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RESEARCH PAPER

In Vitro and *In Vivo* Biological Evaluation of O-Carboxymethyl Chitosan Encapsulated Metformin Nanoparticles for Pancreatic Cancer Therapy

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ABSTRACT

Purpose *In vitro* anticancer effect and *in vivo* biodistribution and biocompatibility of metformin encapsulated *O*-Carboxymethyl chitosan nanoparticles were evaluated for its application as pancreatic cancer therapy.

Methods *In vitro* studies such as cell migration assay, clonogenic assay, cell cycle analysis and qRT-PCR analysis were done in pancreatic cancer cells (MiaPaCa-2) treated with *O*-CMC-metformin NPs for evaluating its anticancer potential. *In vivo* biodistribution studies were carried out by NIR imaging of *O*-CMC-metformin NPs after tagging it with ICG. *In vivo* biocompatibility of the NPs was assessed by histopathology analysis of organs from mice administered with the NPs.

Results *In vitro* cell migration assay showed marginal effect of NPs on migration property of pancreatic cancer cells (MiaPaCa-2). *In vitro* clonogenic assay established that the *O*-CMC-metformin NPs reduced colony formation ability of the cancer cells. While cell cycle analysis showed that the *O*-CMC-metformin NPs had only minor effect on progression of cell cycle in the cancer cells. qRT-PCR analysis exhibited reduced mRNA expression of p21, vanin 1 and MMP9 in pancreatic cancer cells treated with the nanoparticles. *In vivo* NIR imaging study showed normal biodistribution pattern of the intravenously injected *O*-CMC-metformin NPs suggesting normal clearance rate of nanoparticles and no adverse toxicity to the organs.

Conclusions The biocompatible *O*-CMC-metformin NPs with anticancer potential and capability for normal biodistribution can be beneficial for the treatment of pancreatic cancer.

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INTRODUCTION

Pancreatic cancer is the fourth leading causes of cancer related death in US with a 5-year survival rate of only 3–5% (1). Since the current therapies for pancreatic cancer offer very limited survival benefits, novel therapeutic strategies are immediately required. Metformin (*N*',*N*'-dimethylbiguanide) is an FDA approved drug, extensively used for type-2 diabetes treatment. In cancer cells, metformin has both direct and indirect effect. The indirect effect is by normalizing hyperinsulinemia, a phenomenon responsible for aggressive proliferation of cancer cells (2, 3). Meanwhile, direct action of metformin is by affecting intracellular signalling pathways, after metformin has been uptaken by the cancer cells. To date, numerous anticancer effects of metformin have been put forth, including AMPK activation, cyclin D1 down regulation, MAPK inactivation, suppression of HER2 expression, inactivation of nuclear factor-kappaB signalling and cAMP-responsive element binding protein (CREB) *etc.* (4-15). However, the predominant mechanism of metformin action in cancer cell is AMP-activated protein kinase (AMPK) pathway activation and inhibition of its downstream protein mTOR, which plays a key role in regulating cell growth, apoptosis, proliferation and protein synthesis (4, 9, 12, 16, 17).

In pancreatic cancer cells, along with inhibition of mTOR, metformin also disrupts an mTOR mediated crosstalk between insulin signalling and GPCR signalling (18, 19). It has also been shown that metformin re-expressed miRNAs (let-7a, let-7b, let-7c, miR-26a, miR-101, miR-200b, and miR-200c miR-192) which are missing in pancreatic cancer cells and reduced expression of cancer stem-like cells markers (CD44, EpCAM, EZH2, Notch-1, Nanog, and Oct4), suggesting its

anti-neoplastic effect and usefulness to overcome drug resistance (20, 21). Recent studies have shown that in contrast to mTOR inhibitors, metformin eliminated mTORC1 activation devoid of over-stimulating Akt and inhibited ERK activation in pancreatic ductal adenocarcinoma (22). Another study on pancreatic cancer cells demonstrated that the effect of metformin can also be attributed to the downregulation of specificity protein transcription factors (Sp1, Sp3 and Sp4) and specificity protein-regulated genes (bcl-2, survivin, cyclin D1, vascular endothelial growth factor (VEGF) and VEGF receptor, and fatty acid synthase) (23).

Although metformin has potential anticancer effect, its rapid renal clearance and low plasma elimination half life (between 4.0 and 8.7 h) limits its therapeutic application (24). Therefore, an efficient delivery system is required to maintain therapeutic dose of metformin in circulation as well as in the tumour micro environment. For this purpose, in our previous study we have encapsulated metformin into *O*-Carboxymethyl chitosan (*O*-CMC) nanocarrier to finally achieve sustained drug plasma bioavailability as well as Enhanced Permeability and Retention effect of nanoparticles. We have also dealt with *O*-CMC-metformin NPs characterization, hemocompatibilty, *in vitro* cytotoxicity and cell uptake in pancreatic cancer cells (25). The present study is about evaluation of *in vitro* anticancer effect and *in vivo* biodistribution and biocompatibility of *O*-CMC-metformin NPs, to substantiate its potential as a therapeutic alternative for pancreatic cancer.

MATERIALS AND METHODS

Materials

O-CMC [Mwt–122,000 Da; degree of deacetylation (DDA)-61.8%; and degree of substitution (DS)–0.54] was procured from Koyo chemical Co. Ltd., Japan. Metformin, calcium chloride ($CaCl_2$), crystal violet and cardiogreen (Indocyanine green) were purchased from Sigma-Aldrich. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Trypsin-EDTA were supplied by Invitrogen. Human pancreatic cancer cell line (MiaPaCa-2) was provided by National Center for Cell Sciences (NCCS), Pune, India. Acetic acid and methanol were obtained from Merck. The chemicals were used without further purification.

