Development of a bioartificial liver employing xenogeneic hepatocytes

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Abstract

Liver failure is a major cause of mortality. A bioartificial liver (BAL) employing isolated hepatocytes can potentially provide temporary support for liver failure patients. We have developed a bioartificial liver by entrapping hepatocytes in collagen loaded in the luminal side of a hollow fiber bioreactor. In the first phase of development, liver-specific metabolic activities of biosynthesis, biotransformation and conjugation were demonstrated. Subsequently anhepatic rabbits were used to show that rat hepatocytes continued to function after the BAL was linked to the test animal. For scale-up studies, a canine liver failure model was developed using D-galactosamine overdose. In order to secure a sufficient number of hepatocytes for large animal treatment, a collagenase perfusion protocol was established for harvesting porcine hepatocytes at high yield and viability. An instrumented bioreactor system, which included dissolved oxygen measurement, pH control, flow rate control, an oxygenator and two hollow fiber bioreactors in series, was used for these studies. An improved survival of dogs treated with the BAL was shown over the controls. In anticipated clinical applications, it is desirable to have the liver-specific activities in the BAL as high as possible. To that end, the possibility of employing hepatocyte spheroids was explored. These self-assembled spheroids formed from monolayer culture exhibited higher liver-specific functions and remained viable longer than hepatocytes in a monolayer. To ease the surface requirement for large-scale preparation of hepatocyte spheroids, we succeeded in inducing spheroid formation in stirred tank bioreactors for both rat and porcine hepatocytes. These spheroids formed in stirred tanks were shown to be morphologically and functionally indistinguishable from those formed from a monolayer. Collagen entrapment of these spheroids resulted in sustaining their liver-specific functions at higher levels even longer than those of spheroids maintained in suspension. For use in the BAL, a mixture of spheroids and dispersed hepatocytes was used to ensure a proper degree of collagen gel contraction. This mixture of spheroids and dispersed cells entrapped in the BAL was shown to sustain the high level of liver-specific functions. The possibility of employing such a BAL for improved clinical performance warrants further investigations.

Introduction

Every year over thirty thousand people die of liver failure in the United States alone (National Center for Health Statistics, 1991). Hepatic failure can be grossly classified as either acute or chronic, based on the time span required for a given clinical agent to produce clinical symptoms and/or perpetuate clinical conditions. It can further be described as being either total or partial, according to the extent of functional impairment. With improvements in immunological and surgical techniques, chronic liver failure can be treat-

ed with orthotopic liver transplantation (Starzl *et al.*, 1989). Although the same approach can be used with acute fulminant hepatic failure (FHF), where hepatic regeneration is neither rapid nor sufficient enough to keep the individual alive, the mortality rates are much higher (Peleman *et al.*, 1987). Availability of donor organs for transplant is still a major problem, and even those who qualify for a liver transplant often die while awaiting an allograft (Busuttil *et al.*, 1987). Organs suitable for transplant in the pediatric group are even more scarce (Vacanti *et al.*, 1987). Many patients in hepatic failure do not qualify for transplantation

because of metastatic cancer, active alcoholism, concomitant infection, or concurrent medical problems. In addition, the concomitant immunosuppressive therapy in patients offers considerable risk. There is a critical need for improved temporary liver support technology for survival of potential transplant recipients and patients with reversible, acute hepatitis excluded from liver transplantation (Takahasi *et al.*, 1991).

The development of an artificial liver has been a significant challenge. Unlike some other organs which have a single dominant physiological function, the liver is a complex metabolic system with multiple functions essential for survival. A variety of techniques for artificial liver support have been attempted for treatment of hepatic failure over the past four decades. These techniques included hemodialysis for the removal of ammonia (Kiley *et al.*, 1956), hemoperfusion using a direct cation exchanger (Dowex 50-X8) (Schlechter *et al.*, 1958), hemoperfusion through albumin coated Amberlite XAD-7 resin (Tom *et al.*, 1979), and hemodialysis across polyacrylonitrile (PAN) membranes (Opolon *et al.*, 1976) for the removal of toxic metabolites which build up in the plasma during liver failure. Coconut charcoal adsorption (Yatzidis, 1964), and microencapsulated charcoal hemoperfusion (Chang, 1972) were demonstrated to reduce hematological side effects associated with earlier hemoperfusion attempts. These and other studies demonstrated the inability of treating liver failure using purely mechanical devices or processes. Adding a biological component to the treatment was thus deemed necessary by most investigators.

