

Increased Survival after Gemfibrozil Treatment of Severe Mouse Influenza[∇]

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Gemfibrozil, an agent that inhibits production of proinflammatory cytokines in addition to its clinically useful lipid-lowering activity, increased survival in BALB/c mice that were already ill from infection by influenza virus A/Japan/305/57 (H2N2). Gemfibrozil was administered intraperitoneally once daily from days 4 to 10 after intranasal exposure to the virus. Survival increased from 26% in vehicle-treated mice ($n = 50$) to 52% in mice given gemfibrozil at 60 mg/kg/day ($n = 46$) ($P = 0.0026$). If this principle translates to patients, a drug already approved for human use, albeit by a different route for another purpose, might be adapted relatively fast for use against influenza, conceivably including human infection with a derivative of the avian H5N1 strain.

The recent emergence of an H5N1 subtype of influenza A virus that spreads rapidly in birds and causes high mortality in humans has raised concern for the prospect of a pandemic should a related strain develop efficient human-to-human spread. While control, vaccination, and antiviral agents have rightly received much attention, negligible research effort has been focused on novel ways to reduce mortality by ameliorating influenza disease, as distinct from reducing the viral load once illness has begun. The need to reduce mortality is particularly pressing during the early phase of a pandemic, since it is generally accepted that determining the required vaccine strain and generating it in adequate quantities takes 4 to 6 months. A drug already approved for human use and found to treat influenza disease could plausibly become part of the armament against a pandemic with little delay.

In recent years the threat of H5N1 influenza virus infection has generated increased interest in the mechanisms of influenza disease. As with other influenza virus subtypes (19), a number of papers have argued an important causative role for proinflammatory cytokines in H5N1 disease (5, 10, 29), as well as that caused by a reconstructed version of the strain of influenza virus responsible for massive human mortality in 1918–1919 (13).

Accordingly, we have tested a drug known to reduce this cytokine response for its ability to protect against influenza. Here we report that gemfibrozil, a commonly prescribed drug with long-established lipid-lowering properties (18) but only recently recognized as inhibiting the pathways leading to inflammatory cytokine release (3) (and having associated *in vivo* activity [4]), increases survival in mouse influenza virus infection. Moreover, mortality was reduced in mice when daily treatment was commenced after illness was evident.

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MATERIALS AND METHODS

Animals. Animals used were BALB/c female mice, 6 to 8 weeks old, from the Australian National University, Canberra, Australia. Mice were kept in physical containment level 2 housing and given access to standard pellet feed and water *ad libitum*. All experimental procedures and housing were approved by the Australian National University Animal Ethics Committee.

Influenza virus. Stocks of influenza virus A/Japan/305/57 (A/Jap, H2N2) were grown in embryonated eggs. Virus-containing allantoic fluid was harvested and stored in aliquots at -70°C . Virus content was determined by hemagglutination using erythrocytes from *Gallus domesticus*. The stock virus titer used throughout this study was 1.6×10^3 hemagglutination units per ml of allantoic fluid.

Infecting and treating mice. Influenza virus infection was established by inoculating 2,2,2-tribromoethanol (Avertin)-anesthetized mice intranasally with 50 hemagglutination units of virus. Mice were weighed prior to infection and then daily from days 4 to 12 inclusive. Gemfibrozil (Spectrum Chemicals, Gardena, CA) was dissolved in 100% propylene glycol (MP Biomedicals, Solon, OH) and administered to mice at doses between 20 mg/kg and 60 mg/kg by the intraperitoneal (i.p.) route (10 μl /mouse) using BD ultrafine needle insulin syringes, 0.5 ml, 29 gauge (Becton Dickinson, Franklin Lakes, NJ), once daily from days 4 to 10 inclusive after virus exposure. Control mice were given propylene glycol alone (10 μl /mouse) i.p. on the same days. Survival was monitored for 30 days.