Methods

The *O*-CMC-metformin NPs used for the following studies were prepared according to the protocol reported by our group (25). In the previous study it was reported that the prepared *O*-CMC-metformin NPs with 240 ± 50 nm sizes showed preferential anticancer effect in pancreatic cancer cells compared to normal cells, after it has been uptaken. The hemocompatibilty of the NPs were also proved in the study by *in vitro* hemolysis assay.

In Vitro Cell Migration Assay

The antimigratory effect of *O*-CMC-metformin NPs was studied by cell migration assay. MiaPaCa-2 cells (2×10^5 cells/well) were grown to a cell monolayer. Then the monolayer was scratched with p200 pipette tip and washed with PBS to get rid of cellular debris. Subsequently the cells were treated with 1.75 mg/ml *O*-CMC-metformin NPs (containing 3.7 mM metformin), 1.75 mg/ml bare *O*-CMC NPs and media alone (positive control). The scratched cell monolayers were imaged at different time points (0 h, 8 h, 20 h and 30 h) with optical microscope (Leica, Germany) connected to digital camera at 20× magnification. The scratch recovery by migrating cells was calculated as percentage of scratch closure using the equation:

$$\text{Scratch closure}(\%) = (A_t/A_0) \times 100$$

A_t : scratch area covered by cells at the time-point, A_0 : scratch area at the time zero.

In Vitro Clonogenic Assay

Clonogenic assay was carried out to study the effect of *O*-CMC-metformin NPs on survival and colony formation ability of MiaPaCa-2 cells. Briefly, 5×10^4 cells were seeded in a 6-well plate and allowed to attach. The cells were then incubated with 1.75 mg/ml *O*-CMC-metformin NPs (containing 3.7 mM metformin), 1.75 mg/ml bare *O*-CMC NPs and media alone (positive control) for 72 h. After which the cells were trypsinized and 1,000 viable cells were counted using trypan blue dye exclusion methods followed by seeding in 100-mm tissue culture dishes. Cells were then allowed to grow for 9 to 11 days at 37°C in a humidified incubator supplemented with 5% CO_2. Colonies formed were fixed with acetic acid: methanol (1:7 *v*/*v*), stained with 2% (*w*/*v*) crystal violet solution and counted.

Cell Cycle Analysis

Cell cycle progression in MiaPaCa-2 cells treated with 1.75 mg/ml *O*-CMC-metformin NPs (containing 3.7 mM metformin), 1.75 mg/ml bare *O*-CMC NPs and media alone (negative control) were analysed by flow cytometry after staining with propidium iodide (PI). PI binds to DNA stochiometrically which enable identification and quantification of cells in various phases of cell cycle. MiaPaCa-2 cells (80,000 cells/well) cultured for 24 h in 6 well plates were

treated with samples and incubated for 48 h. The cells were then trypsinized and permeabilized with ice cold ethanol (70%*v/v*) for 1 h followed by PBS wash. After incubating the cells with PBS containing RNase A (0.2 mg/mL) for 1 h, the samples were stained with 50 µg/mL PI at 4°C in dark for 2 h. The cells were then flow cytometrically analyzed in FACS Aria II Beckton and Dickinson, Sanjose, CA by measuring the emission at 575 nm after 488 nm excitation.

mRNA Expression Analysis by qRT-PCR

One step MESA GREEN qRT-PCR MasterMix Plus for SYBR® assay kit (Eurogentec, Belgium) was used to determine the expression of mRNAs related to cell cycle regulation (Cyclin D1, p21 and p27) and metastasis (MMP-9 and Vannin-1) in MiaPaCa-2 cells treated with 1.75 mg/ml *O*-CMC-metformin NPs (containing 3.7 mM metformin) and 1.75 mg/ml bare *O*-CMC NPs for 48 h. Cells without treatment acted as positive control. Total RNA extraction was carried out from the cells using Trizol reagent (Invitrogen) according to manufacturer's protocol. One step qRT-PCR was performed with 100 ng of total RNA and 100 nM of primers using 7900HT Fast Real Time PCR System (Applied Biosystems) by following the kit manufacturer's instructions. The primers used for the analysis were human cyclin D1 primers (sense, ATGCTGAAGGCGGAGGAG; antisense, AGGTGGCGACGATCTTCC), human p21 primers (sense, GGGATGAGTTGGGAGGAG; antisense, AGGTCCAC ATGGTCTTCC), human p27 primers (sense, GAGTGG CAAGAGGTGGAGAAGG; antisense, CCGCTGACAT CCTGGCTCTC), human MMP-9 primers (sense, CCAC TGCTGGCCCTTCTACG; antisense, CGATGGCGTC GAAGATGTTCAC), human vannin-1 primers (sense, GGACAAGAAGCCATGCGATACC; antisense, GTAG CGTGCCACCAGTTTTCC) and human β-actin primers (sense, CCACACTGTGCCCATCTACG; antisense, AGGATCTTCATGAGGTAGTCAGTCAG). β-actin was amplified as inner control and each samples were normalized by its mRNA level. Data were expressed in percentages of mRNA level with respect to control cells.

Preparation of Indocyanine Green (ICG) Tagged *O*-CMC-metformin NPs

ICG tagged *O*-CMC-metformin NPs were prepared by incorporating ICG while NPs synthesis. 5 mL of 0.5% (*w/v*) *O*-CMC solution was stirred for 1 h with 70 µl (1 mg/mL) of ICG and NPs were prepared from it following the preparation protocol of *O*-CMC-metformin NPs as described in literature (25).

In Vivo Studies

Animal experiments were carried out under a protocol approved by Institutional Animal Ethics Committee (Approval no: IAEC/2012/2/8), in adult Swiss albino mice weighing 20–30 g. The mice were housed in plastic cages with paddy husk bedding in a room with ambient temperature and light cycle. The mice were given sterilized laboratory feed and filtered water.

