Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 4, Issue, 12, pp. 473-476, December, 2012

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

INVITRO EVALUATION OF METHOTRAXATE LOADED PLA NANOPARTICLES PREPARED BY SOLVENT DISPLACEMENT TECHNIQUE

***[1]Riddhi M. Dave and [2]Rakesh K. Patel**

[1,2]S. K. Patel College of Pharmaceutical Education and Research, Ganpat University, Kherva- 382711. Gujarat, India

ARTICLE INFO

Article History:
Received 09th September, 2012
Received in revised form
20th October, 2012
Accepted 16th November, 2012
Published online 28th December, 2012

Key words:
HELA,
MCF- 7,
A549,
LNCaP Cell line,
IC50.

ABSTRACT

The major objective of the present study is to incorporate a hydrophilic drug methotraxate within biodegradable polymer; poly (lactic acid) for the formulation of nanoparticles by solvent displacement technique. Three organic solvents such as Tetrahydrofuran (THF), Methanol and acetone were used in different ratios with water and all of them were characterized with respect to particle size and entrapment efficiency. Selected batches were studied for cytotoxicity using LNCaP, MCF- 7, A549, HELA cell line and normal Vero cell lines and 50% growth inhibitory concentration (IC_{50}) values were found out for formulations. The activity of both free and nanoparticle-entrapped with methotraxate increased with increasing methotraxate concentration and incubation time from 24 hrs to 72 hrs. Whereas plain nanoparticles showed no change in viability of cancer cells. Based on IC_{50} values it was observed that after 24 hrs of incubation, 50% inhibitory concentration (IC_{50}) for methotraxate loaded nanoparticle was 60.12 mMolar/ml whereas for free methotraxexate it was around 93.42 mMolar/ml which observed after 72 hrs for methotraxate loaded nanoparticle 27.36 mMolar/ml whereas for free methotraxexate 80.54 mMolar/ml. These data indicates that less concentration required for nanoparticulate formulation to kill cancer cells (lesser dose) compared to any conventional formulation.

INTRODUCTION

Pharmaceutical nanoparticles are submicron-sized, colloidal vehicles that carry drugs to the target or release drugs in a controlled way in the body which are made up of biocompatible and /or biodegradable material. The characterization properties, as well as targeting and controlled release, can be affected by nanoparticle material selection and by surface modification. Variety of preparation techniques exist ranging from polymerization of monomers to different polymer deposition methods.[1,2] Among the drugs used in nanoparticle formulations, particularly cancer therapeutics is widely studied because the formulation might reduce toxicity of the drug while improving efficacy of the treatment.[3] By modifying particle surface, e.g., by coating, defence mechanisms of the body can be avoided to some extent leading to longer circulation times of nanoparticles in the blood.[4]

The benefits of nanoparticles include protection of the encapsulated pharmaceutical substance, improved efficacy, fewer adverse effects, controlled release and drug targeting.[5,6,7] As the time approaches when an increasing number of nanoparticulate drug delivery systems reach the market and the systems are transferred from animal tests to human use, concerns about the safety of the products are emerging. More attention will be paid to stability and toxicology of nanoparticles and their constituents. According to recent opinion, proper physicochemical characterization of nanoparticles should be included in risk assessment and toxicology considerations of nanoparticulate systems, in addition to pharmaceutical in vitro and in vivo testing.[8] The effect of such formulations checked by XTT assay on different cell lines.[9, 10]

***Corresponding author:** riddhi_dave2002@yahoo.com

MATERIALS AND METHODS

Materials

Methotraxate was obtained as a gift samples from Astron Research Ltd. Ahmedabad. Poly (lactic acid) (PLA) biodegradable polymer was supplied by Aldrich, USA. THF, Acetonotrile, Methanol, Buffer phosphate and Buffer phosphate saline (pH 7.4) were obtained from Merck (Darmstadt, Germany). All solvents and reagents were of analytical grade. Prostate tumor carcinoma cell lines (LNCaP), HeLa, MCF 7, A549 and Vero (kidney) cell lines were obtained from National cancer research institute, Pune. Ethyl acetate, Methylene chloride, Buffer phosphate and Buffer phosphate saline (pH 7.4), DMEM, EDTA, Trypsin, DPBS solution were Procured from Merck (Darmstadt, Germany). Growth media (RPMI -1640) Penicillin, Amphotericin B, Fetal bovine serum and L-Glutamine were obtained from Hi-Media. Na Carbonate and glucose were obtained from local commercial source. All solvents and reagents were of analytical grade.

