

## Chapter II

### Materials and methods

**Chemicals:** FeCl<sub>3</sub> anhydrous was from Fluka Chemika. Desferroamine mysilate, Tetrazolium salt (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)), zoletil 100 were from VERBAC Lab. France. All reagents were of the highest quality available.

#### Preparation of solutions

Deionized double distilled water was used throughout the experiments for solutions and buffers (Mankhetkorn S; et al, 2008; Kothan. S; et al. 2008; Suttana, W; et al, 2007)

A stock solution of MTT was prepared by dissolving 5 mg of MTT in 1 mL of HEPES-Na<sup>+</sup> buffer and then filtered using a 0.22 μm filter and stored at 4 °C.

The balanced salt PBS solution was composed of 20 mM HEPES-Na<sup>+</sup> buffer plus 132 mM NaCl, 3.5 mM KCl, 1 mM CaCl<sub>2</sub>, and 1.5 mM MgCl<sub>2</sub>, pH 7.4 at 37 °C.

The HEPES-Na<sup>+</sup> buffer solution consisted of 20 mM HEPES buffer plus 132 mM NaCl, 3.5 mM KCl, 1 mM CaCl<sub>2</sub>, and 1.5 mM MgCl<sub>2</sub>, pH 7.25 at 37 °C.

The stock solutions of 0.01 M ferric ion (in 0.4 M H<sub>2</sub>SO<sub>4</sub> and tridistillation water) were freshly prepared before being used. The stock solution was protected from light with an aluminium foil and kept at 4 °C. The concentration of ferric solution was determined by spectrophotometric analysis which was determined using the molar extinction coefficient (Sibmooh, N; et al, 2001)  $\epsilon$  ( $\lambda = 304$  nm) equal to 2204 M<sup>-1</sup>.cm<sup>-1</sup>.

The stock solution of 0.1 M desferoxamine (DFO) was dissolved in deionized double distilled water (De-aerated with N<sub>2</sub> gas for 30 minutes). The stock solution was protected from light with an aluminium foil and kept at 4 °C.

#### **Cell culture and cytotoxicity assay.**

The GLC4 human small cell lung carcinoma cell line was routinely cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C. For the assays, cell cultures were initiated at a density of  $5 \times 10^5$  cells/ml to have cells in the exponential growth phase; the cells were used 24 h later when the culture had grown to about  $8 \times 10^5$  cells/ml. Cell viability was assessed by trypan blue exclusion. The number of cells was determined with a haemocytometer (Reungpatthanaphong and Mankhetkorn 2002; Loetchinat, C; et al, 2005; Dechsupa et al. 2007).

The cytotoxicity assay was performed as follows. Cells ( $5 \times 10^4$ /mL) were incubated in the presence of various concentrations of compounds. The viability of cells was then determined using the MTT assay based on the reduction of MTT to purple-colored formazan by live cells, and not dead cells. The concentration of drugs required to inhibit cell growth by 50% when measured at 72 h (IC<sub>50</sub>) was determined by plotting the percentage of cell growth inhibition versus the drug concentration (Kothan. S; et al. 2008. Dechsupa S; et al 2007; Tungjai M. et al. 2008).

#### **Measurements of T1- and T2-relaxation time of Proton**

The effects of ferrioxamine on T1- and T2-relaxation time of proton was performed in deionized double distilled water, 3.7% agarose gel (tissue equivalent phantom) and RPMI 1640. The final ferrioxamine concentrations were 0,  $5 \times 10^{-6}$ ,  $5 \times 10^{-5}$ ,  $1 \times 10^{-4}$ ,  $5 \times 10^{-4}$ ,  $1 \times 10^{-3}$ ,  $5 \times 10^{-3}$  and  $1 \times 10^{-2}$  M. The gel phase of 3.7% agarose

was obtained after boiling the solution to 95 °C then allowed to cool to 40 °C and then was added to the stock solution of ferrioxamine to obtain the expected final concentrations and was then rigorously mixed to assure a homogenous phase.

The signal intensity of T1-relaxation and T2-relaxation times were measured on 1.5T medical MRI scanner (Archieva, Philips medical instrument) after coronal survey imaging. For measurement of T1-relaxation time, the T1 weighted-spin echo (SE) excitation mode was applied by fixing TE at 10 ms, and varies TR from 100-6000 ms. For measurement of T2-relaxation time, the spin echo (SE) sequence of excitation mode using the Archieva scanner did not allow us to vary the TE. For example, when TR was fixed at 1000 ms, the TE varied from a minimum of 60 to 500 ms. However, the optimal excitation condition was obtained by using T2w-turbo spin echo sequence (T2w-TSE). The T2w-images were obtained by fixing TR at 1000 ms, TE varied from minimum of 60 to 1000 ms and by fixing TR at 4000 ms, TE varied from a minimum of 45 to 1000 ms (Figure 2.1). Operator defined, region of interest (ROI) measurements were also performed on T1w-spin echo and T2w-turbo spin echo images. Mean signal intensities (SI) and standard deviations (StdDev) for the contents of samples and background were obtained with circular ROI that had exactly the same size and shape for each case. The signal-to-noise ratio (SNR) was determined as the mean value of SI for a material divided by the StdDev of the mean SI of the background ( $SNR = SI / \text{StdDev of noise}$ ). In this case the contrast-to-noise ratio (CNR) was calculated between the contrast agent alternatives and the surrounding tissue models such as agarose gel and ferric-desferoxamine complex, this was done using the following formula:  $CNR = (SI1 - SI2) / (\text{StdDev noise})$ , where SI1 and SI2 correspond to the signal intensities of the agarose gel alone and with ferric-

