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Figure S1. Annealing of primers used for the amplification of the E9L gene. The numbers indicate the nucleotide position in the VACV genome. In brown is highlighted the nucleotide sequencing encoding the DNA polymerase. Forward (in green) and reverse (in yellow) primers used for PCR amplification are as well highlighted.

```
53581 tctaatggat aaactgaatc taacaaagag cgacgtacaa ctgttgtaaa ttatttttatg
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54361 tatctacatc ttgactgtct atctctgtaa acacggagtc ggtatctcca tacacgctac
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56581 ttcgacatct ggatttcaga tataaaaatc tgttttcacc gtgactttca aaccaattaa
56641 tgcaccgaac atccattttat agaatttaga aatatatttt catttaaatg aatcccaaac
56701 attgggaag agccgtatgg accattattt ttatagtact ttcgcaagcg ggtttagacg
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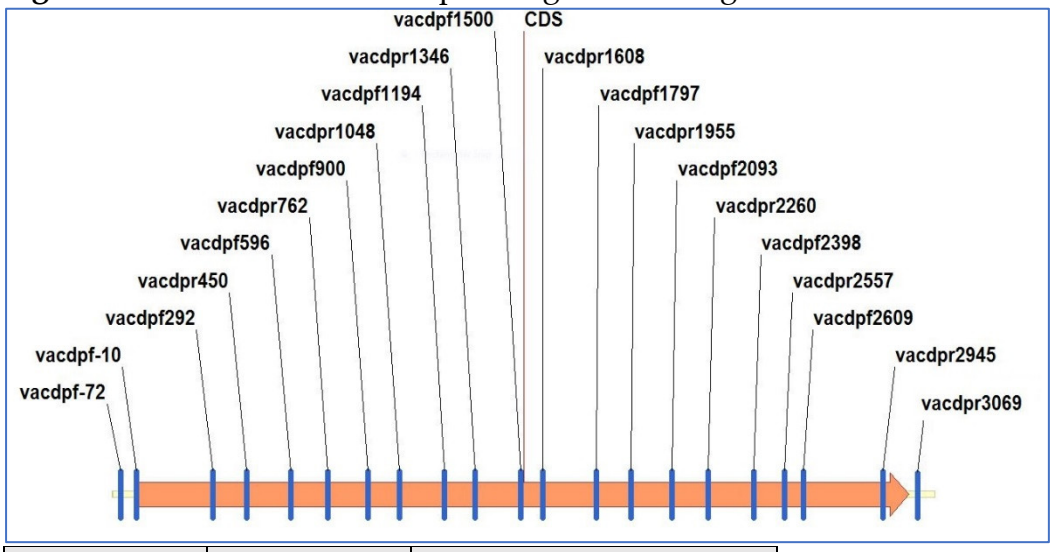
The sets of primers used for amplification were:
Primer set 1: 5′-ATAATGGTCCATACGGCTCTTCCC-3′ & 5′-TGGAGCAAATACCTTACCGCCTTC-3′

Primer set 2: 5-AGTCATCAAGGGTCCACTGTTAAAGC-3′ & 5-GATAAACTGAATCTAACAAAGAGCGACG-3′

Figure S2. Primers used for sequencing of the E9L gene.