In Vivo Biodistribution Study by Imaging

For biodistribution studies, two groups of mice were used with sample size $n=3$ in each group. Two days prior to the study mice diet was switched from a standard laboratory feed to fresh corn to minimize the auto fluorescence of the feed. For imaging and sample administration the mice were anesthetized with intramuscular injection of 0.02 mL of Xylazine and Ketamine (1: 4) in the gluteal area. Kodak *in vivo* multispectral imaging system FX pro (USA) was used to capture the NIR fluorescence of sample from an emission wavelength of 795 nm to 900 nm after exciting the sample at 780 nm. To obtain fine images, fur from the ventral region of the mice was removed. The mice were imaged prior to sample administration in order to normalize sample fluorescence from the background auto fluorescence of the mice. The mice were intravenously administered with 100 µl of either 1.75 mg/ml ICG tagged *O*-CMC-metformin NPs (containing 3.7 mM metformin) or a concentration of ICG (20 µg/mL) corresponding to that contained in the NPs by means of caudal vein injection. Mice from each treatment groups were then imaged at different time intervals (5 min, 30 min, 1 h, 3 h, 6 h, 24 h and 48 h). After which the mice were euthanized by overdose of anesthesia and organs were collected immediately. The organs were then imaged for fluorescence after washing with saline.

In Vivo Biocompatibility Evaluation by Histopathology

The study was performed in three groups of mice ($n=3$), including a control group (administered with 0.9%*w/v* saline) and two experimental groups (administered with 1.75 mg/ml *O*-CMC-metformin NPs (containing 3.7 mM metformin) and 1.75 mg/ml bare *O*-CMC NPs. The mice were intravenously administered with 100 µl of samples by means of caudal vein injection. After 48 h, the mice were euthanized and their organs such as brain, lung, heart, liver, spleen and kidney were harvested immediately. The organs were then fixed using 10% (*v/v*) neutral buffered formalin embedded in paraffin wax and was cut into 5 µm sections. Then the sections were stained with hematoxylin and eosin, and imaged in light microscope at 40× magnification.

RESULTS

In Vitro Cell Migration Assay

Figure 1 shows bright field images (Fig. 1a) and percentage of wound closure (Fig. 1b) of MiaPaCa-2 cell monolayer at different time points after treatment with media alone, 1.75 mg/ml *O*-CMC-metformin NPs (containing 3.7 mM metformin) and 1.75 mg/ml bare *O*-CMC NPs. The results showed that upon 8 h incubation, the percentage of wound closure was 13.5, 7 and 12% for cells treated with media, bare *O*-CMC NPs and *O*-CMC-metformin NPs respectively. Similarly, the percentage of wound closure was 45, 29.5 and 36% at 20 h and 66, 54.5 and 63% at 30 h for respective samples.

In Vitro Clonogenic Assay

Figure 2 shows colonies formed by MiaPaCa-2 cells after 72 h exposure to media alone, 1.75 mg/ml *O*-CMC-metformin NPs (containing 3.7 mM metformin) and 1.75 mg/ml bare O-CMC NPs. The effect of the NPs on the colony formation ability of MiaPaCa-2 cells was assessed in this experiment. The average number of colonies formed from 1,000 cells were 1,202, 1,008 and 785 for untreated, bare *O*-CMC NPs and *O*-CMC-metformin NPs treated cells respectively.

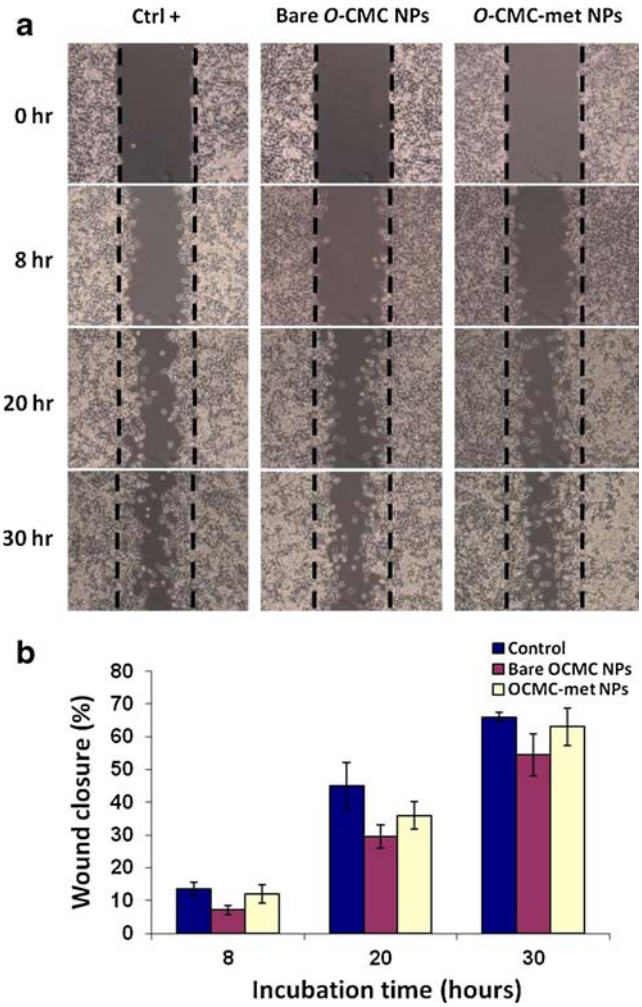

Fig. 1 (**a**) Bright field microscopic images and (**b**) graph showing percentage wound closure of MiaPaCa-2 cells treated with media alone (ctrl +), bare *O*-CMC NPs and *O*-CMC-metformin NPs at different time intervals.