Method

Preparation of nanoparticles by solvent displacement techniques

Solvent displacement technique was used to formulate the nanoparticles. In this method, Attempts were made to prepare stable nanoparticles without any surfactant/stabilizer as residual surfactant is problem and toxic to human body. Various organic solvent of increasing dielectric constant like tetrahydrofuran (THF), acetonitrile and methanol were selected. In this, the solvent: water ratio (1:1, 1:2 and 1:5) were varied while the drug to polymer ratio was constant. In another case, the polymer concentrations in organic phase (5, 10 and 15 %) were varied while the solvent: water ratio was constant at 1:2. In another modification various organic solvents to aqueous phase ratios were selected in order to keep the use of organic solvents at lowest possible limits. Both drug and polymer (PLA) were dissolved in an organic solvents and this mixture was poured in to an aqueous phase containing water under mechanical stirring for 2 to 3 hr. The resultant nanoparticles were in nanosuspension separated by ultracentrifugation at 12000 rpm for 30 min.

Purification of nanoparticles

The resultant nanosuspension was subjected to ultracentrifugation at 2 to 5^{o}C in cooling centrifuge at around 12000 rpm for 30 min. The settled nanoparticles were collected by separating supernatant containing free drug. The supernatant and nanoparticles both were dissolved in phosphate buffer and quantified by spectrophotometer method.

In vitro cytotoxicity study of formulation on LNCaP, HeLa, MCF 7, A549 and Normal Vero cell lines by XTT assay

The cytotoxicity of free methotraxte and methotraxate loaded PLA nanoparticles were investigated using different cancer cell lines LNCaP, HeLa, MCF 7, and A549 by XTT assay. LNCaP cell line seeded and sub cultured in T flask in bio safety cabinet hood. Here growth media, 100 ml RPMI -1640, with L-Glutamine, Sodium Carbonate and glucose were added. The media was supplemented with 10 ml fetal bovine serum for the purpose of generating monolayer. Addition of 1 ml of Penicillin and Amphotericin B prevented the fungal growth. Now T flasks were incubated for 15 days in CO2 incubator with every 3 to 4 days for media change. Here it has been observed that LNCaP cell line was slow growing cell line and cell size were also very small compared to any other cell lines. Now cell viability checked by trypsinization, then LNCaP cells were seeded 96 well plate sat at density of 10,000 cells per well in 100 µl RPMI supplemented with 10% fetal bovine serum along with 50 µl of cell culture. Cytotoxicity was carried out at three fold dilution with concentration range of 100m Molar to 0.41m Molar/ml.

After twenty-four hours plating, incubation at 37^{o}C, 50 µl of XTT die was added into each well, after 24, 48 and 72 hrs, the well plates were read in micro plate reader (Bio-Rad, Hercules, CA,USA) at a wavelength of 490 nm. The experiments were repeated in triplicate. Same method were followed for the other cell lines like HeLa, MCF 7 and A549. Cytotixicity was expressed as % reduction cell viability and the data are tabulated as shown in Result section as Table 2,3,4 and 8. The toxicity of plain methotraxate, and methotraxate loaded PLA nanoparticles on normal cells of normal Vero (kidney) cell lines were evaluated using cell line study. The anatomy and physiology of normal Vero cell line is different from any cancer cell lines. The primary culture was sub cultured and all reagents like FBS (fetal bovine serum), DMEM, EDTA–trypsin solution (Trypsin-EDTA made by diluting the stock 1/10 by adding PBS only) Penicillin and Amphotericin B were brought at room temperature before start of sub culture. Cell lines were handled under cytotoxicity cabinet to prevent cross contamination of cell lines. Cells were split when they were about 80-90% confluence. Cells were washed with 0.1 ml cm^2 / flask (2.5 ml in case of 25cm^2 flask) DPBS- EDTA (1mM EDTA) solution.

The monolayer adhere to flask was gently rinsed by rocking the flask back and forth. After 5 minutes, aspirate offs the excess PBS-EDTA from the flask. To the above flask, 0.1-0.2 ml/cm^2 trypsin was added until the entire monolayer was covered than incubate it for 3-5 minutes at room temperature to detach the cells from monolayer Cells were dispersed into a single cell suspension by pippeting the cell solution up and down. These Cells were added in to media flask (DMEM + BSS) containing FBS in it (FBS inactivates the trypsin, which was why it had to be rinsed off with PBS-EDTA initially).Cells was counted by hemocytometer and dilute to the appropriate concentration for seeding. Finally, the appropriate volume of cell suspension were added in to a new flask containing medium along with 1% antibiotic solution and was placed flask in 5 % CO_2 incubator at 37^{o}C. This splitting/passage was repeated every 3-4 days, in order to prevent dilution or overgrowth. The rest of the process of generation of 96 well plates was same like LNCaP cell lines. The Cytotixicity was expressed as % reduction cell viability and the data of cell viability after 24, 42 and 72hours are tabulated as shown in Result section as table 5, 6 and 7 respectively. From the data obtained IC50 Values were calculated and tabulated in result.

