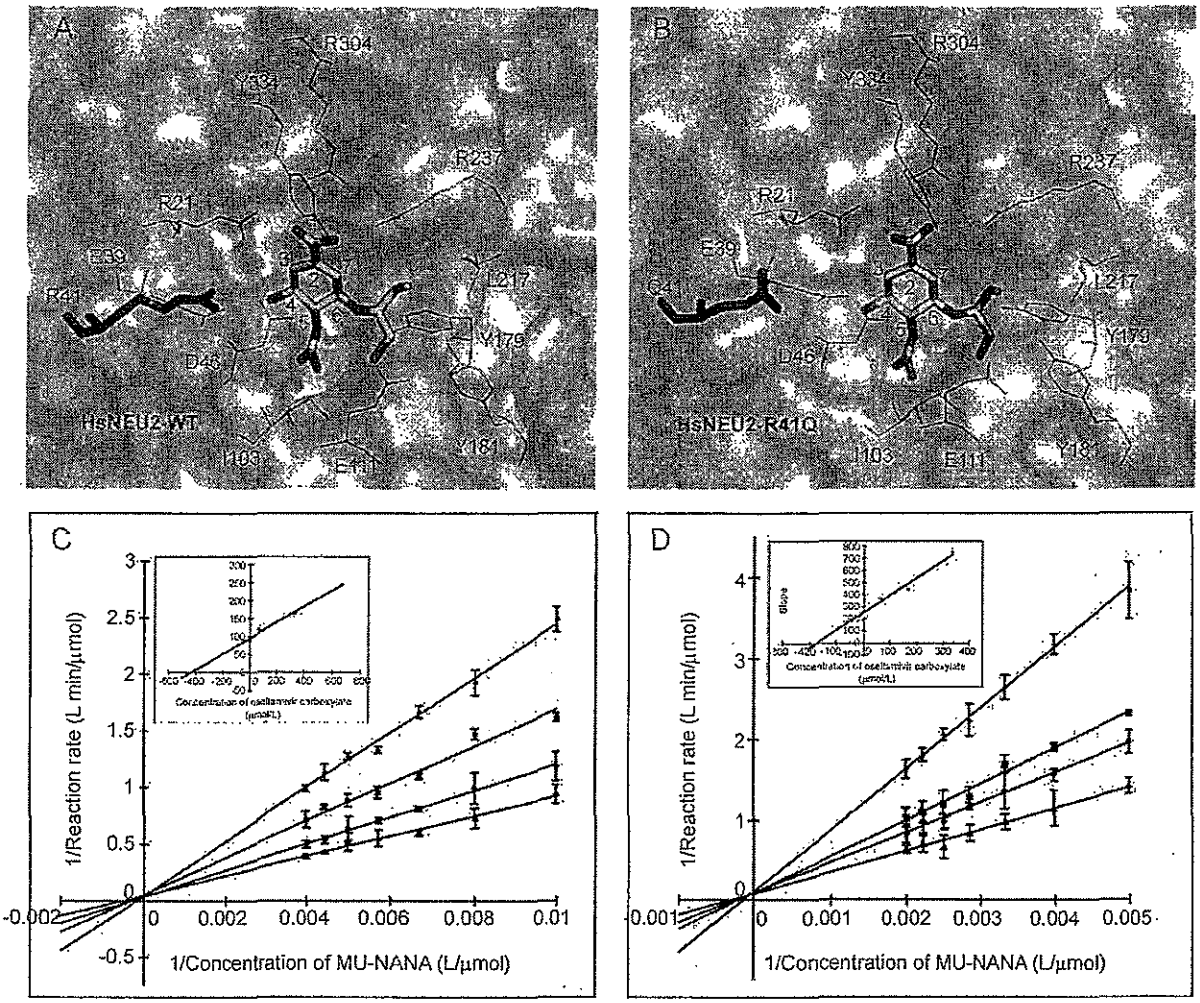

Figure 1 An SNP causing R41Q substitution in HsNEU2, occurring primarily in Asian population, could significantly reduce sialidase activity. **(A, B)** Binding of oseltamivir carboxylate to HsNEU2 wild-type (HsNEU2-WT) **(A)** and HsNEU2 R41Q variant (HsNEU2-R41Q) **(B)**. The structures of HsNEU2 and oseltamivir carboxylate were retrieved from PDB entries 1VCU and 2QWK, respectively. The complex structures were optimized by Insight II. Residues close to the active site were shown as lines while SNP-related residues (Arg in HsNEU2-WT and Gln in HsNEU2-R41Q) were highlighted as sticks. Carbon numbering of oseltamivir carboxylate followed that of the native ligand. **(C, D)** Oseltamivir carboxylate is a competitive inhibitor for HsNEU2. The *Ki* value was 0.175 mM for HsNEU2 R41Q and 0.432 mM for HsNEU2 wild-type, suggesting that HsNEU2-R41Q was more sensitive to oseltamivir carboxylate. In the absence of oseltamivir carboxylate, compared with wild-type HsNEU2, R41Q variation caused *Km* to increase from 2.136 mM to 2.795 mM, and *Vmax* to decrease from 24.272 μmol/L·min to 10.846 μmol/L·min. Data were presented as mean±SD (n=6 independent experiments).

Table 1 Results of sialidase assays

	Km (m mol/L)	*Vmax* (μmol/L min)	*Vmax*/*Km*	*Ki* (m mol/L)
HsNEU2-WT	2.136	24.272	11.363	0.432
HsNEU2-R41Q	2.795	10.846	3.879	0.175
HsNEU2-R41Q/WT	1.308	0.447	0.341	0.405

cleaved by HsNEU2 from the fluorogenic substrate MU-NANA as described by Venerando *et al.*, in the absence or presence of oseltamivir carboxylate, respectively [20]. The reactions were set up in 33 mM MES buffer pH 6.5 containing 4 mM $CaCl_2$. Different amounts of substrate were mixed with 1 μg of wild-type HsNEU2 or HsNEU2 R41Q in the reaction mixture with a final volume of 100 μL. After incubation at 37 °C for 15 min, 300 μL of stop buffer (0.042 M Na_2CO_3, 0.133 M glycin pH 10.0, 0.06 M NaCl) was added to stop the reaction. The fluorescence emission was measured on Synergy HT (BioTek) with excitation at 360 nm and emission at 460 nm. The calibration curve was plotted using different amounts of 4-methylumbelliferyl (4-MU) (Sigma), and 1 nmol of 4-methylumbelliferyl was equal to 6 378 fluorescence units. *Km* and *V*max were determined by the method of Lineweaver and Burk [21].

Sialidase inhibition assay

