Fax: (617) 324-0505, ; Email: mshoulde@mit.edu

relative to wild-type N1 neuraminidase [17]. The associated fitness costs of the H275Y substitution for influenza prevented this variant from circulating prior to 2008, after which permissive secondary mutations that rescue H275Y neuraminidase surface expression appeared [2,18,19]. Significant progress has been made in identifying these compensatory mutations and characterizing their mechanisms of action [18–20]. Beyond the H275Y substitution, it is now known that the I223R/K/T, N295S, and several other amino acid substitutions can also confer oseltamivir resistance, although they simultaneously reduce neuraminidase activity for various reasons [21–24].

Interestingly, reported neuraminidase amino acid substitutions that engender oseltamivir resistance in influenza strains most often occur at active site framework residues, which are residues that interact with functional residues but are not directly involved in the catalytic mechanism of action (Figure 1A) [23–25]. While mutation of functional residues generally abrogates protein function, mutation of framework residues is usually less detrimental to protein function but can still have significant associated fitness costs. Indeed, prior to the work of Jiang et al. [26], the reported oseltamivir-resistant mutations in neuraminidase had associated fitness costs that often required compensatory, fitness-enhancing mutations for efficient viral propagation.

Although computational methods have had success in specific cases [18], the diverse structural locales of oseltamivir-resistance mutations and the associated permissive secondary mutations question the feasibility of using purely theoretical methods to predict amino acid substitutions that could contribute to antiviral drug resistance. Rationally designing drugs that are less susceptible to antiviral drug resistance mechanisms is instead likely to require highly integrated experimental and theoretical studies. With advances in next-generation sequencing technologies, the field has therefore shifted toward high-throughput screening to systematically identify potential resistance-conferring mutations at single nucleotide resolution. Numerous studies have used a variety of experimental methods to introduce mutations, perform selection, and analyze results [27]. A typical strategy involves random mutagenesis of codons or individual nucleotides of influenza genes of interest (often neuraminidase) in plasmids to generate mutant libraries that are then subjected to selection via passaging in tissue culture in the presence or absence of drug (Figure 2) [28–32]. Viral pools are sequenced before and after selection to determine the relative fitness of each variant. The codon mutagenesis method provides good correlation between true biological replicates (R^2 as high as 0.62) [28] for libraries that include every possible codon mutation for an entire gene (>10^4). These studies have produced numerous biomedically significant findings, such as the characterization of the high mutational tolerance of hemagglutinin [28]. Similar methods that generate random mutations at the nucleotide level have also yielded key results, including the identification of several neuraminidase H274Y compensatory mutations and the identification of mutations in the influenza NS1 protein that abolish anti-interferon activity [30–32].

For both nucleotide and codon mutation libraries generated by mutagenesis, the resultant libraries often contain several mutations per gene, which has the advantage of increasing their throughput and exploration of amino acid space but can complicate interpretation of variant fitness [27,31,33–35]. Jiang and colleagues introduce a valuable new experimental

technique to this field that sidesteps the potentially confounding effects of interacting mutations and uneven representation of variants in libraries [26]. Specifically, they adapt the EMPIRIC (Exceedingly Meticulous and Parallel Investigation of Randomized Individual Codons) method [36,37] to study mechanisms of oseltamivir resistance in neuraminidase. EMPIRIC involves the construction of libraries containing all possible single nucleotide mutants in particular regions of a gene (Figure 2). Following a selection step (in this case, influenza replication in the presence or absence of oseltamivir), the relative fitness of each variant is quantified by focused deep sequencing of the small, mutated regions [36–38]. During library generation, EMPIRIC utilizes a cassette ligation strategy that provides equal representation of mutants, maximizing signal during sequencing. Careful construction of these single point mutants enables noise reduction downstream of sequencing by removing reads containing double mutants [36]. EMPIRIC limits the size of the genomic region that is feasible to probe in any given experiment to about 30 base pairs, owing to constraints on sequencing read lengths and base call accuracy [36]. However, exclusively creating single point mutants removes any confounding epistasis or noise from differences in genetic background, contributing to extremely precise measurements of relative fitness and a very strong ($R^2 > 0.9$) correlation between biological replicates. Furthermore, Jiang et al. demonstrate that EMPIRIC is amenable to studying several influenza genomic regions in parallel.

