SPECIAL ARTICLE

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Preclinical Development of Low Toxicity Drugs

Focus on Zanamivir, an Anti-Influenza Drug

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Contents

Abstract

Developing novel compounds with low toxicity may present more difficulties for pharmaceutical companies than developing compounds with known class-related effects. The absence of clearly identified toxicity may be a consequence either of an inadequate or poorly designed toxicity programme or of the very low toxicity of the novel compound. To enable an informed risk assessment to be undertaken prior to registration, regulatory authorities must satisfy themselves that all efforts to fully evaluate the toxicity profile of a novel compound have been made.

Zanamivir is a novel antiviral agent developed for the treatment and prevention of influenza when administered by the oral inhaled route. The toxicology programme for zanamivir was designed to support both a short term treatment indication for patients clinically diagnosed with influenza and a longer term treatment indication for the prevention of influenza. The toxicology studies demonstrated that zanamivir has very low toxicity and no drug-specific toxicities were observed in animal toxicity studies. Systemic plasma concentrations 1336-fold those achieved in clinical use were not associated with significant adverse effects.

In the absence of dose-limiting toxicity in animal studies and in an attempt to identify target-organ toxicity, the high dosage level in all repeat dose studies was selected to be the maximum practicable. In the rat, nonspecific effects were seen

in the respiratory tract following long term inhaled administration and in the kidneys following continuous infusion. However, these nonspecific effects were consequences of the excessive dosages administered and are not related specifically to zanamivir; thus, they are without relevance to the clinical use of this agent.

Drug development programmes are designed to answer the following questions:

- Does the drug have a significant clinical effect on the disease?
- Is the safety profile of the drug compatible with its use in the management of that disease?

An integral part of this process is to ensure the safety of pharmaceuticals for use in humans. For potential new medicines this aspect of development is conducted within a comprehensive package of animal toxicity studies. The aim of these studies is to identify toxic reactions of the potential drug, at dosages in excess of those likely to be used clinically, to allow a risk-benefit assessment to be performed prior to use in humans and to establish an estimate of the safety margin.

Toxicology studies fall into 1 of 3 main categories: genetic toxicology, general toxicology and reproductive toxicology. Data from all of these categories, along with detailed knowledge of the compound's pharmacokinetic and pharmacological profile, are assessed to provide a complete evaluation of its preclinical safety.

Genetic toxicology studies aim to identify any mutagenic or clastogenic potential of a new compound, as a predictor of potential human carcinogenicity, using a variety of *in vitro* and *in vivo* screens. For the majority of new pharmaceutical agents a positive result in any of these screens results in the immediate termination of development of the compound before further more costly studies are initiated.

General toxicology studies look at adverse reactions involving the major organ systems of the body following single and repeated administration of the new compound. These studies aim to mimic the use of the new agent in humans and hence, wherever possible, the compounds are administered to animals using the same route and form as intended for use clinically.

Reproductive toxicology assesses any effects on general reproductive performance of the male and female parents and on the developmental and reproductive capacity of their offspring.

The basic requirements for these types of studies are similar throughout the world. Regulatory guidelines for the development of pharmaceutical agents for human use have been published since the 1930s, primarily in the US after deaths occurred in that country following administration of antibacterials containing toxic solvents. However, it was only after the thalidomide disaster in the 1960s that most European countries also adopted legislation and passed acts of parliament defining requirements for the development of new medicines. Although the type of data required in different countries to meet these requirements is similar, there are several areas where individual regulatory authorities have different views on what specific endpoints they consider suitable to meet their criteria for adequately assessing the potential toxicity of any new medicine. Therefore, an attempt to gain worldwide acceptance of a new drug has often obliged pharmaceutical companies to perform specific tests, just to fulfil the demands of an individual regulatory authority. Recent initiatives between the major regulatory authorities and the pharmaceutical industry to discuss the main areas of disagreement at the International Conference on Harmonisation (ICH) have resulted in many of those individual differences being resolved.