Sialidase inhibition assay by oseltamivir carboxylate obtained by saponification of the oseltamivir phosphate [22] was carried out to determine the *Ki* values following the same sialidase assay procedure but with pre-incubation of wild-type HsNEU2 and HsNEU2 R41Q with oseltamivir carboxylate before adding the MU-NANA substrate. The concentration of oseltamivir carboxylate versus the reaction rate was plotted, and the *Ki* values were determined by the method of Lineweaver and Burk [21].

Results

Close inspection of molecular docking of the HsNEU2 structure and oseltamivir carboxylate showed that HsNEU2 could bind oseltamivir carboxylate but the binding is weakened by the basic side chain of Arg41 in HsNEU2 that could have a repulsive effect on the basic C4 group in oseltamivir carboxylate (Figure 1A). The nonsynonymous SNP from G to A (refSNP ID rs2233385 in dbSNP database at NCBI [15]) causes Arg41 to be substituted by Gln in HsNEU2. The uncharged and shorter side chain of Gln41 would not have the same repulsive effect on the C4 basic group in oseltamivir carboxylate. On the contrary, nearby acidic residues Glu39 and Asp46 might now be able to create a negatively charged pocket to potentially form a stronger interaction with oseltamivir carboxylate. Molecular docking simulation between oseltamivir carboxylate and HsNEU2 wild-type vs. R41Q variant showed enhanced binding affinities between oseltamivir carboxylate and the R41Q variant (Figure 1B).

Sialidase assays with the MU-NANA substrate confirmed the docking prediction. The results of the *Ki* measurements showed that oseltamivir carboxylate was a competitive inhibitor for HsNEU2 (Figure 1C and 1D). The *Ki* constant was 0.175 mM for the HsNEU2 R41Q variant, 2.2 times lower than the *Ki* constant for HsNEU2 wild-type (0.432 mM). Thus the R41Q variant was more sensitive to inhibition by oseltamivir carboxylate than the wide-type was (Figure 1C and 1D). We also compared the sialidase activity of the wide-type and variant enzymes towards the MU-NANA substrate in the absence of oseltamivir carboxylate. The *Km and V*max of wild-type HsNEU2 were 2.136 mM and 24.272 μmol/L·min, respectively, whereas they were 2.795 mM and 10.846 μmol/L·min for the R41Q variant, respectively (Figure 1C and 1D; Table 1). The *V*max/*K*m ratio was 11.363 for wild-type HsNEU2, and 3.879 for the R41Q variant. This result suggested that the activity of the R41Q variant was about three times lower than that of HsNEU2. Taken together, the HsNEU2 R41Q variant had reduced sialidase activity compared to the wild-type enzyme in the presence of oseltamivir carboxylate.