Jiang et al. constructed A/WSN/33 H1N1 neuraminidase libraries containing all possible point mutations for five regions of 30 consecutive bases. The mutated regions were selected to encompass amino acid positions known to contribute to oseltamivir resistance, as well as a control region in a loop far from the neuraminidase active site. After generating comprehensive single mutant libraries for the selected regions, they performed selections as batch competitions in the presence and absence of oseltamivir. Importantly, Jiang et al. are able to identify known oseltamivir-resistant variants such as H275Y and N295S using the EMPIRIC technique. The depth of their mutational scanning also enables the discovery of several novel oseltamivir-resistant variants, including K221N, Y276F, and multiple variants at position 223. Separate fitness analyses on these and other individual variants were performed and the results observed are similar to those from the batch analyses.

As noted above, Jiang and colleagues identify position 223 as a particularly important mutational hotspot for oseltamivir resistance. Indeed, three resistance mutations had been previously identified at this position (resulting in I223R/K/T amino acid substitutions) [23,39]. Jiang et al. discover an additional three using EMPIRIC (I223M/L/V). They further demonstrate that diverse amino acid substitutions at position 223 have an intermediate capacity to attenuate oseltamivir sensitivity. In contrast, variation at other positions, such as 275, results in highly attenuated oseltamivir sensitivity, but only when mutated to one specific codon (i.e., H275Y). Other amino acid substitutions included in the EMPIRIC library do not confer significant oseltamivir resistance. Structural studies show that amino acid residues at position 223 can make direct contact with oseltamivir [39], whereas those at position 275 interfere with the hydrophobic pocket where the pentyloxy group of oseltamivir binds but do not make contact with the native substrate [15]. Cumulatively, these observations suggest that the mutational hotspot characteristics of position 223 relate to its

ability to interact directly with substrate and oseltamivir, which is also the case for analogous mutational hotspots conferring drug resistance in HIV protease [40].

The characterized oseltamivir resistance-conferring mutations in neuraminidase from previous studies and discussed above from this study induce a loss of relative fitness for influenza propagated in the absence of oseltamivir [17–19,21,22,32,39]. The difficulty is that amino acid variation at these positions reduces the enzymatic activity of neuraminidase; a finding that is unsurprising considering that oseltamivir is a substrate analog. Thus, perhaps the most interesting discovery of Jiang et al. is the identification of the oseltamivir-resistant neuraminidase variants Y276F and K221N [26]. Unlike all the other oseltamivir-resistant neuraminidase variants, Y276F and K221N actually enhance influenza fitness both in the absence and the presence of drug. Enzymatic activity studies demonstrate that, unlike the I223M, H275Y, and N295S oseltamivir-resistant variants that decrease neuraminidase activity but also decrease drug affinity, the Y276F and K221N oseltamivir-resistant variants increase neuraminidase activity but do not affect drug affinity. It is apparently via this mechanism that Y276F and K221N manage to provide an adaptive advantage even in the absence of drug, while still conferring drug resistance at low doses of oseltamivir owing to increased substrate processing. This novel mechanism of action represents an intriguing contrast to the view that most drug resistant mutations have an associated fitness cost that can mitigated by their genetic background. These discoveries highlight the continued importance of developing alternative and innovative influenza treatments, and of continuously deepening our understanding of resistance mechanisms so that we can rationally avoid them.

Overall, the application of EMPIRIC to investigate oseltamivir resistance sets the stage for identifying additional mechanisms of antiviral drug resistance. For instance, Jiang et al. report that mutations known to confer resistance in N2 subtypes are often deleterious in the N1 genetic background on which they focused [26]. An improved understanding of how genetic background influences the acquisition of new amino acid substitutions will be extremely useful for identifying probable resistance mutations in circulating strains. Similarly, the libraries used here consist of single mutants, which allow Jiang et al. to perform precise variant fitness measurements. Once the effects of single mutants have been characterized, it will be interesting to study viruses with combinations of these mutations to extract interactions between mutations that do not affect inhibitor binding but do increase substrate processing with mutations that decrease inhibitor binding and compromise viral fitness. In the future, it will be important to evaluate whether amino acid variants that engender drug resistance via enhancing enzyme activity emerge in vivo, and to assess whether these types of drug resistance mechanisms are important for proteins in other rapidly evolving viruses.