Most new drugs developed in the 1950s and 1960s were small chemical molecules designed to act at a target receptor associated with a particular disease state. These receptors were often ubiquitous throughout the body so, as a consequence, activation or deactivation of these receptors was fre-

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quently associated with many adverse effects. The significantly increased scientific knowledge of the last few decades, and in particular the increasing role of genetics and biomolecular structure in drug development, has resulted in the development of more highly selective compounds for medicinal use, and also in the use of proteins and other biological compounds for the treatment of disease. Therefore, classical toxicity programmes set out in the regulatory guidelines may not be relevant to an assessment of the preclinical safety of many of these novel medicines. Consequently, more and more often the preclinical packages supporting the use of these types of compounds are agreed on a case-by-case basis, following discussion between the individual regulators and pharmaceutical companies.

The most valid data for risk assessment prior to registration of new drugs are derived from significant human use. However, for any new drug these data are limited prior to registration. Therefore, an assessment is often based on the toxicity seen in experimental animals extrapolated to the proposed human situation. Animal studies do have limitations in predicting some types of clinical adverse events, in particular those involving alterations in psychological or behavioural response. However, experience has shown that classical pathological lesions observed in animal studies are predictive for similar adverse effects in the clinical situation. Without any identified target-organ toxicity in animal studies, it is difficult to predict possible adverse effects in human use. Therefore, regulatory guidelines have a clear requirement to identify target-organ toxicities in experimental animals, or to demonstrate that all possible efforts have been made to fully evaluate the compound.

The aim of this article is to discuss the programme of toxicology work conducted to support the clinical use of a very low toxicity compound, and to highlight some areas where achieving the requirements of the regulatory guidelines required modifications to the extensive preclinical investigations.

1. Pharmacology of Zanamivir

Zanamivir is a novel antiviral agent in late-phase clinical development indicated for the treatment and prevention of influenza. The compound exerts its pharmacological action by potent and selective inhibition of the influenza viral surface neuraminidase thereby preventing budding of the viral progeny and halting the spread of infection.[1] Zanamivir was designed using a highly specific computer-modelling rationale and is extremely potent as an inhibitor of influenza A and B virus neuraminidases.[2-4] Neuraminidases are found in a wide variety of organisms. Within mammalian cells they have been shown to play an important role in a number of metabolic processes.[5] However, in contrast to its potent inhibitory activity against of influenza neuraminidase, zanamivir is a very weak inhibitor of the human lysosomal neuraminidase with 50% inhibitory concentration (IC_{50}) values in the range of 10^5 to 10^6 higher than its activity against the influenza virus neuraminidase.[2]

Influenza in the general population is a self-limiting disease with a duration typically between 6 and 10 days. However, in epidemic years the excess mortality due to influenza is reported by the US Centers for Disease Control to be approximately 10 000,[6] with certain groups, in particular the elderly and those with underlying chronic disease, being at higher risk.[7-11] Therefore, the treatment and prevention of influenza is likely to require differing durations of drug administration, the exact duration being dependent on the clinical setting. Published data have shown that a 5-day course of zanamivir, administered at a dosage of 10mg twice daily, to be well tolerated and efficacious in the treatment of acute influenza.[12] Prevention during epidemic years may require prolonged administration to certain populations. Therefore, administration may continue for as long as the threat of infection remains.

Since zanamivir exerts its antiviral activity by the extracellular inhibition of the influenza virus neuraminidase within the respiratory tract, administration via the topical route has several advan-

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tages for treatment of influenza. Topical administration to the respiratory tract allows a high concentration of the drug to be delivered to the site of infection without the need for high systemic exposure, thereby reducing the likelihood of adverse effects. Zanamivir is a highly polar molecule and therefore its penetration into cell membranes is poor, with a volume of distribution representing that of extracellular water.[13] However, studies in volunteers have demonstrated the presence of zanamivir in the lungs for prolonged periods after administration. In addition, because of its highly polar nature, any of the drug that does enter the systemic circulation is efficiently cleared through the kidneys in humans and animals, resulting in a short systemic half-life.[13]

2. Toxicology Programme

The toxicology programme for zanamivir was designed to support repeated administration to the respiratory tract. In general, toxicology studies are conducted using the planned clinical route of administration, in the species considered to be most similar to humans with respect to their pharmacokinetics. For zanamivir there was very good correlation between the way the experimental species and humans handled the drug. The Wistar rat and beagle dog were selected for the majority of toxicology studies on the basis of extensive in-house knowledge of these species. The animal work conducted to support the use of zanamivir complied with national legislation and with related codes of practice.