Discussion

Variations in human sialidase activity have long been implicated in serious diseases and symptoms including neuropsychiatric problems such as seizing and convulsions and skin problems [23-26]. Some of the symptoms coincide with several reported side effects of Tamiflu. People with the HsNEU2 R41Q variation might already have dampened sialidase activity, and because this variation also increases the enzyme's sensitivity to oseltamivir carboxylate, administration of oseltamivir could further reduce sialidase activity and cause symptoms similar to those in well-known sialidase-related disorders. We suspect that only homozygotes of this SNP might show adverse oseltamivir reactions because heterozygotes could presumably compensate by the other wide-type allele. Furthermore, most of the known sialidase-related disorders were discovered in people with homozygous mutant sialidase alleles, and the total number of reports of severe adverse reactions that occur with administration of Tamiflu was small.

Our hypothesis was supported by results from structural bioinformatic analyses and *in vitro* enzymatic activity assays. The difference in sialidase activity between wild-type HsNEU2 and the R41Q variant in the presence of oseltamivir carboxylate is likely to hold *in vivo* as well at least partly. Oseltamivir carboxylate is a competitive inhibitor of HsNEU2 (Figure 1C and 1D). Although the plasma concentration of oseltamivir carboxylate is known to be only up to 1.2 μM after an oral dose of 75 mg capsule twice-daily (http://www.rocheusa.com/products/tamiflu/), the concentration in local tissues and cells (in some individuals) may be much higher. Indeed there is evidence from a recent study that administration of oseltamivir could inhibit wild-type human sialidase *in vivo* [28].

We further observed that, based on the data in dbSNP, the frequency of the minor allele (A) of this SNP was 9.29% in Asian, 0.55% in Sub-Saharan African, and none in European and African American. This differential population distribution might offer a clue in explaining why most cases of severe neuropsychiatric side effects of oseltamivir

were reported in Japan and few in America or European countries [27]. However, this circumstantial evidence is confounded by the much higher per capita oseltamivir use in Japan compared to other countries. Further testing of our hypothesis would require population genetic studies to determine whether homozygotes of the minor allele (A) of this SNP were significantly enriched in cases reporting severe adverse reactions to oseltamivir. Because of the relatively low prevalence of the SNP, the population genetic tests would require a large number of cases for statistical significance. We also suggest further genotyping and enzyme assay of all four sialidases to discover any additional SNPs that may be relevant.

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RESEARCH HIGHLIGHT

Side effects of Tamiflu: clues from an Asian single nucleotide polymorphism

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Tamiflu (Oseltamivir phosphate) seems to be a double-edged sword to some in Asia. While it is counted on against influenza and a feared avian influenza pandemic [1], the drug is also associated with side effects, ranging from neuropsychiatric, gastrointestinal, to hyperthermia and skin problems. According to a document from US Food and Drug Administration in 2005 [2], 1184 cases of side effects have been reported. Interestingly 69 out of the 75 pediatric cases were from Japan, including two teen suicides. The situation seemed to have made a gloomier turn recently. It was reported in February, 2007 that two Japanese teenagers jumped from apartment buildings after taking Tamiflu and died, bringing the total number of deaths after taking Tamiflu in Japan to 54 [3,4]. Although no direct causal relationship had been established yet, the Japan Health Ministry warned doctors about giving the drug to teenagers. In comparison, relatively few cases of severe side effects were reported from America and European countries [5]. What is wrong with this picture? It has concerned and bewildered many. The pages in this issue [6] offer one fascinating hypothesis that tries to explain the mystery using an integrated approach combining structural bioinformatic analysis and enzyme assays.