Amplicon	Name	Sequence (5' - 3')
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	vacdpF-10	aattctataaatggatgttcggtgc
	vacdpf292	agcatacaaaatgccaccatggatg
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	vacdpr450	agcgaaacagttacgtggatcgtcac
	vacdpr762	tagttccaacaattgtttagctattcg
	vacdpr1048	atatagaatccaatttgtacgaatcc
	vacdpr1346	agattataatccttatacatctgagc
	vacdpr1608	tggagcaaataccttaccgccttc
VACDP_R	vacdpf1500	agtcatcaagggtccactgttaaagc
	vacdpf1797	tcattgtgaacctagactaccgaacc
	vacdpf2093	tagaatcggtactaaatggagcag
	vacdpf2398	atgcaatcgaagaagaaatatacaacg
	vacdpf2609	gttctttagaaacagatttacgatcc
	vacdpr1955	atggccttttcagttgaactggtag
	vacdpr2260	agtcggtatctccatacacgctacg
	vacdpr2557	tctcagacagtctggtcttgtatgtc
	vacdpR2945	agattgactatttcggacgtcaatcg
	vacdpr3069	gataaactgaatctaacaaagagcgacg

Figure S3. **(A)** Genotyping (sequencing of the E9L gene, DNA polymerase) and phenotyping of viral clones derived from VACV VV1 emerging under pressure with HPMPC. **(B)** *Drug-susceptibility profile of selected VV1 HPMPC-R viral clones to evaluate the impact of the R97H, A314T, and A684V mutations. Drug-resistance properties of the different viral clones were determined using a CPE reduction assay with HEL fibroblasts. The effects of different drugs on viruses encoding the indicated mutations were determined by calculating the EC_{50} values for the parental wild-type strain and clones bearing the specific mutations. Al least two independent experiments were performed for each test compound. Horizontal lines for each drug and mutant viral clones indicate the mean values ± standard deviation. The fold-resistance (ratio of the EC_{50} for the mutant viruses to the EC_{50} for the corresponding wild-type virus is marked at the top of the graph. VACV viral clones showing a ≥2-fold increase (red) were considered drug-resistant (R) and those with a ≤ 0.5-fold decrease (orange) were considered drug-hypersensitive (hs).*

A

Virus clones analyzed	DNA pol mutations		
VV1 / HPMPC^r clone 1	R97H	A314T	A684V
VV1 / HPMPC^r clone 2	R97H	A314T	A684V
VV1 / HPMPC^r clone 3*	R97H	A314T	A684V
VV1 / HPMPC^r clone 4	R97H	A314T	A684V
VV1 / HPMPC^r clone 5	R97H	A314T	A684V

B

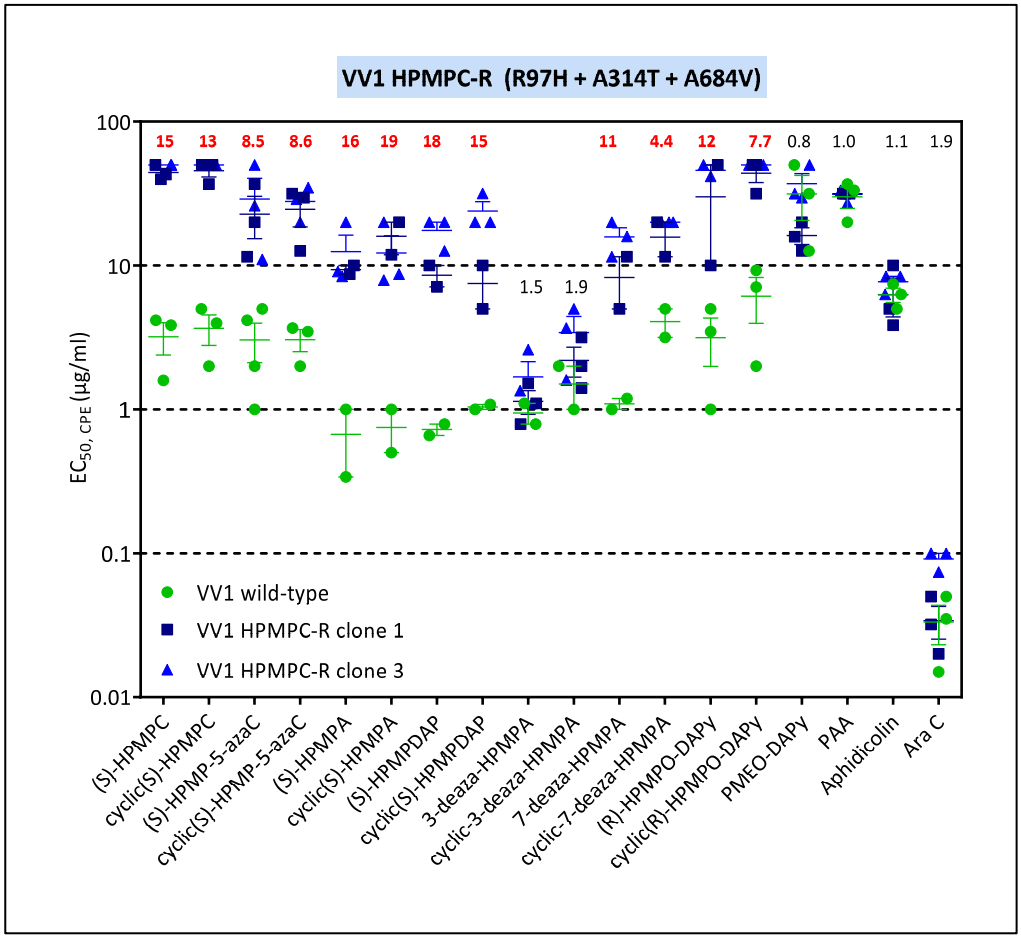
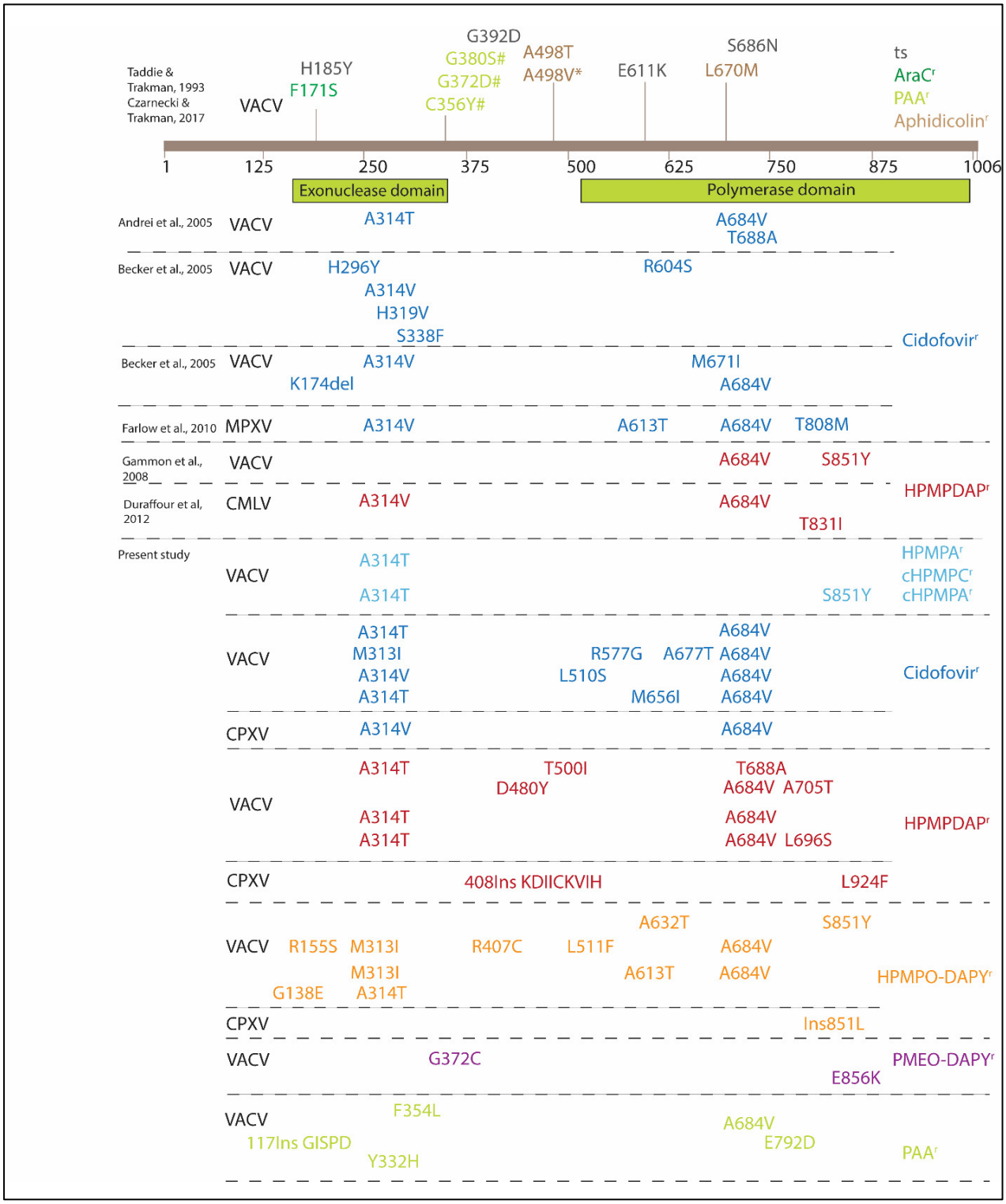