RESULT AND DISCUSSION

The data's obtained from cell line study on LNCaP, HeLa, MCF 7, A549 and Vero cell lines are tabulated in excel sheet here with attached. The in vitro anticancer cytotoxic activity of free methotraxate and methotraxate loaded PLA nanoparticles on LNCaP cells, expressed as % reduction of cell viability was evaluated. The 50% growth inhibitory concentration (IC_{50}) values for free methotraxate and methotraxate-loaded nanoparticles were estimated from the available cytotoxicity data (Table 1). The nanoparticles loaded with methotraxate exhibited more in vitro anticancer activity comparable to that of free methotraxate. The activity of both free and nanoparticle-entrapped methotraxate increased with increasing methotraxate concentration and incubation time from 24 hrs to 72 hrs (Table 2-4). Whereas plain nanoparticles showed no change in viability of cancer cells. Based on IC_{50} values it was observed that after 24 hrs of observation, 50% inhibitory concentration (IC_{50}) for methotraxate nanoparticle was 60.12 mMolar/ml whereas for free methotraxate, it was around 93.42 mMolar/ml indicating less concentration required for nanoparticulate formulation to kill cancer cells (lesser dose) compared to any conventional formulation. Here

to check the effect of plain methotraxate, methotraxate loaded PLA the cytotoxicity study on normal Vero cell lines was performed on normal kidney cells, its anatomy and physiology was different from any cancer cell lines. From the tables and figures we can conclude that plain methotraxate shows more toxicity compared to methotraxate loaded PLA nanoparticles. Regarding plain nanoparticles, it exhibit very minor toxicity on normal cell lines. The main purpose to perform the cytotoxicity on normal Vero kidney cell lines was to evaluate the whether the nanoaparticles show any detrimental effect on healthy cells .As shown in table 5, 6 and 7 the cytotoxicity data showed that there was very minor around 10% cell inhibition observed at 100mMolar/ml concentration, whereas almost double around 22% cell inhibition found with same concentration of free methotraxate concentration. The Plain nanoparticles show very minor cell toxicity of around 2 to 3% found with same concentration. The cytotoxicity were also checked on other cell lines like HeLa, MCF 7, A549 the result tabulated in table no 8,which shows a good inhibition of cells in MCF 7 cell lines.

Table 1. IC50 values (mMolar/ml) for free MTX and MTX loaded PLA nanoparticles

Formulation	24 h	48 h	72 h
Free methotrexate formulation	93.42	87.52	80.54
Methotrexate based PLA nanoparticles (METH 44)	60.12	35.14	27.36

Table 2. % Cell viability of MTX loaded nanoparticles on LNCaP cell line after 24 hrs

Concentration Formulation	% cell viability	% cell viability
	Free MTX formulation	MTX based PLA nanoparticles
100	24.12±1.12	20.35±1.18
33.33	34.15±1.03	30.19±1.87
11.11	46.23±2.12	41.36±1.12
3.703	55.27±1.25	46.22±2.11
1.234	65.26±2.22	59.12±2.06
0.411	86.48±3.08	75.15±2.05

Table 3. % Cell viability of MTX loaded nanoparticles on LNCaP cell line after 48 hrs

Concentration mMolar/ml Formulation	% cell viability	% cell viability
	Free MTX formulation	MTX based PLA nanoparticles (METH 44)
100	20.11±1.46	18.15±2.28
33.33	31.12±1.06	28.29±1.82
11.11	42.23±2.12	39.26±1.22
3.703	52.27±1.25	42.12±2.11
1.234	61.16±2.08	55.24±2.16
0.411	82.18±3.18	72.25±2.15

Table 4. % Cell viability of MTX loaded nanoparticles on LNCaP cell line after 72 hrs

Concentration mMolar/ml	% cell viability	% cell viability
	Free MTX formulation	MTX based PLA nanoparticles (METH 44)
100	17.22±1.08	15.11±2.11
33.33	29.56±1.13	25.19±1.08
11.11	40.43±2.22	35.36±1.22
3.703	50.47±1.35	40.16±2.12
1.234	58.19±2.27	52.27±2.56
0.411	80.28±3.45	68.15±2.35