Treating mice exposed to LPS. To test the ability of gemfibrozil to affect mortality in a standard model of severe systemic inflammation, 30 mg/kg lipopolysaccharide (LPS; from *Escherichia coli* serotype O111:B4; Sigma) was administered i.p. to mice. A single 60 mg/kg dose of gemfibrozil dissolved in propylene glycol was administered i.p. to mice 1 h before or 2 h after LPS. Control mice were given propylene glycol alone. Mice were monitored for 14 days.

Statistics. The effect of gemfibrozil on survival time was tested using the log rank test, and the Cox proportional hazards model was used to estimate hazard ratios (9). R (21) was used for these analyses.

RESULTS

Untreated influenza virus infection. Mice infected with influenza virus began losing weight soon after infection and by day 4 had lost about 15% of their preinfection body weight (Table 1, see the 0-mg/kg gemfibrozil group). Physical signs of illness continued to worsen, with ruffled fur and lethargy becoming apparent on day 5. Deaths began occurring on day 6 but peaked on days 7 and 8, at which time mice had lost about 30% of their body weight, appeared very ruffled and lethargic, and were cool to the touch. By day 10 surviving mice began to

TABLE 1. Weight loss in mice infected with influenza virus (A/Japan/305/57) given gemfibrozil once daily from days 4 to 10 after virus exposure

Gemfibrozil dose (mg/kg/day)	No. of mice that survived/total	Avg % day 0 body wt \pm SD on indicated day p.i. (no. of mice remaining on that day)				
		4	6	8	10	12
0	1/9	86.8 \pm 4.0 (9)	76.0 \pm 3.4 (6)	68.9 \pm 3.7 (3)	69.1 \pm 8.5 (2)	71.1 \pm 12.7 (2) ^a
20	3/10	87.9 \pm 6.3 (10)	77.5 \pm 6.0 (8)	75.1 \pm 3.6 (5)	75.2 \pm 7.7 (4)	79.0 \pm 13.1 (4) ^b
40	4/9	87.0 \pm 5.3 (9)	75.6 \pm 5.3 (9)	72.2 \pm 12.8 (6)	73.4 \pm 16.2 (5)	77.1 \pm 17.2 (4)
60	5/10	86.5 \pm 4.6 (10)	77.1 \pm 4.5 (9)	73.2 \pm 9.3 (7)	78.0 \pm 11.9 (5)	81.8 \pm 14.4 (5)

^a After day 12 there was one more death, on day 17.

^b After day 12 there was one more death, on day 14.

gain weight. Mice had regained their original weight by the end of the 30-day monitoring period.

Pilot study with gemfibrozil. Gemfibrozil, given on days 4 to 10 after infection, apparently alleviated weight loss (Table 1) and increased survival of mice with influenza (Fig. 1), from 11% survival in vehicle-treated mice to 30%, 44%, and 50% in mice treated with 20 mg/kg, 40 mg/kg, and 60 mg/kg gemfibrozil, respectively. These data appear to reflect a trend of improved survival with increased dose, but the small numbers used in this pilot study precluded statistical analysis. We used the highest of these doses for further study, since it appeared to be well tolerated and conferred the greatest survival advantage.

Influenza treatment with gemfibrozil at a dose of 60 mg/kg. Gemfibrozil treatment increased survival in influenza virus-infected mice from 26% ($n = 50$) to 52% ($n = 46$) (Fig. 2). There was a highly significant (log rank test, $P = 0.0026$) estimated hazard reduction of 0.46 (95% confidence interval, 0.26–0.76). The median survival time was 8 days for vehicle-treated mice but could not be determined for gemfibrozil-treated mice, since more

than half survived (Table 2). Gemfibrozil also appeared to decrease the body weight loss in these infected mice (Table 2).

Prevention of experimental systemic inflammatory illness with gemfibrozil. Gemfibrozil was also tested in mice treated with LPS, which generates a severe systemic inflammatory illness. Administration of a single 60-mg/kg dose of this drug 1 h prior to LPS increased survival from 0% in vehicle-treated mice ($n = 20$) to 25% in gemfibrozil-treated mice ($n = 20$) (log rank test, $P < 0.001$) (Fig. 3), a result which was maintained for the 14 days of monitoring. A single dose of 60 mg/kg gemfibrozil given 2 h after LPS changed survival from 0% ($n = 20$) to 10% ($n = 20$) (Fig. 3), although this difference was not significant ($P = 0.098$).