Figure S4. Diagram of poxvirus DNA polymerase with previously described mutations known to be associated with altered drug-



susceptibility and mutations reported in the present study.

Table S1. **Starting and ending drug concentrations used for the drug escalations**

Drug	Starting concentration (μg/ml)	End concentration (μg/ml)
Cidofovir (HPMPC)	1	50
cHPMPC	1	50
HPMPA	0.2	30
cHPMPA	0.2	4
HPMPDAP	1	50
HPMPO-DAPy	1	50
PMEO-DAPy	20	200
PAA	20	150

Table S2. Genotyping (sequencing of the E9L gene, DNA polymerase) of viral clones derived from VACV VV2 emerging under pressure with HPMPC.

Virus clones analyzed	DNA pol mutations				
VV2 / HPMPC^r clone 1	W8C	M313I	R577G	A677	A684V
VV2 / HPMPC^r clone 2*	W8C	M313I	R577G	A677T	A684V
VV2 / HPMPC^r clone 3	W8C	M313I	R577G	A677	A684V
VV2 / HPMPC^r clone 4	W8C	M313I	R577G	A677T	A684V
VV2 / HPMPC^r clone 5	W8C	M313I	R577G	A677	A684V

Table S3. Genotyping (sequencing of the E9L gene, DNA polymerase) of viral clones derived from VACV VV11 emerging under pressure with HPMPC.

Virus clones analyzed	DNA pol mutations					
VV11 / HPMPC^r clone 1*	L90S	R234L	A314T	M656I	A684V	**S898T**
VV11 / HPMPC^r clone 2	L90S	R234	A314T	M656I	A684V	**S898T**
VV11 / HPMPC^r clone 3	L90	R234	A314T	M656I	A684V	**S898**
VV11 / HPMPC^r clone 4	L90	R234	A314T	M656I	A684V	**S898**
VV11 / HPMPC^r clone 5	L90	R234	A314T	M656I	A684V	**S898**
VV11 / HPMPCr clone 6	L90S	R234	A314T	M656I	A684V	**S898T**
VV11 / HPMPCr clone 7	L90	R234	A314T	M656I	A684V	S898T

Table S4. Summary of the known and new DNA polymerase mutations in poxviruses associated with altered drug-sensitivity and their putative mode of action

E9L mutation	Virus	Selected under pressure of:	Sensitivity / Hypersensitivity (hs) / resistance			Putative mechanism
			HPMP's & HPMPO-DAPy's	3-deaza-HPMPA HPMP-5-azaC	PMEO-DAPy, PAA, aphidicolin & AraC	
F171S (44)*	VACV	AraC	Not available	Not available	AraC-R AraA-R Aphidicolin-hs	**F171S** (3′-5′ exonuclease domain, Exo-I): modifying the 3′ nucleotide binding at the exonuclease site (45)
K174 del (39)	VACV	Cidofovir (HPMPC)	HPMPC-R - no data availale for other ANPs	Not available	Not available	**K174 del** (3′-5′ exonuclease domain): modifying the 3′ nucleotide binding at the exonuclease site (45)
H185T (45, 52)	VACV	*ts*	Not available	Not available	Not available	**H185T** (3′-5′ exonuclease domain): Destabilisation of the 3′-5′ exonuclease domain (45)