desferoxamine complex, respectively. The mean signal intensity was plotted as a function of TR or TE and the data was quantitatively analyzed using Block's equation.

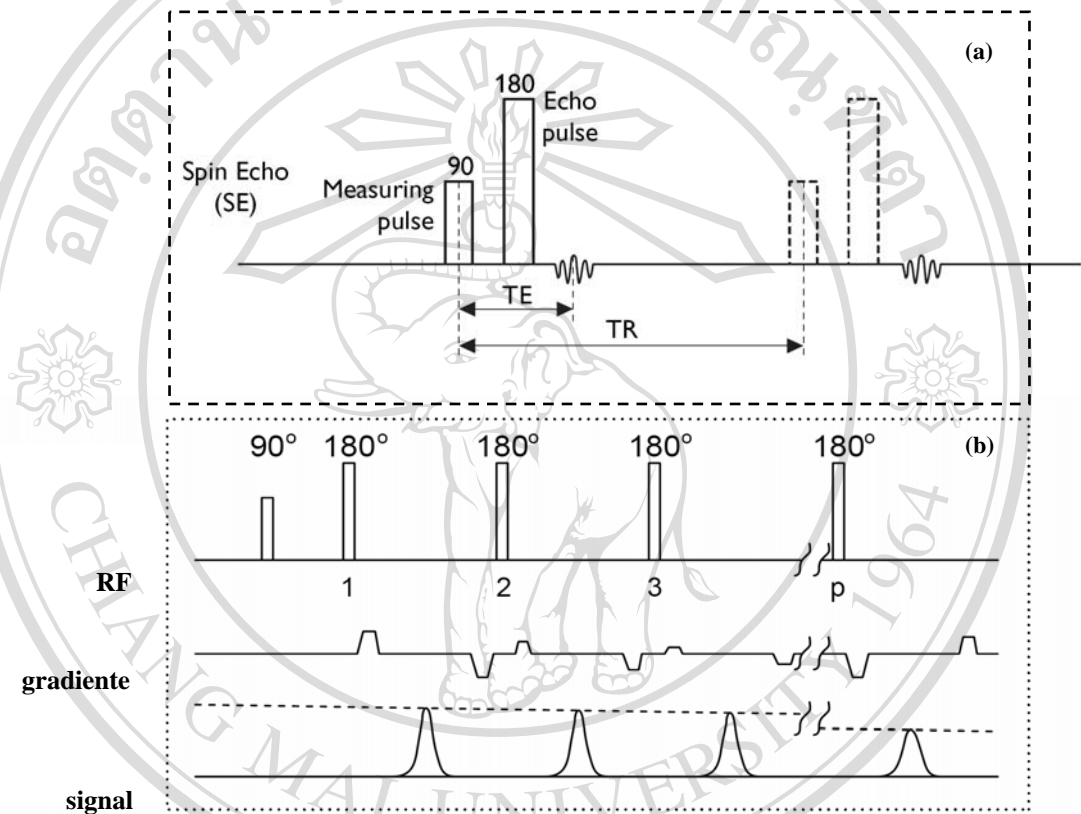


Figure 2.1 Diagram of excitation sequence of (a) Spin Echo (SE) by fixing TE constant at 15 ms and flip angle at 90° and varied TR and (b) Turbo Spin Echo (TSE) by fixing TR constant at 1000 ms or 4000 ms, TSE-factor at 15 and flip angle at 90° and varied TE. (Archieva, Philips medical instrument manual)

### **MR Imaging of ferrioxamine distribution in Wistar rats**

A total of 3 male Wistar rats (Outbred: National Laboratory Animal Center, Mahidol University, Thailand) of 8 months of age were housed and fed rodent chow and water ad libitum and treated in accordance with institutional guidelines for animals.

Rats were anesthetized by intra-peritoneal injection with  $2 \text{ mg.kg}^{-1}$  of nembutal (OVATION Pharmaceutical Inc., Deer field Illinois, USA). The tail was intravenously punctured with catheter no. 24g and then a drip of NSS was administered (Dechsupa et al. 2007). The anesthetized rats were placed and fixed in a prone position inside the head coil. The coronal and sagittal plan surveys were obtained as the same scout techniques used for both of T1w-SE (TR = 535 ms; TE = 10 ms) and T2w-TSE (TR = 2500 ms; TE = 115 ms).