The first generation of studies employing a biological component involved attempts including xenogeneic cross-hemodialysis with a healthy dog (Hore *et al.*, 1967), extracorporeal perfusion of homologous isolated livers (Otto *et al.*, 1958), and use of isolated hepatocytes on an extracorporeal circuit (Matsumara, 1973; Eiseman *et al.*, 1976). Limited success was reported in human trials using extracorporeal perfusions with livers from pigs (Eiseman *et al.*, 1965; Norman *et al.*, 1966), dogs (Kimoto, 1959), baboons (Fischer *et al.*, 1981; Lie, 1981), and other animal species (Abouna *et al.*, 1973). Based on the limited success of the previous efforts, the concept evolved that liver functions essential for survival would be best provided by a mammalian liver, or part thereof, allowing for sustained and repetitive application. The advent of extracorporeal perfusion against whole livers (Lie, 1981; Fischer *et al.*, 1981) and liver tissue (Nose *et al.*, 1963), and the use of isolated hepatocytes (Eiseman *et al.*, 1976; Mat-

sumar, 1973) catalyzed the development of systems containing biological components within a synthetic network: hybrid 'bio-artificial' liver systems.

When compared to cellular components and isolated enzyme systems, the bioartificial liver systems provide greater numbers of liver functions since they utilize intact, metabolically active liver cells (Hager *et al.*, 1983). Hepatocyte-based systems are less constrained by mass transfer limitations compared to chunks of liver tissue, and are therefore amenable to scale up (Matsumura *et al.*, 1987). A major advantage of bioartificial livers is that the system can be constructed using a semipermeable material to protect the hepatocytes from the patient's immune system. This allows for therapy through employment of hepatocytes from a different species (xenocytes) within the bioartificial liver without need for severe immunomodulation (Neuzil *et al.*, 1991; Nyberg *et al.*, 1992). The main disadvantages lie in the material-membrane interactions, similar to those encountered in blood contacting devices (Galletti, 1987), and in the maintenance of hepatocyte viability and function at the high cell densities required for clinical applications. Hepatocytes, when cultured on traditional cell culture surfaces, flatten, become agranular and lost their differentiated state and tissue-specific function within one to three days. As a result, improved cell culture techniques are essential for maintaining the functional state of hepatocytes within bioartificial liver systems. Methods to prolong hepatocyte viability and function include addition of growth factors and hormones to the culture medium (Lanford *et al.*, 1987; Dich *et al.*, 1988), cultivation of hepatocytes in the presence of attachment factors and extracellular matrix components (Akaike *et al.*, 1993; Dunn *et al.*, 1989; Dunn *et al.*, 1992; Spray *et al.*, 1987), co-culture with liver-derived nonparenchymal and endothelial cells (Yanamoto *et al.*, 1989; Shimaoka *et al.*, 1987) and cultivation within a polymeric/biologic membrane or gel matrix (Mikos *et al.*, 1993; Cima *et al.*, 1991; Vacanti *et al.*, 1988).

Hepatocyte entrapment BAL

A unique feature of our BAL is that the cells are entrapped within the lumen of the hollow fibers instead of in the extracapillary space (Shatford *et al.*, 1991; Nyberg *et al.*, 1993) (Figure 1). Because of the scarcity of human hepatocytes, xenogeneic hepatocytes were used. For initial studies, rat hepatocytes were harvested using a collagenase perfusion method. Harvested

Figure 1. A schematic diagram of a gel-entrapment hollow fiber bioreactor employing xenogeneic hepatocytes as a bioartificial liver.