M313I	VACV	HPMPC HPMPO-DAPy	Low resistance	HPMP-5-azaC-S 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs Aphidicolin-S Arac-S	**M313I:** (3′-5′ exonuclease domain, beta-hairpin): Modulation of guidance from elongation to editing mode
M313I + A613T	VACV	HPMPO-DAPy	Higher resistance than M313I	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs Aphidicolin-S Arac-S	**A613T** (polymerase domain): Destabilization of insert 3 or of the elongation site
M313I + A613T + A684V	VACV	HPMPO-DAPy	Higher resistance than M313I	HPMP-5-azaC-R 3-deaza-HPMPA-R	PMEO-DAPy-R PAA-S Aphidicolin-S Arac-R	**A684V** (polymerase domain): indirect effect on the binding of the template backbone in the elongation site (45)
M313I + R577G + A684V	VACV	HPMPC	Higher resistance than M313I	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-S Aphidicolin-hs Arac-hs	**R577G** (polymerase domain) catalytic impairment of the enzyme
M313I + R577G + A677T + A684V	VACV	HPMPC	Higher resistance than M313I + R577G + A684V	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-S Aphidicolin-S Arac-hs	A677T (polymerase domain): catalytic impairment of the enzyme

A314T (37-42, 45)	VACV CMLV MPXV CPXV	HPMPC cHPMPC HPMPA HPMPDAP HPMPO-DAPy	Higher resistance than A314V	HPMP-5-azaC-R 3-deaza-HPMPA-R	PMEO-DAPy-hs PAA-hs Aphidicolin-S Arac-S	A314T (3′-5′ exonuclease domain, beta-hairpin): modulation of guidance from elongation to editing mode (45)
A314V (37-42, 45)	VACV CMLV MPXV CPXV	HPMPC HPMPDAP	Low resistance	HPMP-5-azaC-S 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs Aph-hs AraC-hs	
A314T + A684V (37)	VACV	HPMPC HPMPDAP	~ resistance as A314T	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-S PAA-S Aphidicolin-S Arac-S	Combination of mutations
A314T + L696S	VACV	HPMPDAP	~ resistance as A314T	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs Aphidicolin-S Arac-S	Combination of mutations **L696S** (polymerase domain): catalytic impairment of the enzyme
A314T + A684V + L696S	VACV	HPMPDAP	Higher resistance than A314T + L696S	HPMP-5-azaC-R 3-deaza-HPMPA-R	PMEO-DAPy-R PAA-S Aphidicolin-S Arac-S	Combination of mutations
A314T + M656I + A684V	VACV	HPMPC	Higher resistance than A314T + A684V	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs Aphidicolin-S AraC-hs	Combination of mutations **M656I** (polymerase domain):