The story behind the work by Li *et al.* [6] exemplifies the old saying "Curiosity is the mother of invention" (and indeed a mother was involved!). In 2005 while the fear of a bird flu pandemic was looming, especially in Eastern Asia, computational biologist Liping Wei at Peking University, the senior author of this paper, was concerned for her one-year old baby son after reading the tragic news of the Japanese children who died after taking Tamiflu. Out of concern and curiosity, she decided to investigate whether and why Tamiflu might have caused the side effects and why Japan was hit the hardest. Her group focused on human sialidases which were homologues of the virus neuraminidase (NA), the intended target of the drug. Interestingly, they found that the minor allele of a nonsynonymous single nucleotide polymorphism (SNP) in human cytosolic sialidase (HsNEU2) occurs in 9.2% of Asian and, in striking contrast, none in European and African American, according to the NCBI dbSNP database.

A hypothesis came into being that the human enzymes might also be able to bind to oseltamivir, and the identified nonsynonymous SNP may impact on the binding potential with the drug. Could this interaction with oseltamivir be the reason for the drug's side effect? The authors noticed the similarity between the reported severe side effects of oseltamivir and the known symptom of sialidase variations—several lines of evidence showed that variations of sialidase activity has significant impact on the functions and development of neurons in brain [7, 8]. The detected genetic variation of HsNEU2, if it reduces sialidase activity in the presence of oseltamivir, could be related to the severe side effects of oseltamivir.

The author's hypothesis made two testable predictions: (i) the binding ability of oseltamivir carboxylate, the active metabolite of oseltamivir, to HsNEU2 would be enhanced by the SNP and (ii) subsequently the activity of the HsNEU2 enzyme would be reduced. This hypothesis was first tested using just the published structure of HsNEU2 [9]. HsNEU2 contains the same active site as virus neuraminidase in terms of sequence and structure. The replaced residue as a result of the SNP is located nearby the active site. Molecular docking simulation showed that the amino acid change from Arg41 to Gln could increase human sialidase's binding affinity to oseltamivir carboxylate. Then, in collaboration with biochemist Xiaofeng Zheng's group at Peking University, the authors expressed both the regular and the mutant protein product of HsNEU2 in *E. coli*. Measuring the enzymatic activities of these HsNEU2 proteins in both the absence and

presence of oseltamivir carboxylate, the authors confirmed that the SNP could increase human sialidase' unintended binding affinity to oseltamivir carboxylate, reducing sialidase activity. Interestingly, they also found that this SNP itself could reduce sialidase activity in the absence of oseltamivir. Thus people with this SNP likely already have dampened sialidase activity. Administration of oseltamivir might further reduce the sialidase activity in some patients to a dangerously low level.

To this point it may be tempting to jump to the conclusion that oseltamivir's interaction with the HsNEU2 SNP variant is causally related to the severe side effects reported in Japan through regulating the neuronal functions. Not so fast. The authors cautioned that their experiments were conducted *in vitro* and further population genetic tests with a large sample size would be required. It would also be important to genotype all four human sialidases to identify any other SNPs that may be involved in a similar mechanism. Side effects are complicated and often cannot be explained by one single factor. Nevertheless, this work offers the first testable hypothesis for the sobering and bewildering cases of reported Tamiflu side effects in Japan. The results provide a likely mechanistic cause for how the drug may impact functions of sialidases through changed structure of the enzyme in the SNP variant. Supported by structural, enzymatic, and circumstantial evidence, this study sheds first light on a mystery and points to a direction to pursue.

Patients in a high-risk population, e.g., the ones who suffered in Japan, could be tested before the drug is administered. It will be also valuable to investigate the nucleotide divergence of human sialidase genes in the Japanese population and other Asian populations and their relevancy to the side effects of Tamiflu. It may even be good news for Roche, the maker of Tamiflu, as the test may separate high-risk patients from the remaining vast majority of the population for whom the drug is generally safe and effective. The methodology adopted by this study is an integration of bioinformatics, population genetic survey, and biochemical analysis. The efficient collaboration between computational biologists and biochemists was essential.

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1: Cell Res. 2007 Apr;17(4):357-62. nature publishing group Links

A nonsynonymous SNP in human cytosolic sialidase in a small Asian population results in reduced enzyme activity: potential link with severe adverse reactions to oseltamivir.

Li CY, Yu Q, Ye ZQ, Sun Y, He Q, Li XM, Zhang W, Luo J, Gu X, Zheng X, Wei L.