Table 5. % Cell viability of MTX loaded nanoparticles on normal vero cell line after 24 hr

Concentration Formulation	% cell viability	% cell viability
	Free methotrexate formulation	Methotrexate based PLA nanoparticles (METH 44)
100	75.32±3.22	93.75±1.27
33.33	87.85±3.56	95.57±3.74
11.11	90.67±2.22	97.54±3.14
3.703	95.65±3.12	98.74±2.33
1.234	94.42±1.21	98.53±2.85
0.411	95.15±1.14	99.23±3.25

Table 6. % Cell viability of MTX loaded nanoparticles on normal vero cell line after 48 hr

Concentration Formulation	% cell viability	% cell viability
	Free methotrexate formulation	Methotrexate based PLA nanoparticles (METH 44)
100	77.52±3.12	92.17±2.25
33.33	88.85±3.56	94.23±2.24
11.11	90.57±2.22	96.75±2.26
3.703	96.65±3.12	97.31±2.47
1.234	95.42±1.21	97.26±2.41
0.411	96.15±1.14	98.33±3.78

Table 7. % cell viability of MTX loaded nanoparticles on normal vero cell line after 72 hrs

Concentration Formulation	% cell viability	% cell viability
	Free methotrexate formulation	Methotrexate based PLA nanoparticles (METH 44)
100	74.32±3.22	92.17±2.33
33.33	87.85±3.56	93.22±2.22
11.11	91.67±2.22	95.75±2.21
3.703	94.65±3.12	95.89±2.43
1.234	94.38±1.21	96.00±2.32
0.411	94.15±1.14	94.33±3.33

Table 8. % Growth inhibition of MTX loaded nanoparticles (METH 44) on different cell lines after 72 hr

Time (hr)	LNCaP	MCF- 7	A549	HELA
0	0.0	0.0	0.0	0.0
2	2.15±0.05	09.22±1.34	08.36±2.38	12.56±3.12
4	6.12±0.12	12.26±1.30	16.35±1.35	22.18±2.36
8	16.22±1.11	26.08±1.16	25.22±3.11	32.12±2.24
12	28.10±2.12	42.52±4.32	36.12±3.24	42.15±2.35
24	36.45±2.09	60.10±1.20	40.10±1.30	56.25±2.51
48	52.12±2.24	70.14±3.28	56.45±4.15	60.56±3.12
72	62.17±2.34	82.15±1.30	64.14±5.28	68.12±1.24

Acknowledgement

The authors are thankful to the Astron Pharmaceuticals, Ahmadabad for providing gift sample of methotraxate drug.

Conclusion

The Present study demonstrates that In vitro antitumor activity indicated a nanoparticulate formulation is therapeutically more effective compare to conventional system.

REFERENCES

1. Kreuter J. Nanoparticles. In: Swarbrick J, Boylan JC, editors. 1994. Encyclopedia of Pharmaceutical Technology. Vol. 10. New York: Marcel Dekker Inc; p. 165-90.

2. Pinto RC, Neufeld RJ, Ribeiro AJ, Veiga F, 2006. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. Nanomedicine; 2: 8-21.
3. Brigger I, Dubernet C, Couvreur P, 2012. Nanoparticles in cancer therapy and diagnosis. Adv Drug Deliv Rev. 17: 1-28.
4. Storm G, Belliot SO, Daemen T, Lasic. 1995. DD Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system. Adv Drug Deliv Rev, 17: 31-48.
5. Couvreur P, Vauthier C, 2006. Nanotechnology: intelligent design to treat complex disease. Pharm Res. 7:1417-50.
6. Pinto RC, Neufeld RJ, Ribeiro AJ, Veiga F, 2006. Nanoencapsulation II Biomedical applications and current status of peptide and protein nanoparticulate delivery systems. Nanomedicine 2: 53-65.
7. Galindo-Rodríguez SA, Puel F, Briançon S, Allémann E, Doelker E, Fessi H, 2005 Comparative scale-up of three methods for producing ibuprofen-loaded nanoparticles. Eur J Pharm Sci. 25:357-67.
8. Oberdörster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K et al. ILSI 2005. Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol.6:2:8.
9. Lager R, Siegel R, Brown L, Leong K, Kost J, Edelman E. 1986. Controlled release three mechanism, Chem Tech, 2: 108-10.
10. Szymanski MS, Porter RA. 2012. Preparation and Quality Control of Silver Nanoparticle - Antibody Conjugate for Use in Electrochemical Immunoassays J Immunol Methods. 12: 35-6.

****