hepatocytes were suspended in type I collagen solution at 4 °C, and this hepatocyte-collagen suspension was injected into the hollow fiber lumen. Subsequently, cell culture medium at 37 °C was perfused in the extracapillary space to bring the temperature of the hepatocyte-collagen suspension to 37 °C. Collagen gelation occurred in approximately 15 minutes, effectively entrapping the hepatocytes within a collagen gel. The extracapillary space was continuously perfused with culture medium for the next 24 hours to supply nutrients and oxygen to the hepatocytes. During this period the collagen gel contracted to 60% of its initial diameter. Intraluminal flow was then initiated to supply hepatocytes with the nutrients and growth

factors required to sustain their function, and the bioartificial liver was connected to the test animal in liver failure. The patient's blood was continuously recirculated in the extracapillary space. Toxic components in the patient's blood diffused through the hollow fiber membrane into the luminal space and were metabolized by the entrapped hepatocytes. The detoxified metabolites could either diffuse back into the patient's blood stream or be washed out with the intraluminal stream. A membrane with a molecular weight cut off (MWCO) of 100,000 Daltons was used in the BAL. The membrane excluded the patient's immunoglobulin and complement proteins from the intraluminal space, thus providing immunoprotection for the xenogeneic

Table 1. *In vitro* performance of dispersed cell BALL*

Function	Rat hepatocytes ^a	Porcine hepatocytes ^b
Albumin (pg cell ⁻¹ hr ⁻¹)	0.12 ± 0.01	0.1
Ureagenesis (pg cell ⁻¹ hr ⁻¹)		1.2
MEGX production (pg cell ⁻¹ hr ⁻¹)	0.08 ± 0.04	
Oxygen consumption	0.40 ± 0.03	0.2

* From Ph.D. Thesis, Scott L. Nyberg, University of Minnesota, 1993.

^a Average over 4 days.

^b Average over 9 days.

hepatocytes. However, the membrane allowed free diffusion of serum albumin, which is a carrier protein for many of the molecules which build up during liver failure and need to be detoxified. In early studies, a polysulfone membrane was used. Later, in dog trials, a cellulose diacetate membrane was used for its higher permeability in the models we tested.

In the first phase of studies, our primary goal was to demonstrate liver-specific functions for the entrapped hepatocytes (Nyberg *et al.*, 1993; Shatford *et al.*, 1991; Nyberg *et al.*, 1993) (Table 1). Oxygen consumption by entrapped hepatocytes was calculated by measuring the difference in the dissolved oxygen concentration between the inlet and the outlet of the recirculating extracapillary medium stream. Stable oxygen consumption was observed for over 7 days of culture. Synthetic activity of entrapped hepatocytes was evaluated by measuring the production of rat albumin using ELISA. Liver-specific cytochrome P450 activity was demonstrated by the clearance of exogenously added lidocaine and the appearance of its hydroxylated product. The conjugation activity for entrapped hepatocytes was demonstrated by measuring conjugated and unconjugated bilirubin levels. All functions were demonstrated for over 7 days of culture.

Demonstration of liver-specific activities in anhepatic rabbit model

Once liver-specific functions had been demonstrated for entrapped hepatocytes, an anhepatic rabbit model was developed to evaluate the *in vivo* function of the BAL (Nyberg *et al.*, 1993; Nyberg *et al.*, 1996). Twenty-two hours prior to surgically removing the liver from the test animal, rat hepatocytes were inoculated into the bioreactor, and a medium stream was recirculated in the extracapillary space. During this time, the

Table 2. BAL function in anhepatic rabbit model

Function	Hepatocyte BAL	Control
Albumin synthesis ($\mu\text{g hr}^{-1}$) ^a	18 ± 11.4	<0.1
Alanine index ^b	1.54 ± 0.65	2.7 ± 0.6
Proline index ^b	1.4 ± 0.58	1.9 ± 0.4
Aromatic amino acid	1.46 ± 0.4	2.6 ± 0.8
Index ^{b,c}		
Ammonia index ^b	2 ± 0.7	3.4 ± 1.2
Lactate index ^b	1.4 ± 0.4	2.1 ± 0.4

^a Rat albumin production measured by ELISA.

^b Ratio of concentration 4 hr post-hepatectomy to concentration pre-hepatectomy.

^c Sum of aromatic amino acids: tyrosine, phenylalanine, tryptophan.

viability of hepatocytes was monitored by measuring their oxygen consumption, and the collagen gel contracted to create space for intraluminal stream. A total hepatectomy was performed on the test animal at 22 hr. At this time, medium was drained from the extracapillary circuit, and the BAL was connected to the animal on an extracorporeal circuit using an arterio-venous shunt.