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The use of oseltamivir, widely stockpiled as one of the drugs for use in a possible avian influenza pandemic, has been reported to be associated with neuropsychiatric disorders and severe skin reactions, primarily in Japan. Here we identified a nonsynonymous SNP (single nucleotide polymorphism) in dbSNP database, R41Q, near the enzymatic active site of human cytosolic sialidase, a homologue of virus neuraminidase that is the target of oseltamivir. This SNP occurred in 9.29% of Asian population and none of European and African American population. Our structural analyses and Ki measurements using in vitro sialidase assays indicated that this SNP could increase the unintended binding affinity of human sialidase to oseltamivir carboxylate, the active form of oseltamivir, thus reducing sialidase activity. In addition, this SNP itself results in an enzyme with an intrinsically lower sialidase activity, as shown by its increased Km and decreased Vmax values. Theoretically administration of oseltamivir to people with this SNP might further reduce their sialidase activity. We note the similarity between the reported neuropsychiatric side effects of oseltamivir and the known symptoms of human sialidase-related disorders. We propose that this Asian-enriched sialidase variation caused by the SNP, likely in homozygous form, may be associated with certain severe adverse reactions to oseltamivir.

PMID: 17426694 [PubMed - in process]

タミフルは中枢抑制
(呼吸抑制,dyscontrol=脱制御)により
突然死や異常行動死を起こす
因果関係はすでに明瞭である

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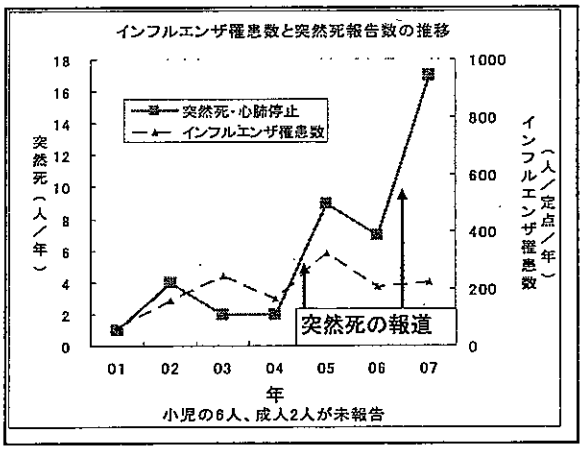
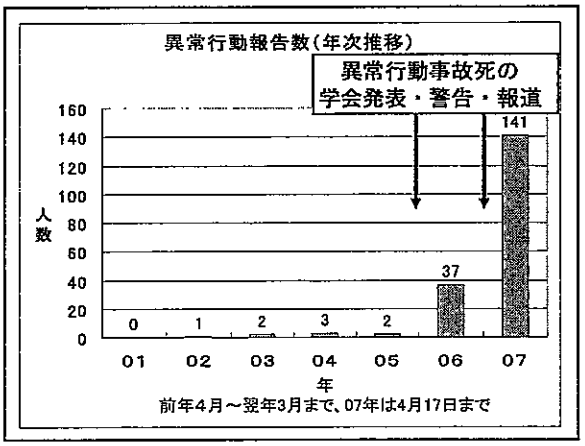


害反応(副作用)死亡例内訳 (2007.4.25現在)

	ADR害反応(副作用)の種類	<10	10代	成人	合計
	突然死(厚労省公表例)	14		31	45
	突然死(浜による独自収集例)	3		2	5
1	突然死 小計 *a	17	0	33	50
2	異常行動・事故死 *a	0	5	3	8
3	呼吸抑制・肺炎・敗血症が疑われる例			4	4
4	感染症が増悪したと考えられる例	2		9	11
5	その他(肝障害、腎障害、詳細不明の死亡)	1		8	9
	合 計 *b	20	5	57	82

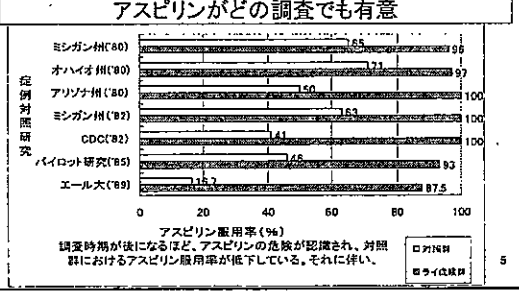
*a:9歳以下(特に5歳以下)と、20歳以上は突然死しやすい。
10歳代は突然死はないが異常行動・事故死しやすい。

*b:07.4.25厚労省発表70人もまだ過少、厚労省把握で77人、他も含めすでに82人が死亡。06年11月30日以降、厚労省が新たに追加した死亡例は24人、うち突然死・心肺停止は17人!



従来の「インフルエンザ脳症」「ライ症候群」の原因の大部分は非ステロイド抗炎症剤(NSAIDs)

米国におけるライ症候群の疫学調査の結果、
アスピリンがどの調査でも有意



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