Acknowledgments

M.D.S. is supported by the Mallinckrodt Foundation Faculty Scholar Award, the Smith Family Foundation Excellence in Biomedical Research Award, and by Grants 1DP2GM119162 and 1R03AR067503 from the National Institutes of Health. A.M.P. is supported by a National Science Foundation Graduate Research Fellowship.

References

1. Horimoto T, Kawaoka Y. Influenza: Lessons from past pandemics, warnings from current incidents. Nature Reviews Microbiology. 2005; 3:591–600. [PubMed: 16064053]
2. Nguyen HT, Fry AM, Gubareva LV. Neuraminidase inhibitor resistance in influenza viruses and laboratory testing methods. Antivir Ther. 2012; 17:159–73. [PubMed: 22311680]
3. Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, Hayden FG, Sugaya N, Kawaoka Y. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet. 2004; 364:759–65. [PubMed: 15337401]
4. Deyde VM, Xu XY, Bright RA, Shaw M, Smith CB, Zhang Y, Shu YL, Gubareva LV, Cox NJ, Klimov AI. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. Journal of Infectious Diseases. 2007; 196:249–257. [PubMed: 17570112]
5. Moscona A. Global Transmission of Oseltamivir-Resistant Influenza. New Engl J Med. 2009; 360:953–956. [PubMed: 19258250]
6. Hay AJ, Wolstenholme AJ, Skehel JJ, Smith MH. The Molecular Basis of the Specific Anti-Influenza Action of Amantadine. EMBO J. 1985; 4:3021–3024. [PubMed: 4065098]
7. Air GM, Laver WG. The neuraminidase of influenza virus. Proteins. 1989; 6:341–356. [PubMed: 2482974]
8. Wagner R, Matrosovich M, Klenk HD. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Rev Med Virol. 2002; 12:159–166. [PubMed: 11987141]
9. Lentz MR, Webster RG, Air GM. Site-directed mutation of the active site of influenza neuraminidase and implications for the catalytic mechanism. Biochemistry. 1987; 26:5351–5358. [PubMed: 3314986]
10. Varghese JN, McKimm-Breschkin JL, Caldwell JB, Kortt AA, Colman PM. The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. Proteins. 1992; 14:327–32. [PubMed: 1438172]
11. von Itzstein M, Wu WY, Kok GB, Pegg MS, Dyason JC, Jin B, Phan TV, Smythe ML, White HF, Oliver SW, Colman PM, Varghese JN, Ryan DM, Woods JM, Bethell RC, Hotham VJ, Cameron JM, Penn CR. Rational design of potent sialidase-based inhibitors of influenza virus replication. Nature. 1993; 363:418–423. [PubMed: 8502295]
12. Kim CU, Lew W, Williams MA, Liu H, Zhang L, Swaminathan S, Bischofberger N, Chen MS, Mendel DB, Tai CY, Laver WG, Stevens RC. Influenza Neuraminidase Inhibitors Possessing a Novel Hydrophobic Interaction in the Enzyme Active Site: Design, Synthesis, and Structural Analysis of Carbocyclic Sialic Acid Analogues with Potent Anti-Influenza Activity. J Am Chem Soc. 1997; 119:681–690. [PubMed: 16526129]
13. Lew W, Chen X, Kim CU. Discovery and development of GS 4104 (oseltamivir): an orally active influenza neuraminidase inhibitor. Curr Med Chem. 2000; 7:663–72. [PubMed: 10702632]
14. Collins PJ, Haire LF, Lin YP, Liu J, Russell RJ, Walker PA, Martin SR, Daniels RS, Gregory V, Skehel JJ, Gamblin SJ, Hay AJ. Structural basis for oseltamivir resistance of influenza viruses. Vaccine. 2009; 27:6317–23. [PubMed: 19840667]
15. Collins PJ, Haire LF, Lin YP, Liu J, Russell RJ, Walker PA, Skehel JJ, Martin SR, Hay AJ, Gamblin SJ. Crystal structures of oseltamivir-resistant influenza virus neuraminidase mutants. Nature. 2008; 453:1258–61. [PubMed: 18480754]
16. Blick TJ, Sahasrabudhe A, McDonald M, Owens IJ, Morley PJ, Fenton RJ, McKimm-Breschkin JL. The Interaction of Neuraminidase and Hemagglutinin Mutations in Influenza Virus in Resistance to 4-Guanidino-Neu5Ac2en. J Virol. 1998; 246:95–103.
17. Ives JA, Carr JA, Mendel DB, Tai CY, Lambkin R, Kelly L, Oxford JS, Hayden FG, Roberts NA. The H274Y mutation in the influenza A/H1N1 neuraminidase active site following oseltamivir phosphate treatment leave virus severely compromised both in vitro and in vivo. Antiviral Res. 2002; 55:307–17. [PubMed: 12103431]
18. Bloom JD, Gong LI, Baltimore D. Permissive Secondary Mutations Enable the Evolution of Influenza Oseltamivir Resistance. Science. 2010; 328:1272–1275. [PubMed: 20522774]