3. Route of Administration

The inhaled route was used for the majority of toxicity studies. Exposure by the inhaled route allowed systemic exposure via the lungs and an assessment of the local tolerability of the respiratory tract. In order to ensure that during clinical trials the distribution of zanamivir to the upper and lower respiratory tract could be assessed, toxicity studies were designed to ensure adequate exposure of the whole respiratory tract.

Rodents are obligate nasal breathers and hence inhaled administration results in significant exposure of the nose and the lower respiratory tract to the inhaled agent.[14] In the dog, where preliminary investigations showed that administration of powder aerosol from a facemask did not give extensive exposure to the nasal passages, spray packs and drops were used in addition to inhaled dosing to administer zanamivir directly to the nose.

Clinical use suggests that the amount of zanamivir within the systemic circulation is likely to be low. However, in order to fully assess the toxicity of zanamivir, the studies conducted were designed to maximise systemic exposure. Intravenous administration allows high concentrations of drug to be given to the animals to fully evaluate any unwanted toxicity. Hence, in order to fully examine the target-organ toxicity of zanamivir, studies were conducted using this route of exposure. In addition, the full reproductive package was performed using the intravenous route to ensure exposure of the pregnant dams and fetuses. The effects of zanamivir on fertility, general reproductive performance and pre- and post-natal development were investigated in the rat. The effects on embryo/fetal development were assessed in the rat and the New Zealand White rabbit.

4. Dosage Selection

Preliminary studies with zanamivir demonstrated that it had low potential for irritancy to the respiratory tract and low systemic toxicity. Therefore, in the absence of dose-limiting toxicity, the high dosage group in all main studies with zanamivir was set as the maximum practicable.

5. Inhaled Administration

In inhalation studies, the dosage is a consequence of the concentration of drug in the exposure chamber and the duration of exposure. Therefore, in the absence of dose-limiting toxicity, the maximum achievable aerosol concentration that could be generated from a dry powder formulation, using current technology and prolonged exposure times, was selected.

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Administration of aerosols to rodents during toxicology studies is generally done using snout-only inhalation chambers. The aerosolised drug is passed into a cylindrical chamber around which the rodents sit in restraint tubes, with their snouts projecting into the airstream. As the aerosol is drawn through the chamber at a slight vacuum the rodents breath from the drug-laden atmosphere. Hence in these types of studies the animals essentially dose themselves and the exact dose each animal receives is a consequence of the concentration of drug in the atmosphere and its individual respiratory pattern.

Dog inhalation systems work on a similar principle. Aerosol is passed from a conditioning chamber and to an individual facemask from which the dog breathes.

6. Study Design

Zanamivir has been administered by inhalation to B6C3F1 mice for up to 13 weeks, to Wistar rats for up to 26 weeks, and to beagle dogs for up to 52 weeks. In addition, 2 lifetime inhaled oncogenicity studies were conducted in the rat and mouse to support the prophylaxis indication.

With the exception of the preliminary studies, where a nebulised solution was used, inhalation toxicology studies used a dry powder blend of zanamivir with a lactose carrier. In order to maximise the aerosol concentration of the drug substance within the exposure chambers, the toxicology studies used a powder blend that had a higher drug ratio than that used for clinical studies. All definitive studies included an additional lactose vehicle control group to assess any nonspecific effects of delivering particulate matter to the lung, even though lactose is a commonly used carrier for inhaled products and wide clinical experience indicates no toxicity associated with its use.