A314T + T688A (37)	VCAV	HPMPC	Resistance to ANPs	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs AraC-hs Aphidicolin-S	Combination of mutations **T688A** (polymerase domain): indirect effect on template backbone binding (45)
A314V + T500I	VACV	HPMPDAP	~ A314V	HPMP-5-azaC-S 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs AraC-hs Aph-hs	Combination of mutations **T500I:** changes in enzyme structure
A314V + T500I + T688A	VACV	HPMPDAP	Higher resistance than A314V	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs AraC-hs Aph-hs	Combination of mutations

A314V + A684V	VACV	HPMPC	Higher resistance than A314V	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs AraC-hs Aphidicolin-hs	Combination of mutations
A314V + L510S + A684V	VACV	HPMPC	Higher resistance than A314V + A684V	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs AraC-hs Aph-hs	**L510S** (N-terminal domain): changes in enzyme structure
Y332H	VACV	PAA	Sensitive	HPMP-5-azaC-S 3-deaza-HPMPA-S	PMEO-DAPy-S PAA-R Aphidicolin-S AraC-S	**Y332H** (3′-5′ exonuclease domain): Destabilization of the 3′-5′exonuclease domain
S338F (40)	VACV	HPMPC	HPMPC-R - no data availale for other ANPs	Not available	Not available	**S338F** (3′-5′ exonuclease domain): indirect effect on complementary strand binding or on strand switching between elongation and editing mode (45)
F354L	VACV	PAA	Sensitive	HPMP-5-azaC-S 3-deaza-HPMPA-R	PMEO-DAPy-R PAA-R Aphidicolin-hs AraC-R	**F354L** (DNA polymerase family B viral insert): altering the interaction between insert 2 and finger domain
C356T** **G372D**** **G380S** (44)**	VACV	PAA	Not available	Not available	PAA-R Aphidicolin-hs AraC-R +/-	**C356T, G372D, G380S**: (DNA polymerase family B viral insert): altering the interaction between insert 2 and finger domain (45)
F171S + C356T **F171S + G372D** **F171S + G380S (44)**	VACV	-	Not available	Not available	PAA-R Aphidicolin-hs AraC-R	Combination of mutations
G372C	VACV	PMEO-DAPy	Sensitive	HPMP-5-azaC-S 3-deaza-HPMPA-R	PMEO-DAPy-R PAA-S Aphidicolin-S AraC-R	**G372C:** (DNA polymerase family B viral insert): altering the interaction between insert 2 and finger domain