19. Butler J, Hooper KA, Petrie S, Lee R, Maurer-Stroh S, Reh L, Guarnaccia T, Baas C, Xue L, Vitesnik S, Leang SK, McVernon J, Kelso A, Barr IG, McCaw JM, Bloom JD, Hurt AC. Estimating the fitness advantage conferred by permissive neuraminidase mutations in recent oseltamivir-resistant A(H1N1)pdm09 influenza viruses. PLoS Pathog. 2014; 10:e1004065. [PubMed: 24699865]
20. Renzette N, Caffrey DR, Zeldovich KB, Liu P, Gallagher GR, Aiello D, Porter AJ, Kurt-Jones EA, Bolon DN, Poh YP, Jensen JD, Schiffer CA, Kowalik TF, Finberg RW, Wang JP. Evolution of the influenza A virus genome during development of oseltamivir resistance in vitro. J Virol. 2014; 88:272–81. [PubMed: 24155392]
21. Kiso M, Ozawa M, Le MtQ, Imai H, Takahashi K, Kakugawa S, Noda T, Horimoto T, Kawaoka Y. Effect of an Asparagine-to-Serine Mutation at Position 294 in Neuraminidase on the Pathogenicity of Highly Pathogenic H5N1 Influenza A Virus. J Virol. 2011; 85:4667–4672. [PubMed: 21367898]
22. Abed Y, Baz M, Boivin G. Impact of neuraminidase mutations conferring influenza resistance to neuraminidase inhibitors in the N1 and N2 genetic backgrounds. Antivir Ther. 2006; 11:971–6. [PubMed: 17302366]
23. Huang L, Cao Y, Zhou J, Qin K, Zhu W, Zhu Y, Yang L, Wang D, Wei H, Shu Y. A conformational restriction in the influenza A virus neuraminidase binding site by R152 results in a combinational effect of I222T and H274Y on oseltamivir resistance. Antimicrob Agents Chemother. 2014; 58:1639–45. [PubMed: 24366752]
24. Marjuki H, Mishin VP, Chesnokov AP, De La Cruz JA, Davis CT, Villanueva JM, Fry AM, Gubareva LV. Neuraminidase Mutations Conferring Resistance to Oseltamivir in Influenza A(H7N9) Viruses. J Virol. 2015; 89:5419–26. [PubMed: 25740997]
25. Colman PM, Hoyne PA, Lawrence MC. Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase. J Virol. 1993; 67:2972–2980. [PubMed: 8497041]
26. Jiang L, Liu P, Bank C, Renzette N, Prachanronarong K, Yilmaz LS, Caffrey DR, Zeldovich KB, Schiffer CA, Kowalik TF, Jensen JD, Finberg RW, Wang JP, Bolon DN. A balance between inhibitor binding and substrate processing confers influenza drug resistance. J Mol Biol. 2016
27. Fowler DM, Fields S. Deep mutational scanning: a new style of protein science. Nat Methods. 2014; 11:801–7. [PubMed: 25075907]
28. Thyagarajan B, Bloom JD. The inherent mutational tolerance and antigenic evolvability of influenza hemagglutinin. Elife. 2014; 3
29. Bloom JD. An experimentally determined evolutionary model dramatically improves phylogenetic fit. Mol Biol Evol. 2014
30. Wu NC, Young AP, Al-Mawsawi LQ, Olson CA, Feng J, Qi H, Chen SH, Lu IH, Lin CY, Chin RG, Luan HH, Nguyen N, Nelson SF, Li X, Wu TT, Sun R. High-throughput profiling of influenza A virus hemagglutinin gene at single-nucleotide resolution. Sci Rep. 2014; 4:4942. [PubMed: 24820965]
31. Wu NC, Young AP, Al-Mawsawi LQ, Olson CA, Feng J, Qi H, Luan HH, Li X, Wu TT, Sun R. High-throughput identification of loss-of-function mutations for anti-interferon activity in the influenza A virus NS segment. J Virol. 2014; 88:10157–64. [PubMed: 24965464]
32. Wu NC, Young AP, Dandekar S, Wijersuriya H, Al-Mawsawi LQ, Wu TT, Sun R. Systematic identification of H274Y compensatory mutations in influenza A virus neuraminidase by high-throughput screening. J Virol. 2013; 87:1193–9. [PubMed: 23152521]
33. Fowler DM, Araya CL, Fleishman SJ, Kellogg EH, Stephany JJ, Baker D, Fields S. High-resolution mapping of protein sequence-function relationships. Nat Methods. 2010; 7:741–6. [PubMed: 20711194]
34. Heaton NS, Sachs D, Chen CJ, Hai R, Palese P. Genome-wide mutagenesis of influenza virus reveals unique plasticity of the hemagglutinin and NS1 proteins. Proc Natl Acad Sci U S A. 2013; 110:20248–20253. [PubMed: 24277853]
35. McLaughlin RN Jr, Poelwijk FJ, Raman A, Gosal WS, Ranganathan R. The spatial architecture of protein function and adaptation. Nature. 2012; 491:138–42. [PubMed: 23041932]