The animal's blood was perfused through the extracapillary space of the BAL at 35 mL min⁻¹, and hepatocyte-supporting medium was perfused intraluminally at a flow rate of 9 mL h⁻¹. A total of 18 rabbits were divided into three treatment groups: (1) no BAL control, (2) BAL without cells, and (3) BAL with entrapped hepatocytes. In all these groups a variety of biochemical functions were tested, and the results have been summarized in Table 2. The group of animals which received treatment with the hepatocyte-BAL exhibited marked reversal of liver failure as measured by decreased levels of toxic metabolites in the jaundiced animal plasma.

Survival in dog model and the use of porcine hepatocytes

As a step to scaling up for human trials, a large animal liver failure model was developed once *in vivo* function had been established in the rabbit model. D-galactosamine, at a dosage which caused 100% fatality within 48 hr, was used to induce liver injury in canines (Sielaff *et al.*, 1995). Relevant clinical parameters, including intracerebral pressure (ICP) and assessment of comotosis, were established. However, for

such large animal trials rats were no longer an ideal source of hepatocytes because of the large number of cells needed for treatment. In order to harvest large quantities of hepatocytes from one source, ten kilogram pigs were used. We developed a sequential *in situ* portal vein perfusion method using ethylene glycinetetraacetic acid and 0.1% collagenase (Sielaff *et al.*, 1995). To increase the viability of the hepatocytes the perfusion solution was oxygenated. In a typical pig liver perfusion procedure, 19.5 billion cells with 94% viability were routinely obtained.

To hold such a large number of cells, the hollow fiber bioreactor used previously in rabbit trials was not adequate. A number of hollow fiber bioreactors with a designated molecular weight cut-off of 100,000 Daltons were selected for testing. The permeability to serum albumin, the leakage of collagen prior to its gelation, and the passage of immunoglobulin to the luminal side from the shell side were evaluated. A cellulose diacetate-based hollow fiber bioreactor was selected for its high permeability to albumin. Hepatocytes cultured in this bioreactor also sustained higher albumin and urea production, as well as higher oxygen consumption. Because a high flow rate during injection appeared to create a shear field sufficient to cause substantial damage to hepatocytes suspended in a viscous collagen solution, a modified cell loading technique was developed to ensure a high viability after injection of the cell suspension into the lumen. In addition, a protocol was developed to minimize uneven cell distribution. A long stretch of collagen gel without cells would result in an uncontracted gel, which in turn would block the intraluminal flow and cause the viability of hepatocytes to decrease downstream of the blockage.

Two hollow fiber bioreactors, containing 10,000 fibers with an inner diameter of 185 μm and an effective length of 18 cm, were placed in series along the path of the extracapillary loop, while separate intraluminal streams were fed into each hollow fiber bioreactor and discarded after a single pass. Each was loaded with approximately 30–35 g of porcine liver cells to achieve a total liver cell mass of 60–70 g. The right external jugular vein was cannulated for BAL access. The hollow fiber bioreactors were housed in an incubator along with pumps, pH and dissolved oxygen electrodes, and a hollow fiber oxygenator. An emergency battery power supply was provided to allow for the convenient transfer of the BAL from the surgery room where the BAL was prepared to the patient's bedside without the interruption of medium flow and oxygen supply. To ensure

Table 3. Survival in dog model

	Number of dogs	% survival	Mean survival time
Control	3	100	48 hr
D-Gal	5	0	36 hr
HepBAL	5	80	47 hr

sufficient oxygen supply for the hepatocytes, the blood was passed through the oxygenator before entering the BAL to increase oxygen tension to at least 250 mm Hg. In the first 24 hr after inoculation of hepatocytes, medium was circulated on the shell side only. Oxygen consumption and ureagenesis were monitored to determine metabolic activities of hepatocytes. During this time period the test animal was also induced with liver failure and entering coma. The experimental animals which received D-galactosamine were divided into two groups: one which received BAL treatment, and one which received no BAL treatment. Control dogs were included to which no D-galactosamine was injected. Results indicated increased survival rates for injured canines subsequently treated with the BAL ($n=5$) compared to injured animals which were not treated ($n=5$) (Sielaff *et al.*, 1995) (Table 3).