Cell Cycle Analysis

Cell cycle analysis showed the percentage of cells in different phases of MiaPaCa-2 cells without treatment and after treatment with 1.75 mg/ml bare *O*-CMC NPs and 1.75 mg/ml *O*-CMC-metformin NPs. Figure 3a shows flow cytometry histogram and Fig. 3b represents the percentage of cells in different cell cycle phases. In untreated cells, the percentage of cells at G1/G0, S and G2-M phases were 53.4, 11.8 and 7% respectively. Likewise, the percentage of cells were 54.1, 9.8 and 6.9% for bare *O*-CMC NPs and 53.7, 9.5 and 7.4 for *O*-CMC-metformin NPs treated cells, at respective G1/G0, S and G2-M phases.

mRNA Expression Analysis by qRT-PCR

The effect of *O*-CMC-metformin NPs on mRNA levels of different cell cycle regulatory proteins (Cyclin D1, p21 and p27) and metastasis associated proteins (MMP-9 and vanin-1) were assessed by quantitative Real time-PCR. Figure 4 shows fold change in the mRNA expression level of genes after treatment with media alone, 1.75 mg/ml *O*-CMC-metformin NPs (containing 3.7 mM metformin) and 1.75 mg/ml bare *O*-CMC NPs. In bare *O*-CMC NPs treated cells the mRNA level of Cyclin D1, p21, p27, MMP-9 and vanin-1 were 1, 0.59, 0.47, 0.32 and 0.29 fold respectively. Whereas, in *O*-CMC-metformin NPs treated cells the mRNA levels were 1.14, 0.42, 0.92, 0.36 and 0.48 fold for respective Cyclin D1, p21, p27, MMP-9 and vanin-1.

In Vivo Biodistribution Study by Imaging

In vivo biodistribution of ICG tagged O-CMC-metformin NPs was imaged by tracking NIR fluorescence of the dye at different time intervals (Fig. 5a). Within 5 mnts of NPs administration, the NIR fluorescence was detected from an area corresponding anatomically to liver. Then the fluorescence intensity has got increased and expanded further down towards gastrointestinal tract in less than 1 h. The NPs continued gastrointestinal transit through a course of time between 6 and 24 h, subsequently reaching intestinal area. Meanwhile, fluorescent intensity of the NPs got reduced by 24 h and became undetectable by 48 h. Images of *ex vivo* mice organs (Fig. 5b) harvested 48 h after treatment showed presence of NPs in liver, stomach and intestine. Fluorescence image of

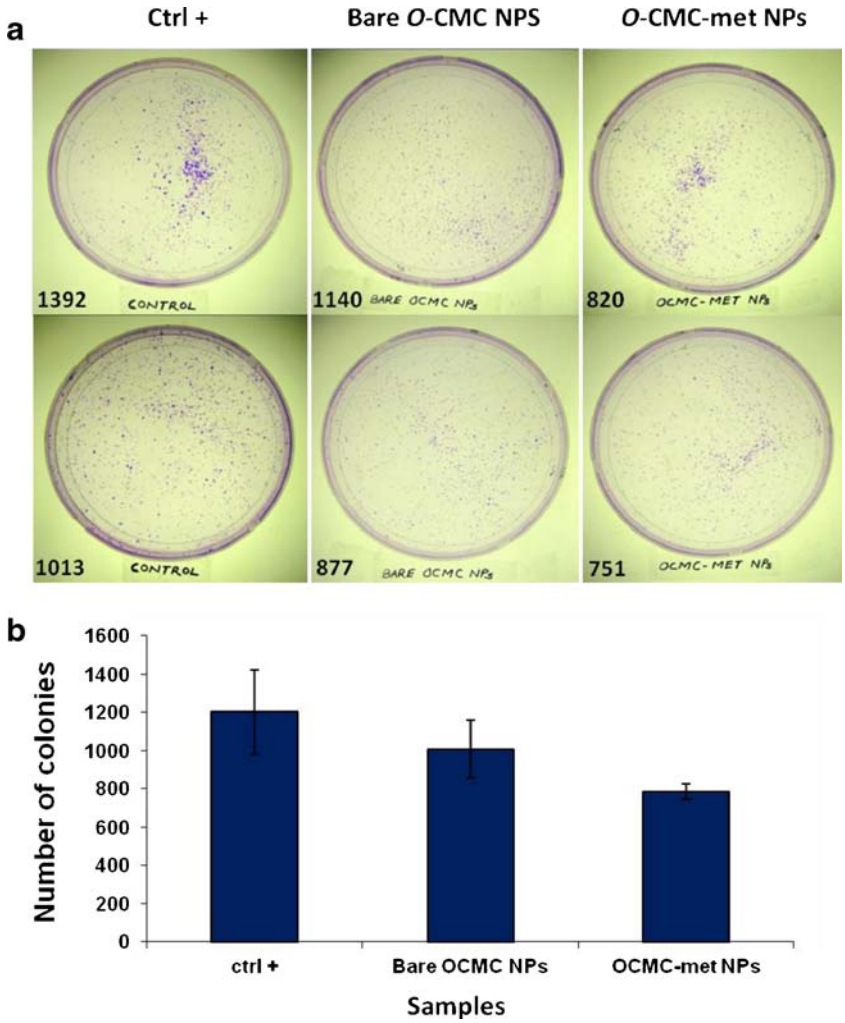

Fig. 2 Colonies formed from MiaPaCa-2 cells treated for 72 h with media alone (ctrl +), bare *O*-CMC NPs and *O*-CMC-metformin NPs are shown with numerations indicating number of colonies (**a**). Graph showing number of colonies formed *versus* samples (**b**).

fecal matter collected after 24 and 48 h (Fig. 5c) indicated excretion of the NPs.