7. Oncogenicity Studies

Studies to assess the potential for tumour induction are required for the majority of novel drugs. They are mandatory where any of the following conditions apply: the compound has shown a positive result in one or more of the genetic toxicology screens; the class of compound is one where there is a reason for concern, or the compound is the first of a novel class; or the drug is likely to be used over a long period in humans. Zanamivir will be administered both for a short time to treat influenza and intermittently for the prevention of influenza. In this situation it is unlikely that the drug will be used for long periods in humans. Zanamivir was not found to be mutagenic in any of the *in vitro* or *in vivo* systems used to detect genetic hazard or clastogenic effect. Thus, for a treatment indication only, it was considered that oncogenicity studies would not be required.

However, for a prophylactic indication there is always the possibility that healthy patients receiving the drug may never have gone on to develop the disease. In this situation the risk-benefit assessment is more difficult, and administration of any compound that may not give clinical benefit requires an additional element of safety. For this reason, 2 lifetime inhaled oncogenicity studies in the rat and mouse were conducted with zanamivir.

Traditionally, the standard method for defining the high dosage in oncogenicity studies has been based on use of the maximum tolerated dose (MTD). This is usually taken to be the dose which is predicted to produce a minimum toxic effect over the course of the oncogenicity study. However, for low toxicity compounds such as zanamivir, use of the MTD would result in administration of very large doses in the oncogenicity studies that have no relevance to the clinical use of the compound. Therefore, following the ICH initiative, guidelines were published which proposed that any one of several approaches may be more appropriate. These include toxicity-based end-points, saturation of absorption, pharmacodynamic end-points, maximum feasible dosage and other end-points that could be set on a case-by-case basis.

In the absence of toxicity, the high dosage selected for use in the zanamivir oncogenicity studies was that achieved following exposure to the maximum achievable aerosol concentration. Rats and mice were exposed for 1 (mice) or 2 (rats) hours

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Table I. Achieved dosages and plasma exposure levels from the long term inhalation toxicity studies

Species	Study duration (wks)	No toxic-effect exposure concentration (mg/L)	No toxic-effect dosage level (mg/kg/day)	Overage from clinical exposure[a]
Rat	26	1.53	44.5	21
Rat	104	0.943	53.1	20
Mouse	104	1.86	105	17
Dog	26	1.36	10.5	39
Dog	52	1.45	11.2	40

a Calculated by comparison of area under the curve data derived from end of study data at 'no toxic-effect' dose level in animal studies with daily exposure in human participants using the clinical dosage (10mg twice daily).

daily for 104 weeks. In the rat study, the duration of exposure was increased from 1 to 2 hours daily during week 16 so that systemic exposure was increased in order to comply with the requirements of some regulatory authorities. However, concerns with animal welfare limited the duration of exposure in the mouse.

8. Results

Zanamivir was very well tolerated by all species at all dosages. No treatment-related clinically relevant effects were observed in any study and there were no deaths seen that were attributed to zanamivir administration. Achieved dosages are given in table I.

Long term administration of zanamivir was associated with reduced bodyweight gain in male rats and in male and female dogs at the highest dosages of 44.5 mg/kg/day and 11.2 mg/kg/day, respectively.

8.1 Pathological Changes

Minor changes were observed pathologically in the nasal passages, trachea and lung following long term administration of zanamivir. All of the changes were exacerbations of background pathological lesions and were considered to be a consequence of prolonged inhalation exposure to high aerosol concentrations.

Increased incidences of eosinophilic droplets (inclusions) were observed in the nasal passages of female mice exposed to zanamivir for 13 weeks. Similar findings were observed in rats and mice following 104 weeks' administration. Inclusions were seen in both the respiratory epithelium and the sustentacular cells of the olfactory epithelium in both species, and despite the great sensitivity of the rat there were no degenerative or inflammatory changes observed in the nasal cavity. Vehicle control animals exposed to lactose concentrations intermediate between those observed in the air control and the zanamivir-treated groups showed a corresponding incidence of nasal inclusions.

Eosinophilic droplets are present in the nasal epithelium as a background feature at low to moderate incidence in the strains of mouse and rat used in the zanamivir toxicology programme. Increases in severity and incidence of these have been described for a number of unrelated compounds administered by the inhalation route to rodents.[15,16] The exact nature of the eosinophilic material is unknown, but electron microscopy has shown the material to be contained within endoplasmic reticulum, and an increase in this material is thought to be a nonspecific defence response.