The BAL used in canine trials had a total priming volume of 800 ml, of which 400 ml was attributed to the extracapillary space of the BAL, 250 ml to the oxygenator, and the remainder to the tubing. For anticipated clinical application, it is desirable to increase the liver-specific functions as much as possible. However, scale-up of reactor size from that used for dog trials will be limited by the amount of blood that can be drawn from a patient. Further increase of cell concentration in the collagen gel is not an option either since the loading density was already approaching its maximum. An alternative approach is to manipulate the hepatocytes such that their liver-specific activities are enhanced. One method to achieve this is to culture the hepatocytes as spheroids.

Hepatocytes cultured as spheroids

Primary rat hepatocytes, when plated on certain modified surfaces, form into spheroids that exhibit enhanced liver-specific functions (Koide *et al.*, 1989; Tong *et al.*, 1992; Peshwa *et al.*, 1994). Given equal loading of cells, a BAL with entrapped spheroids can potentially be more active than one containing dispersed cells.

After being plated between 30–80% confluency onto positively charged polystyrene surfaces (Koide *et al.*, 1990), hepatocytes first spread out and moved around randomly (Peshwa *et al.*, 1994). During the first 24 hours, cells began to form patches on the surface. In the next 24 hr these patches of cells continued to increase in size as they collided with each other and merged into multicellular islands. These multicellular masses became increasingly spherical in shape and eventually shed off into suspension as freely floating spheroids (Peshwa *et al.*, 1994). An average spheroid has a diameter of approximately 100 μm and is 6–8 cell layers thick. Spheroids appeared to have smooth undulating surfaces, and individual cells were not readily distinguishable when viewed by scanning electron microscopy (Figure 2). Under transmission electronic microscopy an extensive internal network of microvilli-lined bile canaliculus-like channels can be seen throughout the spheroid (Peshwa *et al.*, 1996).

These spheroids sustained a high viability in culture over a week, while hepatocytes cultivated as a monolayer on a collagen-coated surface or on a plastic surface typically lost more than 50% of viability over the same period. Hepatocytes cultivated as spheroids also had high activities of albumin biosynthesis, urea production, and cytochrome P450 enzyme activity. The P450 enzyme system is responsible for the detoxification of a wide variety of endogenous and exogenous compounds. As a result, we felt it was important to evaluate cytochrome P450 activity in spheroids more closely.

Cytochrome P450IA1 (CYP1A1) activity was assessed *in situ* using confocal laser scanning microscopy to detect resorufin, the highly fluorescent product of P450-mediated O-dealkylation of 7-ethoxyresorufin. The early stage of culture, marked by dispersed patches of monolayers, was associated with relatively low CYP1A1 activity. As cells formed multicellular structures and aggregated into spheroids, the level of CYP1A1 activity increased over time. In addition, the distribution of activity within spheroids was heterogeneous (Figure 3). At least a five-fold higher fluorescence intensity was observed in spheroids compared to that of monolayers maintained on collagen. The higher CYP1A1 activity in spheroids was confirmed by measuring activity in microsomes prepared from monolayer and spheroid cultures. The higher P450 activity within spheroids may be associated with their ability to maintain a greater degree of differentiation compared to monolayers.

When plated on a surface, only about 35–40% of cells formed into spheroids, while the rest remained small cell patches. If spheroids were to be prepared from monolayer culture for use in the BAL, the task of cultivating such a large number of cells would be very labor intensive. A method of preparing porcine hepatocyte spheroids in a stirred tank bioreactor was developed to alleviate this problem (Lazar *et al.*, 1995). Freshly harvested porcine hepatocytes were inoculated at a moderate cell concentration of $3\text{--}5 \times 10^5$ cells ml^{-1} into spinner flasks containing a defined culture medium. These hepatocytes agglomerated quickly to form small clumps within hours. Further agglomeration of these clumps resulted in spheroid formation 24 hr after cell inoculation. A similar spheroid preparation technique was also developed for rat hepatocytes (Wu *et al.*, 1996). Approximately 80–90% of inoculated hepatocytes form spheroids with a mean diameter of about 135 μm . Scanning electron microscopy revealed numerous microvilli projecting from the entire surface of the spheroids. Transmission electron revealed differentiated hepatocytes which displayed well-developed cytoplasmic structures separated by bile canaliculus-like structures. Ureagenesis by spheroids was twice as high and was sustained for a longer culture period than that by hepatocytes cultured as monolayers. Their morphological features, ultrastructural characteristics, prolonged viability and sustained higher liver-specific functions all resembled those of spheroids prepared from monolayer culture. Preparation of hepatocyte spheroids in an agitated vessel is simple, efficient, and reproducible, and it allows for preparation of large quantities of spheroids required for use in a BAL (Table 4).