In Vivo Biocompatibility Evaluation by Histopathology

Histopathological assessment was performed in our studies (Fig. 6) to explore whether *O*-CMC- metformin NPs induces any structural alterations and toxicity to organs. We have assessed major organs such as spleen, liver, kidney, brain, lung and heart from mice treated with saline, bare *O*-CMC NPs and *O*-CMC- metformin NPs. Interestingly, *O*-CMC- metformin NPs group showed similar morphological features compared to control groups indicating that the metformin encapsulated *O*-CMC NPs did not produce any adverse effect to the organs. All the treated groups of spleen showed without alterations of Red Pulp, White Pulp, Lymphocytes, Central arteriole and liver showed presence of Kuppfer Cell, Portal Vein, Bile Duct, Hepatocyte. Interestingly, main structural unit of kidney revealed that Distal convoluted tubule formations, Proximal convoluted tubule, Glomerulus. Moreover, brain sections shown the clear Glial cells, Neuron and Lung showed architecture of Alveoli, Respiratory epithelium, Pulmonary vein. Finally, the heart staining resulted Blood vessel, Cardiac myocyte (Fig. 6) altogether suggesting that the *O*-CMC-metformin NPs does not produce any structural alteration to any organs.

DISCUSSION

Metformin or *N*,*N*-dimethyl biguanide is an oral hypoglycemic drug with a remarkable record of safety that has been prescribed worldwide for treatment of Type II diabetes. Metformin also protects against many other diseases especially pancreatic cancer (26-28). It was reported that diabetic patients using metformin had a significantly lower risk for pancreatic cancer than patients using other antidiabetic drugs (29). The typical characteristics of the abnormal proliferation of tumors are out-of-control cell reproduction and growth. Therefore, inhibition of tumor cell proliferation is the key to control tumor development. *In vitro* and *in vivo* studies have

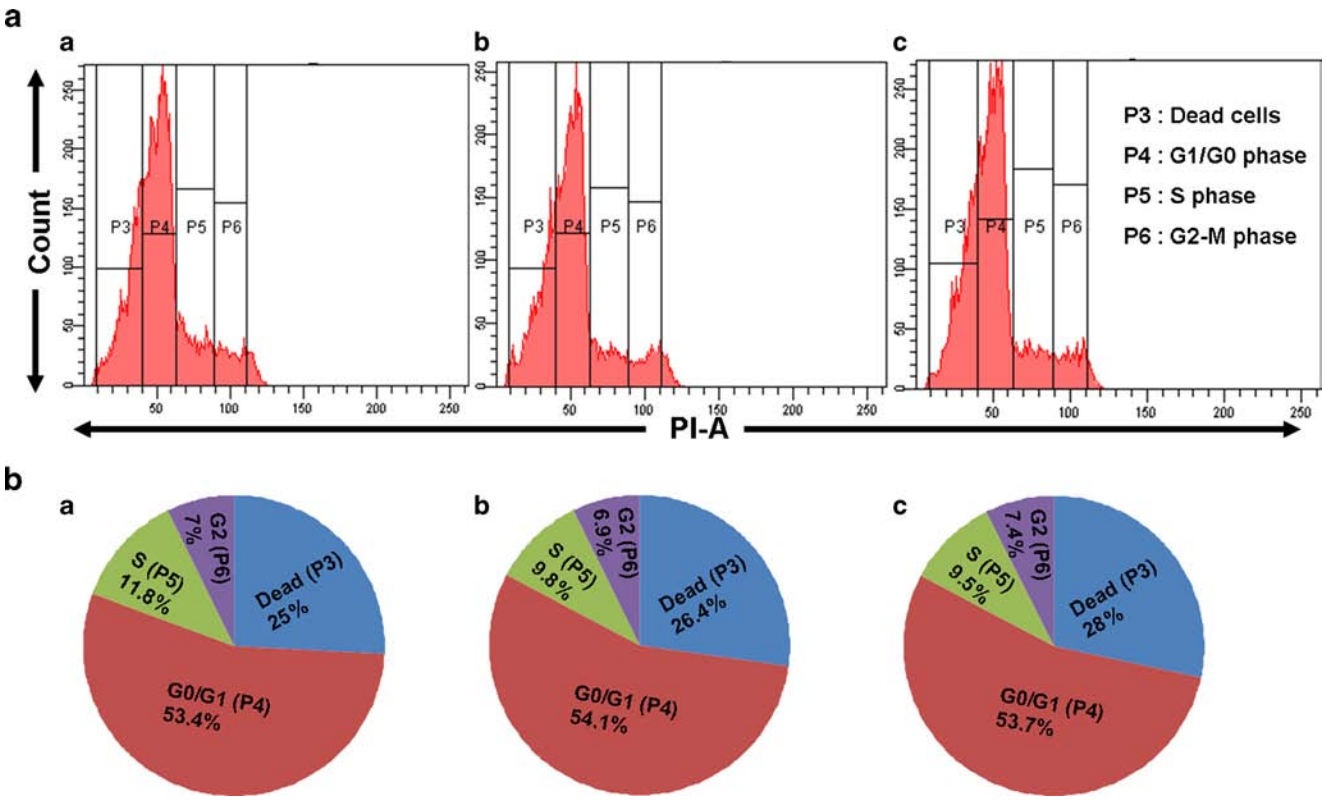

Fig. 3 Flow cytometry histogram showing number of cells in G1/G0, S and G2-M phases after 48 h treatment with (**a**) media alone (ctrl +), (**b**) bare *O*-CMC NPs and (**c**) *O*-CMC-metformin NPs (**a**). Percentage of cells in different phases *versus* samples is represented as pie chart (**b**).