G392D (44, 53)	VACV	*ts*	Not available	Not available	Not available	**G392D**: (DNA polymerase family B viral insert): desestabilization of insert 2 (45)
R407C + L511F	VACV	HPMPO-DAPy	Low resistance	HPMP-5-azaC-S 3-deaza-HPMPA-S	PMEO-DAPy-S PAA-S Aphidicolin-S AraC-S	Combination of mutations **R407C** (changes in enzyme structure) **L511F** (changes in enzyme structure)

M313I + R407C + L511F	VACV	HPMPO-DAPy	Higher resistance than R407C + L511F	HPMP-5-azaC-S 3-deaza-HPMPA-S	PMEO-DAPy-S PAA-hs Aphidicolin-S AraC-hs	Combination of mutations
R155S + M313I + R407C + L511F + A684V	VACV	HPMPO-DAPy	Higher resistance than M313I + R407C + L511F	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-S PAA-S Aphidicolin-S AraC-S	Combination of mutations
D480Y	VACV	HPMPDAP	Low resistance	HPMP-5-azaC-S 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-S Aphidicolin-S AraC-hs	**D480Y** (3′-5′ exonuclease domain): indirect effect on complementary strand binding or on strand switching between elongation and editing mode
D480D + A684V + A705T	VACV	HPMPDAP	Higher resistance than D480Y	3-deaza-HPMPA-S HPMP-5-azaC-R	PMEO-DAPy-S PAA-R Aphidicolin-hs AraC-hs	Combination of mutations **A705T** (palm domain): impaired catalytic activity
A498T A498V* (44)	VACV	Aphidicolin	Not available	Not available	PAA-hs Aphidicolin-R AraC-hs & AraA-hs	**E498T/V:** Interference with the rotation of a base in the template requirred for aphidicolin binding (45)
E611K (44)	VACV	*ts*	Not available	Not available	Not available	**E611K:** Destabilization of insert 3 or of the elongation site (45)
L670M (44)	VACV	Aphidicolin	Not available	Not available	Aphidicolin-R	**L670M:** Indirect effect on the binding of the template backbone next to the aphidicolin-binding site (45)
A684V (37, 39, 41, 42)	VACV CMLV CPXV MPXV	HPMPC HPMPDAP HPMPO-DAPy PAA	Low resistance	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-R PAA-R Aphidicolin-hs AraC-S	**A684V:** Indirect effect on the binding of the template backbone in the elongation site (45)
S686N	VACV	*ts*	Not available	Not available	Not available	**S686N:** Destabilization of insert 3 or the elongation site (45)

117Ins GSPD + E792D	VACV	PAA	Sensitive	HPMP-5-azaC-S 3-deaza-HPMPA-S	PMEO-DAPy-R PAA-R Aphidicolin-hs AraC-S	Combination of mutations **117Ins GSPD:** (3′-5′ exonuclease domain): modifying the 3′ nucleotide binding at the exonuclease site **E792D:** Modulation of domain movements by a modification at the thumb-palm domain connection and a changed interaction with the complementary strand