In the lung, increased numbers of enlarged, diffusely distributed alveolar macrophages were seen in the alveoli of a small number of rats exposed long term to zanamivir. In the 26-week study this effect was only seen at the highest dosage of 44.5 mg/kg/day. In the oncogenicity study this effect was seen in the intermediate and high dosage groups.

In the literature, administration of high concentrations of low toxicity particulate matter to the lung is reported to be associated with a condition of 'lung overload'.[17] This term refers to the situation where respirable, poorly soluble particles are

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Table II. Mean lung zanamivir concentrations in the high dosage group in repeat dose inhalation studies

Species	Study duration	Exposure concentration (mg/L)	Dosage of zanamivir (mg) per m^2 of lung surface[a]	Overage from clinical dose[b]
Rat	26 wks	1.53	34.6	119
Rat	104 wks	0.943	57.2	197
Mouse	104 wks	1.86	54.4	187
Dog	26 wks	1.36	2.26	8
Dog	52 wks	1.46	2.26	8
Human[c]	5 days	NA	0.29	NA

a Data were calculated from the following values for lung surface area: mouse lung = $0.068m^2$;[14] rat lung = $0.39m^2$;[14] human lung = $70m^2$;[14] dog lung = $51m^2$.[18]

b Calculated by comparison of area under the curve data derived from end of study data at 'no toxic-effect' dose level in animal studies with daily exposure in human participants using the clinical dosage (10mg twice daily).

c Two 10mg doses twice daily to 50kg person.

NA = not applicable.

deposited in the lung at a rate exceeding the rate at which alveolar macrophages can clear particles from the pulmonary region. Pathological sequelae may include increased numbers or accumulation of particle-filled macrophages in the alveoli and interstitium and, in certain circumstances, progression to chronic inflammation, fibrosis and hyperplasia.[17] Alveolar macrophages are the primary mechanism for the clearance of particulate matter from the lungs and their increased prominence is merely a reflection of this function.

The concentrations of particulate material administered to the lung in terms of milligrams of zanamivir per m^2 for the main inhalation studies have been calculated and are shown in table II.

The minor effects on the nasal passage and lungs were considered to be due to excessive amounts of zanamivir in these regions and as such have no relevance for the clinical use of zanamivir.

However, the risk with these types of lesion caused by excessive particle administration is that subtle drug-induced effects in the tissues may be masked. Therefore, the need to push the top dose to the maximum to fulfil regulatory requirements must be balanced with the scientific consequences of excessive particle administration.

8.2 Intravenous Administration

Parenteral administration requires the test compound to be formulated in a vehicle suitable for administration directly to the systemic circulation. In most situations, simple solutions in water (for injection) or saline are the preferred vehicles as they are without effect to the animal at the amounts administered.

The maximum intravenous dose is limited by the maximum solution concentration of the compound under test and maximum volumes that can be repeatedly administered to animals.

The clinical route for zanamivir is inhaled, so it was considered necessary to perform only general

Table III. Plasma zanamivir exposure at the no toxic-effect dose level in repeat dose toxicity studies

Species	Route	Study duration (days)	No toxic-effect dosage level (mg/kg/day)	Overage from clinical exposure[a]
Rat	Intravenous	35	90	345
Dog	Intravenous	35	36	259
Human	Inhaled	5	20[b]	NA

a Calculated by comparison of area under the curve data derived from end of study data at 'no toxic-effect' dose level in animal studies with daily exposure in human participants using the clinical dosage (10mg twice daily).

b Total daily dosage.

NA = not applicable.

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toxicology studies for only 1 month using the intravenous route in the rat and dog. However, reproductive studies were performed using the intravenous route to ensure exposure of the fetuses. In these studies, the maximum dosage of zanamivir was limited to 90 mg/kg/day because of the aqueous solubility of zanamivir. However, at this dosage zanamivir was not teratogenic to rat and rabbit fetuses and had no adverse effects on fertility, reproductive performance, or the physical development of offspring in the rat.

The dosages in the 1-month studies in the rat and dog were sufficiently high from a bolus dose to give plasma exposures of 345 and 259 times those proposed from clinical use in the rat and dog, respectively. However, in neither study was any toxicity observed and the details are shown in table III.