shown that metformin possess potent antitumor activity, including inhibition of cell growth and induction of apoptosis in various tumor cells. The fact that anti-diabetic drug metformin can inhibit molecular pathways such as that of mTOR, particularly during pancreatic carcinogenesis had been reported (4, 9, 12, 16, 17). Nanotechnology has already advanced cancer detection and treatment. Abraxane is a major success story in the treatment of metastatic breast cancer, and has extended the scope of nanomedicine to other cancers and chemotherapeutic drugs (30). Our group studied the delivery of metformin encapsulated in *O*-CMC-polymeric nanoparticles, together with its hemocompatibilty, *in vitro* cytotoxicity and cell uptake in pancreatic cancer cells (25). Another typical characteristic of pancreatic cancer is aggressive metastasis (31), including local invasion to adjacent structures and metastasis to lymph nodes and liver in the very early stages. Therefore, efforts must be focused not only on targeting the primary tumor but also controlling metastasis of pancreatic cancer cells.

In this study, we have explored therapeutic efficacy of metformin encapsulated *O*-CMC nanoparticles on pancreatic

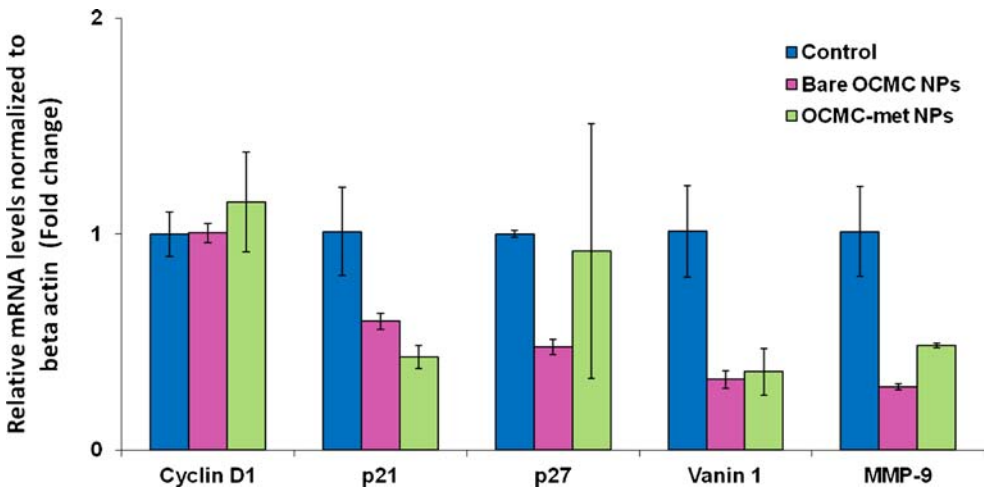

Fig. 4 qRT-PCR graph showing genes *versus* mRNA level fold change after exposure of MiaPaCa-2 cells to media alone, bare *O*-CMC NPs and *O*-CMC-metformin NPs for 48 h.

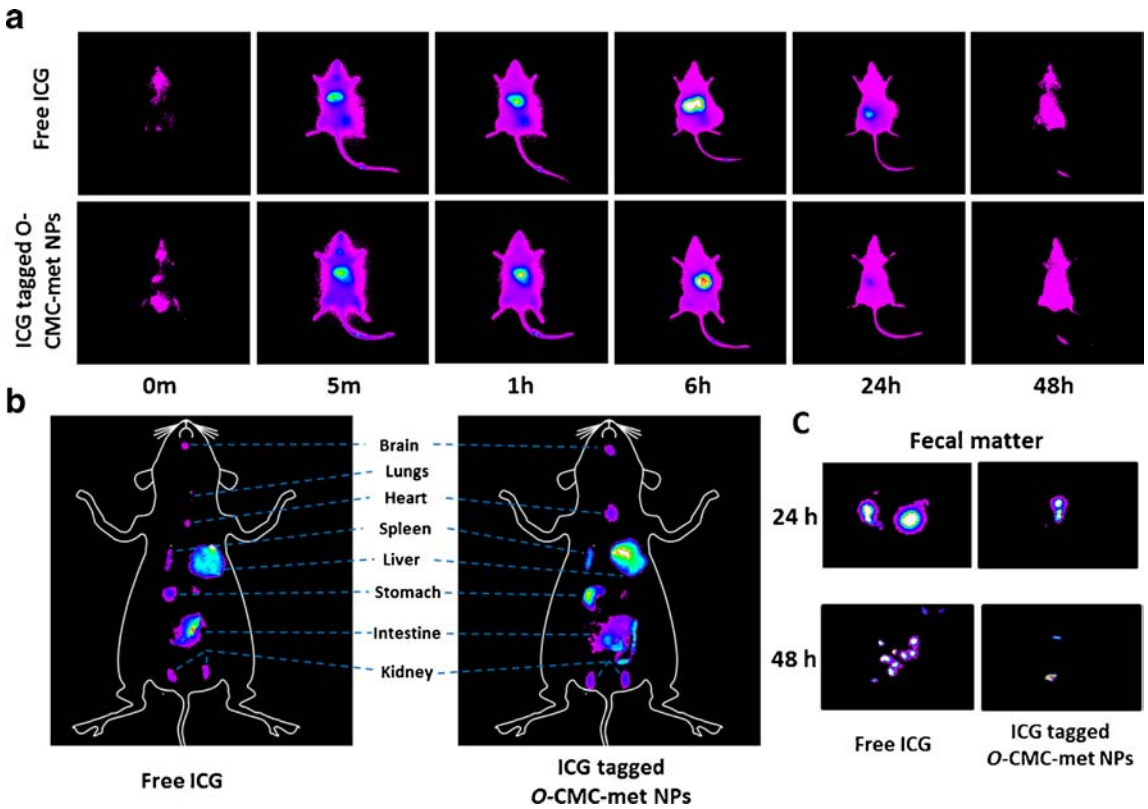

Fig. 5 *In vivo* NIR images of Swiss albino mice after intravenous administration of free ICG (*upper panel*) and Indocyanine green (ICG) tagged *O*-CMC- metformin NPs (*lower panel*) (**a**), *ex vivo* NIR images of organs harvested from mice after 48 h (**b**) and NIR images of fecal matter collected from mice after 24 h and 48 h (**c**).