DISCUSSION

We have shown that gemfibrozil, when administered i.p. on days 4 to 10 after exposure to virus, after the onset of illness, significantly increased survival in mice with severe influenza. Gemfibrozil belongs to the fibrate family of drugs, which were discovered to lower plasma lipids and cholesterol 40 years ago (18) and now have widespread use as a treatment for hypertriglyceridemia. They are one of several known synthetic ligands to the peroxisome proliferator-activated receptors (PPARs), known as PPAR- α agonists (2).

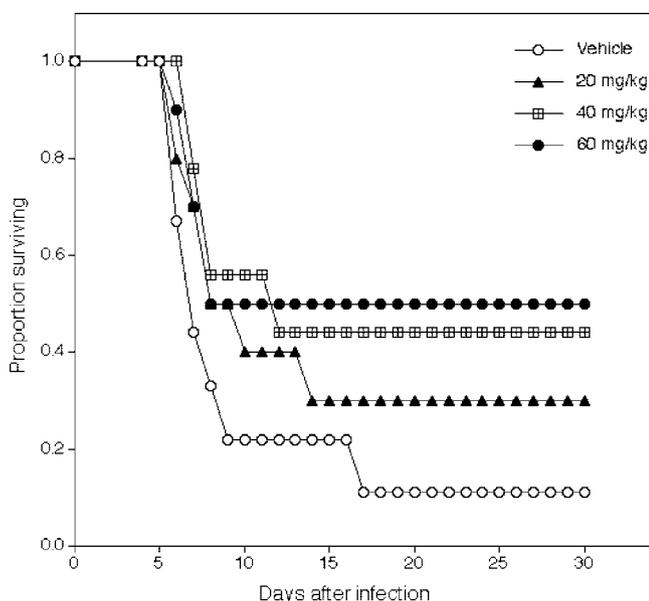


FIG. 1. Effect of gemfibrozil on survival of BALB/c mice with severe influenza. Mice were infected with influenza virus A/Japan/305/57 and treated with vehicle or 20 mg/kg, 40 mg/kg, or 60 mg/kg gemfibrozil once daily from days 4 to 10 after virus exposure. These results are from a single experiment (vehicle, $n = 9$; 20 mg/kg, $n = 10$; 40 mg/kg, $n = 9$; 60 mg/kg, $n = 10$).

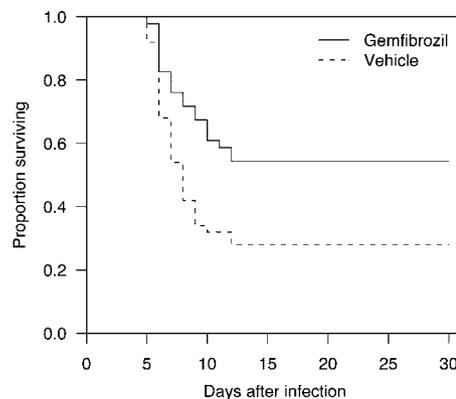


FIG. 2. Effect of 60 mg/kg gemfibrozil on survival of BALB/c mice with severe influenza. Mice were infected with influenza virus A/Japan/305/57 and treated with vehicle or 60 mg/kg gemfibrozil once daily from days 4 to 10 after virus exposure. These results are pooled from four separate experiments (vehicle, $n = 50$; gemfibrozil, $n = 46$). Statistical analysis using the log rank test determined the increase in survival time with gemfibrozil treatment to be significant ($P = 0.0026$).