Systemic exposure to zanamivir was limited in the inhalation studies by the relatively low inhaled bioavailability. The half-life of zanamivir following intravenous administration is approximately 15 minutes. Therefore, it was possible that the lack of target-organ toxicity in these studies was a consequence of its rapid clearance.[15]

Estimates of clinical safety are usually based on the difference in systemic exposure at the no-effect level in animal studies and that obtained following clinical use. This difference is expressed as a safety margin or overage from clinical use and is used in the risk benefit assessment. Zanamivir will be used for a short duration, with intermittent use over the influenza season. Thus, the exposure overages in the range achieved in the toxicology studies should provide reassurance that its use would be well tolerated in humans, despite a lack of identified target-organ toxicity.

However, discussions with several regulatory authorities identified unease at the lack of target organ toxicity for a compound with a novel pharmacological action. Therefore, to significantly increase the systemic exposure and hence the opportunity for toxicity, intravenous infusion studies were initiated in the rat and dog. In the dog, slow intravenous administration enabled systemic exposures equivalent to 552 times that proposed for human use to be achieved. No target-organ toxicity was observed following 14 days' administration. For ethical reasons no further work was conducted in the dog. Instead, a rat continuous infusion system was selected to further explore the systemic toxicity. A 2-phase study was conducted, with an initial 48-hour dosing phase to identify the MTD, followed by a 14-day continuous dosing phase. Selected animals were retained following 14 days' administration to assess reversibility of any induced toxicity.

Table IV. Dosages of zanamivir administered by continuous infusion to the rat

Infusion volume (ml/kg/h)	Solution concentration (mg/ml)	Achieved dosage (mg/kg/day)
2	18	864
4	18	1 728
8	18	3 456
16	18	6 912
32	18	13 824

Rats were cannulated with an internal catheter through which zanamivir solution was administered. The dose was varied by infusion of increasing volumes of the maximum practicable concentration of zanamivir. Matched saline controls were used to assess the effects of fluid overload. Dosages and volumes administered are shown in table IV.

Dosages of 13 824 mg/kg/day were administered to rats for 48 hours without adverse clinical signs. The infusion rates necessary to achieve these high dosages were associated with minor disturbances in fluid balance, characterised by increased urinary volume with corresponding reductions in specific gravity and water consumption. Therefore, to prevent these types of nonspecific effects following longer term administration, lower infusion rates were selected.

Administration of dosages >864 mg/kg/day were associated with changes in the kidneys characterised by vacuolation of the proximal tubules. The severity was dose related and reversible upon withdrawal. These types of changes have been previously documented following administration of

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hypotonic solutions such as dextrose and mannitol. These situations are considered to be nonspecific changes due to changes in osmolarity.[19] In this study, isotonic solutions were administered but the effects on the rat kidney of handling such extreme volumes of fluid and a renally excreted compound are difficult to assess. In the absence of similar findings in the saline-only animals, the kidney effects were attributed to zanamivir administration, but it is possible that the increased workload required to clear both the fluid volume and the compound may have contributed to this lesion.

9. Conclusion

The preclinical development of zanamivir has been designed to maximise exposure in all toxicology species in an attempt to demonstrate target-organ toxicity. Despite conducting very extensive preclinical work by the intravenous and inhaled routes of administration, no significant toxicity has been observed in any species. Therefore, whilst every effort to comply with regulatory requirements to demonstrate toxicity was made, it was not possible to demonstrate clear target-organ toxicity with zanamivir due to its inherent low toxicity. The comprehensive toxicology programme conducted to support the clinical use of zanamivir has demonstrated that it is very well tolerated following repeated administration and no clinically relevant effects have been seen in animal species. The no effect levels show high margins of safety over the maximum clinical exposure. Furthermore, the very low order of toxicity in animals has been substantiated in humans where a very low level of adverse events has been noted to date. Therefore, in these situations, both pharmaceutical companies and regulatory authorities should carefully consider the need for extensive animal studies that are unlikely to generate clinically relevant data.

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