cancer cells, using different biological assays. First, we examined the effect of *O*-CMC-metformin NPs, on metastasis of pancreatic cancer cells (MiaPaCa-2) by wound healing assay. The results showed time dependent marginal closure of wound in MiaPaCa-2 cells treated with *O*-CMC-metformin NPs compared to the untreated cells for a period of 20 h (Fig. 1). This simple reduction *in vitro* approaches for sample like bare NPs *O*-CMC cannot rely on the complexity of *in vivo* pancreatic tumor migration and invasion that lead to angiogenesis. We further explored effect of *O*-CMC-metformin NPs on proliferation of pancreatic cancer cells by clonogenic assay. We found that metformin *O*-CMC nanoparticles inhibits cell survival by time dependent manner in MiaPaCa-2 cells after 72 h treatment. Therefore, our results showed that the cell migration assay, the marginal reduction in an area covered by the cancer cells after 20 h incubation with *O*-CMC-metformin NPs (36%) compared to untreated sample (45%) showed the less effect of NPs on migration property of the cancer cells on time dependent manner, whereas scratch assay results suggest that the *O*-CMC-metformin NPs could elicit pronounced cell growth inhibitory effect on pancreatic cells which correlates with other studies (20).

Metformin was also reported to have inhibitory effect on cancer cell cycle events (32). Our results showed that the proportions of cells in each phase was not changed in MiaCaPa-2 cells treated with metformin *O*-CMC nanoparticles compared to the controls. However, we hypothesized that the metformin resistance of MiaPaCa-2 caused by minimal changes in cell cycle progression. This indicated that metformin nanoparticles could induce apoptosis of MiaPaCa-2 cells mainly through initiating the endogenous apoptotic pathway. However, it remains unclear how metformin nanoparticles induces apoptosis of MiaPaCa-2 cells by initiating the endogenous apoptotic pathway instead of the exogenous pathway. To understand the effect of *O*-CMC-metformin NPs on the proliferation and tumor progression of pancreatic cancer we investigated mRNA gene expression on MiaPaCa-2 cells after treatment with the NPs. Studies shown that pancreatic cancer proliferation and tumor progression by modulating cell cycle machinery through various genes such as cyclin D1 (an important regulator in the transformation of G1 to S Phase (33), p21, p27 along with candidate gene Vanin1 and matrix metalloproteinase 9 (MMP9) reported to be upregulated in pancreatic cancer associated with type II diabetes patients (34) were selected for the studies.

We therefore examined expression of mRNA levels of cyclinD1 and p27 were not altered whereas p21, vanin, MMP9 expression level was down regulated compared to the native pancreatic cancer cells (MiaPaCa-2) and our results are in line with previous findings (34) suggesting an important role of metformin nanoparticles in cell cycle progression. This *O*-CMC-metformin NPs on gene expression studies merits further investigation in the future.