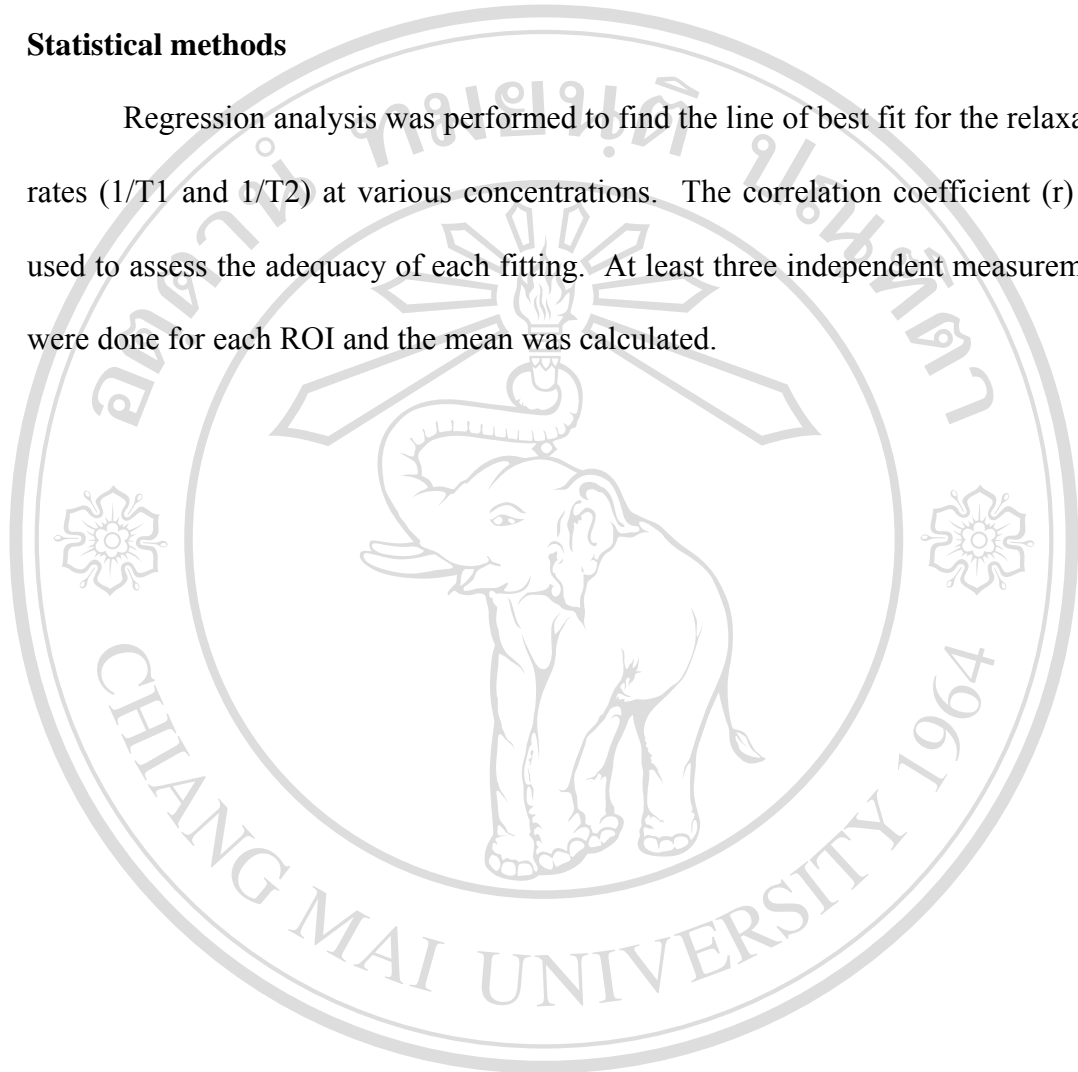
### **Magnetic resonance angiography**

The dynamic magnetic resonance angiography and distribution of ferrioxamine was performed in comparison with a known intravascular MRI contrast agent, Gd-DTPA, which consistently showed no significant leakage through the vascular wall after remaining in circulation for more than 30 min. MR angiography of the rats was performed with an injection of  $0.5 \text{ mmol Fe(III)/kg}$  of ferrioxamine compared with an injection of  $0.066 \text{ mmol Gd/kg}$  of Gd-DTPA. All of the images were obtained with head coils. The turbo spin echo technique (T2W-TSE; TR/TE, 120/2910; flip angle,  $90^\circ$ ) with chemical fat-suppression was used for all rats. The images were acquired before injection of the contrast agents and at 0 (immediately post-injection), 1, 2, 3, 5, 8, 10, 15, and at 30 min post-injection. The coronal images

were reconstructed with 1.0 mm section thickness and 0.5 mm overlap. The FOV was 279 mm.

### **Statistical methods**

Regression analysis was performed to find the line of best fit for the relaxation rates ( $1/T_1$  and  $1/T_2$ ) at various concentrations. The correlation coefficient ( $r$ ) was used to assess the adequacy of each fitting. At least three independent measurements were done for each ROI and the mean was calculated.



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