T831I	VACV	HPMPDAP	High resistance	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs Aphidicolin: not available AraC: not available	**T831I:** Modulation of domain movements by a modification at the thumb-palm domain connection and a changed interaction with the complementary strand (45)
S851Y (41)	VACV	HPMPDAP HPMPO-DAPy cHPMPA	Low resistance	HPMP-5-azaC-S 3-deaza-HPMPA-hs	PMEO-DAPy-hs PAA-hs Aphidicolin-S AraC-hs	**S851Y:** Modulation of domain movements by a modification of the thumb-palm domain interface (45)
S851Y + A353V	VACV	cHPMPA	Higher resistance than S851Y	HPMP-5-azaC: not available 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs Aphidicolin-S AraC-hs	**A353V:** (DNA polymerase family B viral insert): altering the interaction between insert 2 and finger domain
S851Y + A632T	VACV	HPMPO-DAPy	Higher resistance than S851Y	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs Aphidicolin-S AraC-S	Combination of mutations **A632T** (polymerase domain): impaired catalytic activity
S851Y + A684V (41)	VACV	HPMPDAP	Higher resistance than S851Y	HPMP-5-azaC-R 3-deaza-HPMPA-R	PMEO-DAPy-S PAA-R Aphidicolin-S AraC-S	Combination of mutations
Ins851L	CPXV	HPMPO-DAPy	Higher resistance than S851Y	HPMP-5-azaC-R 3-deaza-HPMPA-S	PMEO-DAPy-hs PAA-hs Aphidicolin-R AraC-S	**S851Y** Modulation of domain movements by a modification of the thumb-palm domain interface (45)
E856K	VACV	PMEO-DAPy	Sensitive	HPMP-5-azaC-S 3-deaza-HPMPA-R	PMEO-DAPy-R PAA-S Aphidicolin-S AraC-R	**E856K** (polymerase domain): impaired catalytic activity

L924F	CPXV	HPMPDAP	High resistance	HPMP-5-azaC-R 3-deaza-HPMPA-R	PMEO-DAPy-hs PAA-hs Aphidicolin-S AraC-S	**L924F** (polymerase domain): impaired catalytic activity and/or effect on 3′-5′ exonuclease activity
L924F + Ins408	CPXV	HPMPDAP	~L924F	3-deaza-HPMPA-S HPMP-5-azaC-R	PMEO-DAPy-hs PAA-hs Aphidicolin-S AraC-S	**Ins408** (DNA polymerase family B viral insert): altering the interaction between insert 2 and finger domain

Abreviatons: ts (temperature sensitive), hs (hypersensitive), S (sensitive), R (resistance), NA: not available

* Mutator phenotype

** Antimutator