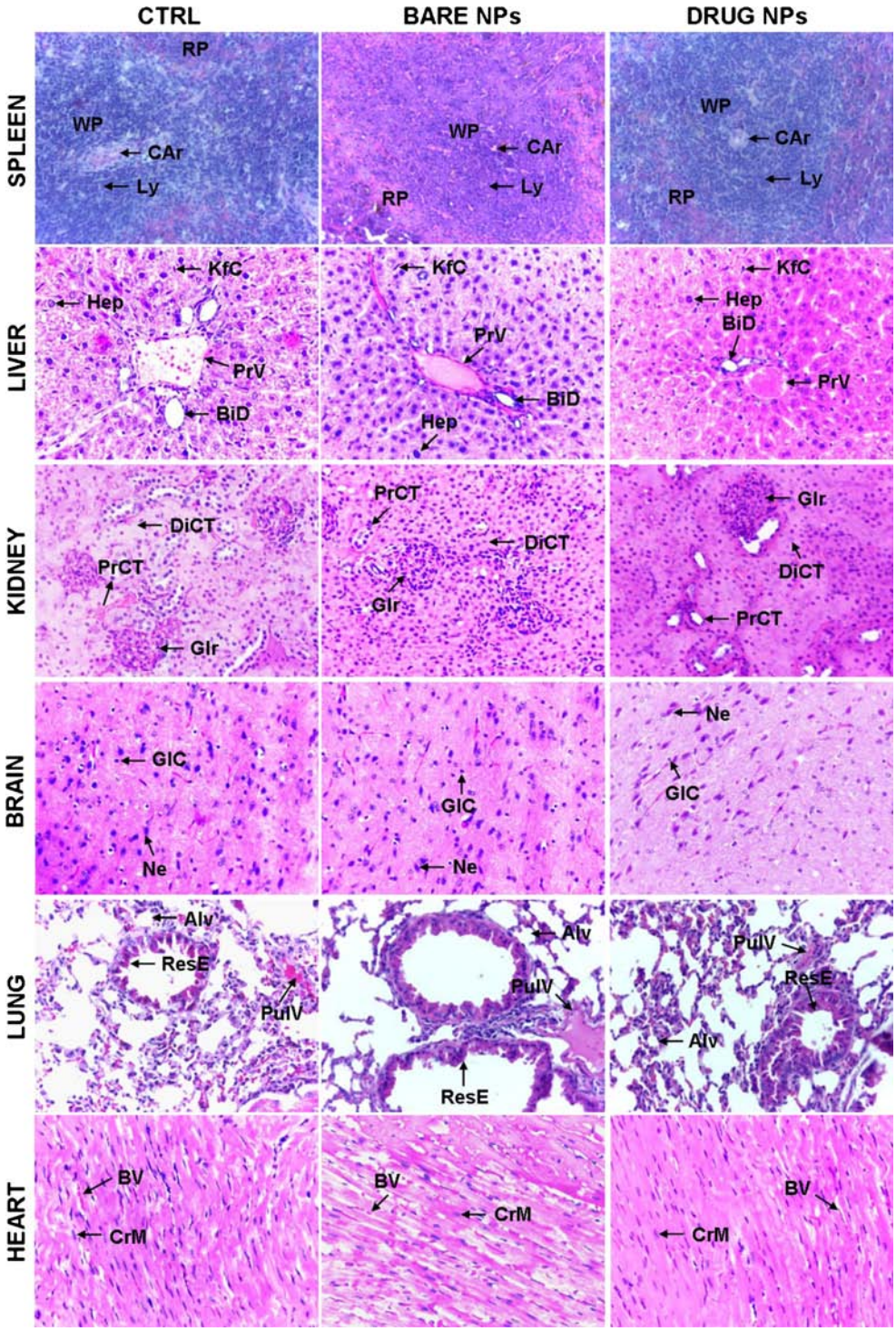

Fig. 6 The light microscopic images of hematoxylin and eosin stained organ sections from mice treated with saline, bare *O*-CMC NPs and *O*-CMC- metformin NPs. Abbreviations in the image indicates: *RP* red pulp, *WP* white pulp, *Ly* lymphocytes, *CAr* central arteriole, *KfC* kuppfer cell, *PrV* portal vein, *BiD* bile duct, *Hep* hepatocyte, *DiCT* distal convoluted tubule, *PrCT* proximal convoluted tubule, *Glr* glomerulus, *GlC* glial cell, *Ne* neuron, *Alv* alveoli, *ResE* respiratory epithelium, *PulV* pulmonary vein, *BV* blood vessel, *CrM* cardiac myocyte.

Polymeric nanoparticles have potential applications in various medical applications (30) yet drug loaded nanoparticles raise biodistribution and toxicity concerns because they can remain in organs such as liver and spleen for prolonged periods of time without being cleared from the body through reticuloendothelial system. Therefore, we investigated *in vivo* biodistribution of *O*-CMC-metformin NPs in normal Swiss albino mice and compared with the literature where majority of ICG got cleared from mice within 20 h of intravenous administration (35). The study results showed that similar to free ICG, the *O*-CMC-metformin NPs passed through Hepatobiliary route, followed by excretion through faecal matter. In short, *O*-CMC-metformin NPs had normal biodistribution pattern which was comparable to that of free ICG with no significant *in vivo* accumulation.

Several studies proved *in vivo* biocompatibility of bare metformin towards organs such as liver, kidney, heart in animal

models (36-39). We performed *in vivo* biocompatibility evaluation of metformin NPs by histopathology which showed normal cellular architecture of mice organs such as spleen, liver, kidney, brain, lung and heart. Moreover, we observed the absence of structural alteration or toxic effect in mice organs including kidney substantiated *in vivo* biocompatibility of the *O*-CMC- metformin NPs suggesting similar structural morphology and integrity with well formed glomerular tuft, distal convoluted tubule and proximal convoluted tubule were normal compare to control groups indicates that Metformin *O*-CMC NPs is not producing any adverse effect to the functional units of organs.

CONCLUSIONS

Our findings of *in vitro* evaluations of metformin encapsulated *O*-Carboxymethyl chitosan nanoparticles revealed (1) marginal delay in wound closure properties compared to untreated cells and decreased colony formation in MiaPaCa-2 (pancreatic cancer cells) with the treatment of *O*-CMC-metformin NPs. Rather than antimigratory effect, inhibition on clonogenic ability of pancreatic cancer cells might play a specific role in the therapeutic applications of *O*-CMC metformin nanoparticles against pancreatic cancer. Furthermore, (2) there is no changes in the cell cycle events such as G1/G0, S and G2-M phases after 48 h treatment with *O*-CMC-metformin NPs could induce apoptosis of MiaPaCa cells mainly through initiating the endogenous apoptotic pathway. Interestingly, (3) at the mRNA level, expression of certain genes such as p21 and vanin 1, MMP9 were downregulated in comparison to that of cyclinD1, p27 in the pancreatic cancer cells after 48 h treatment with *O*-CMC-metformin NPs suggesting an important role of metformin nanoparticles in cell cycle progression. (4) The results of *in vivo* biodistribution study with metformin encapsulated *O*-CMC nanoparticles suggested normal distribution of the NPs in mice. (5) Histopathology analysis revealed that the NPs had no adverse effect to the major organs (spleen, liver, kidney, brain, lung and heart) of mice. Therefore, our results suggest that the *O*-CMC-metformin NPs addressing the role of clonogenicity and gene expression pattern on pancreatic cancer cells opened an avenue to be explored further on the cell cycle protein levels and various kinases and phosphorylation events. Furthermore, combination studies with metformin and chemo drug have to be pursued in the hopes of increased antitumor effects attributable to each individual drugs broad range of clinical activity and different mechanism of action may demonstrate the concept of cell cycle mediated drug effects on pancreatic cancer cells. In future, this metformin nanoparticles may be useful to explore preclinical animal models for assessing its efficacy and can be explored further with combination of chemo drugs in preclinical studies and subsequently in the management of pancreatic cancer.

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