36. Hietpas R, Roscoe B, Jiang L, Bolon DN. Fitness analyses of all possible point mutations for regions of genes in yeast. Nat Protoc. 2012; 7:1382–96. [PubMed: 22722372]

37. Hietpas RT, Jensen JD, Bolon DN. Experimental illumination of a fitness landscape. Proc Natl Acad Sci U S A. 2011; 108:7896–901. [PubMed: 21464309]

38. Bank C, Hietpas RT, Wong A, Bolon DN, Jensen JD. A bayesian MCMC approach to assess the complete distribution of fitness effects of new mutations: uncovering the potential for adaptive walks in challenging environments. Genetics. 2014; 196:841–52. [PubMed: 24398421]

39. van der Vries E, Collins PJ, Vachieri SG, Xiong X, Liu J, Walker PA, Haire LF, Hay AJ, Schutten M, Osterhaus AD, Martin SR, Boucher CA, Skehel JJ, Gamblin SJ. H1N1 2009 pandemic influenza virus: resistance of the I223R neuraminidase mutant explained by kinetic and structural analysis. PLoS Pathog. 2012; 8:e1002914. [PubMed: 23028314]

40. Ali A, Bandaranayake RM, Cai Y, King NM, Kolli M, Mittal S, Murzycki JF, Nalam MNL, Nalivaika EA, Özen A, Prabu-Jeyabalan MM, Thayer K, Schiffer CA. Molecular Basis for Drug Resistance in HIV-1 Protease. Viruses. 2010; 2:2509–2535. [PubMed: 21994628]