TABLE 2. Weight loss in mice infected with influenza virus (A/Japan/305/57) given 60 mg/kg gemfibrozil once daily from days 4 to 10 after virus exposure

Gemfibrozil dose (mg/kg/day)	No. of mice that survived/total	Median survival time (days)	95% confidence interval (days)	Avg % day 0 body wt ± SD on indicated day p.i. (no. of mice remaining on that day)			
				6	8	10	12
0	13/50	8	7–10	76.1 ± 4.2 (47)	70.3 ± 3.8 (29)	68.3 ± 8.7 (19)	70.8 ± 13.0 (16) ^b
60	24/46	NA ^a	10+	76.7 ± 5.2 (45)	71.1 ± 7.1 (36)	71.7 ± 11.1 (30)	77.0 ± 13.4 (25) ^c

^a NA, median survival time cannot be reported, as more than half the mice survived the experiment.

^b After day 12 there were three more deaths, two on day 13 and one on day 17.

^c After day 12 there was one more death, on day 13.

These agents have recently been shown to have an additional activity. They reduce inflammation at its source, decreasing serum levels of tumor necrosis factor (TNF) and gamma interferon (17), as well as interleukin-6 (IL-6) (24) in patients with atherosclerosis, with more recent studies demonstrating that lower serum TNF is a reflection of reduced secretion of TNF from peripheral blood mononuclear cells (28) and adipocytes (27). They also promote production of the anti-inflammatory cytokine IL-4 and protect against experimental autoimmune encephalitis, the animal model of multiple sclerosis (16). In the atherosclerosis of experimental diabetes, fibrates reduced superoxide production and expression of nuclear factor κB (NF-κB), which regulates inflammation (4). Further, LPS-stimulated astrocyte production of TNF, IL-1β, IL-6, and nitric oxide was inhibited by fibrates (25).

In recent years, the threat of H5N1 influenza virus infection has generated increased interest in the mechanism of illness and fatality in influenza disease. Nearly 20 years ago (7) it was proposed, because of its clinical similarities to malaria, that influenza disease arose from excessive systemic release of proinflammatory cytokines. Gradually the idea took root for various influenza virus subtypes (19), particularly for more pathogenic strains. For example, H5N1/97 viruses induce much higher transcription of the TNF gene than do H3N2 or H1N1 viruses (5), TNF-related ap-

optosis-inducing ligand and TNF mRNA are up-regulated in human monocyte-derived macrophages infected with H5N1/97 virus (29), and high levels of inflammatory cytokines and chemokines are associated with a fatal outcome (10). Moreover, a reconstructed version of the strain of influenza virus responsible for massive human mortality in 1918–1919 has recently been reported to induce a strong proinflammatory cytokine response, including TNF, during the fatal infections it causes in mice (13) and monkeys (14).

Although we used an influenza virus subtype that is no longer circulating, the H2N2 influenza virus subtype caused a pandemic in 1957 with clinical manifestations similar to those of the 1968 (H3N2) pandemic. Further, human H2N2 infections are clinically indistinguishable in the early phase of infection from those caused by the prevailing human influenza virus subtypes H3N2 and H1N1 (26). In this sense, the clinical similarity between H2N2 and H3N2 infections makes our model relevant to the current circulating H3N2 influenza virus subtypes and thus relevant to the treatment of severe seasonal influenza. With the threat of an H5N1 influenza pandemic, finding novel ways to reduce mortality in pathogenic avian influenza is a priority. While our model might also be relevant to avian influenza, since the early phase of human H2N2 infections is also clinically indistinguishable from early-phase H5N1 infection (26), we have plans to extend the findings of this study to determine if gemfibrozil also decreases mortality in H5N1 influenza virus infections in mice.

Although gemfibrozil is as yet untried in human influenza, it could have two major advantages. First, it is already approved for daily human use, albeit by a different route, to lower plasma lipids and cholesterol (18, 23). Second, from our data, enhanced survival did not depend on giving gemfibrozil before the onset of illness, since treatment begun 4 days after exposure to virus, when mice were already sick and had lost weight, was effective. This implies that gemfibrozil has the potential to be a treatment rather than a preventative in human disease, allowing limited stocks of the drug to be focused where required in a pandemic. The idea that severe systemic inflammatory disease arises through overproduction of proinflammatory cytokines in influenza (6, 8) now also has general acceptance. This made it attractive to test the effects of gemfibrozil on influenza, a disease acknowledged to operate through these cytokines. No fibrate appears to have been previously tested against an infectious disease.