While these spheroids exhibited higher activities for a number of liver specific functions compared to hepatocytes cultivated as monolayers, these activities decreased over time in culture. Metabolic activities of spheroids entrapped in collagen gel were maintained at a stable level for over 21 days (Lazar *et al.*, 1995). Production of albumin and urea by hepatocyte spheroids entrapped in collagen gels were 2–3 times higher than those by freshly isolated single cells. P450 activity was demonstrated by metabolism of lidocaine to its main metabolite, monoethylglycinexylidide (MEGX). Phase II drug metabolism was demonstrated by glucuronidation of 4-methylumbelliferone (4-MU). This work showed that porcine hepatocyte spheroids entrapped in collagen maintained differentiated functions for an extended time period.

Figure 2. Scanning electron micrograph of rat hepatocyte spheroids.

Table 4. Conditions for spheroid formation in stirred tanks

	Rat hepatocytes	Porcine hepatocytes
Inoculation density	3×10^5 cells ml ⁻¹	$5\text{--}10 \times 10^5$ cells ml ⁻¹
Agitation speed	100 rpm	80 rpm
Efficiency of formation	80–85%	80%
Albumin synthesis	3.6 ± 0.7 pg cell ⁻¹ h ⁻¹	2.1 pg cell ⁻¹ h ⁻¹
Ureagenesis	3.9 ± 0.5 pg cell ⁻¹ h ⁻¹	2.1 pg cell ⁻¹ h ⁻¹
Oxygen consumption	10^{-9} mmol cell ⁻¹ h ⁻¹	

Spheroid entrapment BAL

We explored the possibility of entrapping spheroids into the BAL in order to improve its performance. Rat hepatocyte spheroids were entrapped in collagen gel within the lumen fibers of the BAL (Wu *et al.*, 1995). The morphology and ultrastructure of collagen-entrapped spheroids resembled those of suspended spheroids formed on petri dishes. Albumin synthesis and P450 enzyme activity were measured as mark-

ers of liver-specific functions of spheroids entrapped in the BAL. At least a four-fold improvement in these functions was observed compared to BAL devices with dispersed hepatocytes entrapped in collagen gels.

In a subsequent studies with porcine hepatocytes a mixture of spheroids and dispersed cells was used (Wu *et al.*, 1996). Both spheroids and dispersed cells were able to contract collagen gel. However, the degree of contraction by dispersed hepatocytes was significantly higher than that by hepatocyte spheroids. Spheroids

Figure 3. Micrograph of confocal laser scanning microscopy of a spheroid showing EROD activity. Spheroid was optically sectioned after incubating with ethoxyresorufin. The fluorescence intensity of the reaction product, resorufin, was measured. White color represents highest activities whereas black is the background. The micrograph is a composite of central 20 sections with a $1\ \mu\text{m}$ step size in optical sectioning.

entrapped into the BAL also demonstrated approximately two-fold increased metabolic activity on a per cell basis compared to devices entrapped with dispersed cells.

Perspectives of BAL and hepatocyte bioreactors

In the last few years we have made significant progress on the BAL. The ultimate test of the BAL we developed will be trials on humans. Efforts to employ this BAL for fulminant liver failure patients has been initiated. Its safety and efficacy in humans are yet to

be demonstrated. The long-term effect of the patient's exposure to porcine proteins crossing the membrane is still not known. Furthermore, whether the two- to five-fold increase in measured liver-specific functions exhibited by spheroids warrants the chore of preparing them needs to be evaluated. Regardless of whether spheroids are eventually used in a BAL, their ability to self-assemble to form structures which resemble liver cells *in vivo* and to maintain higher functions is attractive to many liver researchers. This process of self-assembly reverses when spheroids are transferred to highly adhesive surfaces and allowed to spread to monolayers. Interestingly, the reversal of the spheroidal structure and morphology coincides with the loss of cytochrome P450 activities measured *in situ*. With their closer resemblance to liver cells *in vivo*, and their ability to retain liver properties over a longer duration, spheroids, either entrapped in BAL or in suspension culture, can be an attractive vehicle for drug metabolic research and transient gene expression studies.

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