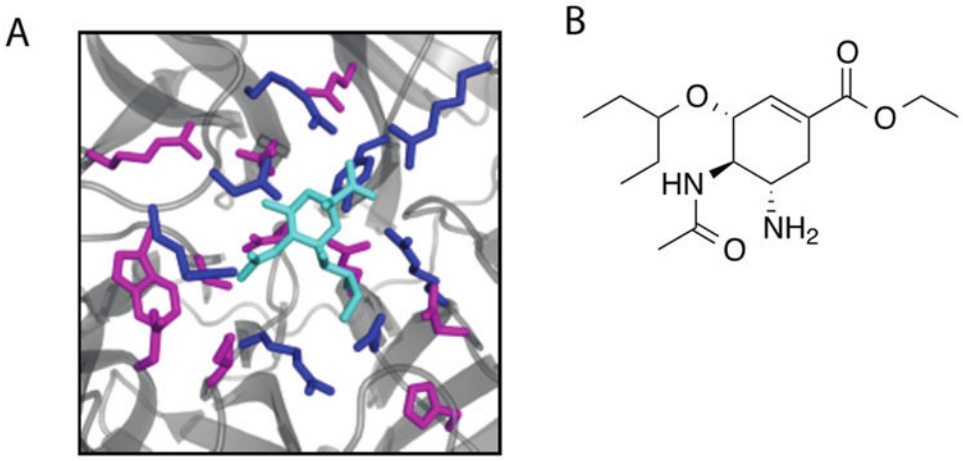

Figure 1.

(A) Structure of N1 neuraminidase with sialic acid bound in the active site. Sialic acid is shown in cyan, functional residues are shown in blue, and framework residues are shown in magenta (PDB 2BAT) [10]. **(B)** Structure of oseltamivir [12].

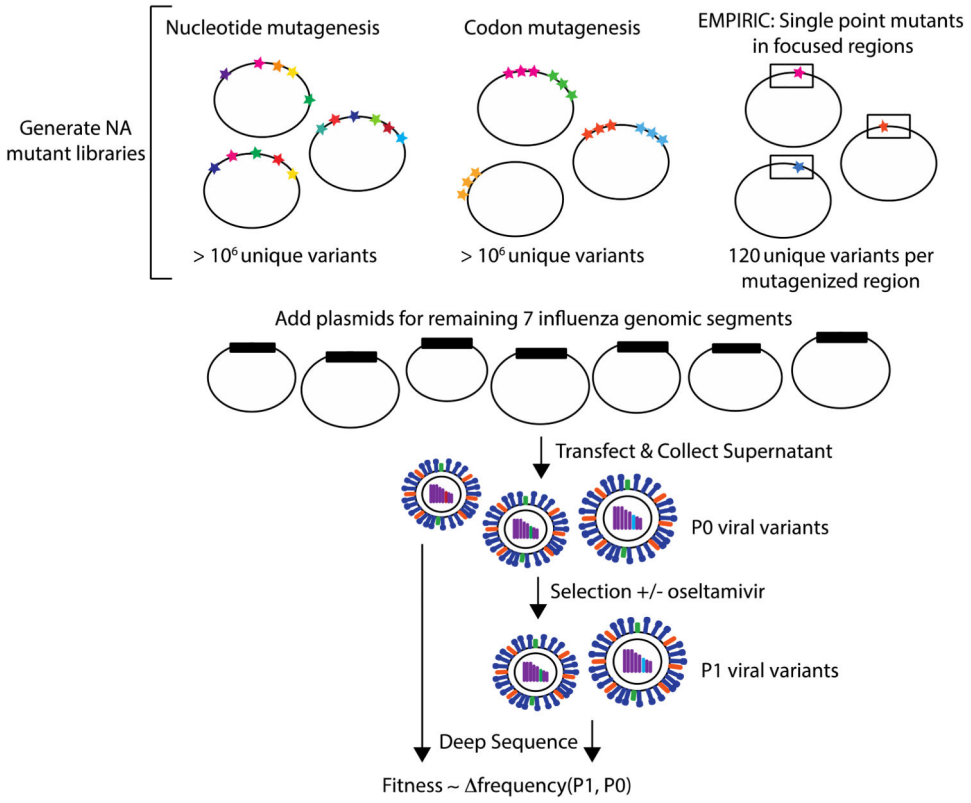

Figure 2. Comparison of methods for constructing neuraminidase mutant libraries

Random nucleotide mutagenesis [27,32,33], random codon mutagenesis [27,28,33], and the EMPIRIC strategy of focused mutagenesis [36,37]. Methods are illustrated in the context of generating mutant libraries to perform selections and quantify variant fitness using deep sequencing.