Using anti-inflammatory agents against influenza is a recent suggestion, with Fedson proposing statins as a prophylaxis and treatment for an influenza pandemic (11, 12). While the con-

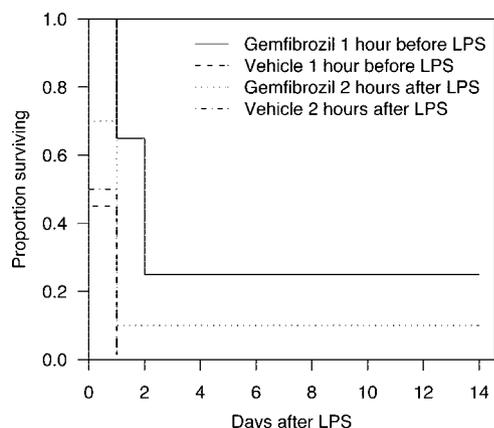


FIG. 3. Effect of 60 mg/kg gemfibrozil on survival of BALB/c mice with severe systemic inflammation. Mice were given 30 mg/kg LPS (*E. coli* serotype O111:B4) and treated with a single dose of vehicle or 60 mg/kg gemfibrozil either 1 h before or 2 h after LPS injection. These results are from a single experiment; *n* = 20 for each group. Statistical analysis using the log rank test determined that a single dose of 60 mg/kg gemfibrozil 1 h before LPS significantly increased survival (*P* < 0.001).

cept has been gaining favor (20), no direct data are as yet available. Since the aim of this study was to find an agent useful in animals already sick from influenza, our initial trials included simvastatin at the human daily maintenance dose for lowering blood lipids. Unlike gemfibrozil, simvastatin had a negligible effect on sick animals under these conditions. Nevertheless, the use of statins in influenza, including as prophylactic agents, warrants closer examination, since the epidemiological data that support the protective effects of statins arose from sepsis patients who were already taking these drugs at the time they became ill (1).

We also found that gemfibrozil gave some protection, in survival terms, against the severe systemic inflammatory illness that results from administering LPS (Fig. 3). This suggests that it could also protect against other similar inflammatory conditions as well as influenza. In addition, activity against LPS implies that at least a major effect of gemfibrozil against influenza is to inhibit inflammatory cytokines, not the virus. Nevertheless, the mechanisms by which gemfibrozil exerts the effects we have observed are yet to be elucidated. Our next priorities are to examine whether inflammatory cytokine production is inhibited and to determine if gemfibrozil has any direct antiviral action.

Although the published mouse 50% lethal dose of gemfibrozil is 3,162 mg/kg for a single orally administered dose, and 300 mg/kg/day of gemfibrozil, given for 3 or 12 months, is well tolerated in rats (15), we are wary of extrapolating this to pathogen-infected mice without further study. Also, it is yet to be ascertained how outcomes of oral and i.p. dosages compare. As noted elsewhere (3), gemfibrozil reduces the production of inflammatory cytokines, molecules that are involved in the host response against pathogens as well as disease pathophysiology (6). The literature on the effects of anti-TNF agents in rheumatoid arthritis provides an example of this useful and harmful duality of inflammatory cytokines (22). This possibility in influenza will be examined in several ways, including further parallel studies using gemfibrozil against influenza and LPS toxicity. The LPS model is useful because it involves no cytokine-susceptible infectious agent and causes pathology only through excessive cytokine production.

In summary, if these results translate to human infections, gemfibrozil may prove to be a readily testable and useful treatment for influenza, both in high-risk individuals with the currently circulating influenza virus strain and in any pandemic resulting from an antigenic shift in a subtype, including the current avian H5N1 influenza virus.

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