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Research Article

New Oral Delivery System to Improve Absorption of Berberine: Likely Interaction of Cationized Chitosan with PG-P Pump.

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ABSTRACT
Berberine Chloride (BC) is an isoquinolinic alkaloid that is extracted from plants of generis Berberis. During the last decade, researchers and clinicians have paid increasing attention to BC, because of its impressive hypoglycemic and blood lipids lowering properties. Several clinical studies gave proof of evidence regarding BC efficacy in humans and pharmacological mechanisms of action, related to the previously mentioned activities, have been proposed and substantially confirmed. On the other side, BC shows a very poor oral bioavailability mainly because of the interaction with P-Glycoprotein (Pg-P) pump, which extrudes BC from inside to outside of the enteric cell. This paper describes a novel oral delivery system containing a Chitosan-N-AcetylCystein salt capable to interact with Pg-P, partially inhibiting BC extruding process. Preliminary data confirming the aforementioned postulated mechanism on Caco-2 in vitro model have been herewith reported and discussed.
Key words: Berberine chloride; Chitosan; N-Acetylcysteine; Pg-P; Tight Junctions; Bioavailability

INTRODUCTION

Berberin in an isoquinolinic alkaloid that is extracted from several plants of generis Berberis such as Berberis vulgaris and aristata. Traditionally, the use of Berberis extracts to treat several diseases, especially in traditional Chinese medicine, can't be traced back. Currently Berberis extract and especially BC, the main active component identified, are widely employed to approach several diseases and clinical conditions characterized by inflammation and immune-based inflammatory pattern. Nevertheless, BC has been receiving increasing attention by the scientific community as blood lipids lowering and glucose tolerance increasing molecule[1,2].

Despite the numerous further clinical indications mentioned for BC and reported in more or less serious papers, these latter are the most scientifically proved and substantiated by impressive pharmacological and clinical data.

Lipid lowering activity of BC

Several papers published during the last years postulated plausible pharmacological mechanisms of action for lipid lowering activity and glucose tolerance enhancing activity of BC. Concerning lipids lowering action, BC is believed to enhance Low Density Lipoprotein Receptors (LDLR) mRNA expression post-trascriptionally[3,4,5]. This mechanism, almost partially, could explain the synergistic effect of BC with statins[6] and with other vegetal derived actives such as polycosanols, vegetal sterols and red rice yeast extract in lowering blood lipids as some clinical investigations reported[7,8]. The mechanism involved in BC lipids lowering action, indeed, does not regard the partial inhibition of HMG CoA reductase as statins and red yeast extract do[9], neither concern to the competition mechanism of cholesterol enteric absorption like vegetal sterols show[10]. According to this plausible mechanism in blood lipids lowering, BC can potentially represent an interesting weapon to reduce blood LDL in patients who experienced muscular side effects during statins based therapy or showing different pharmacological contraindications. More investigations are however needed to clarify the real mechanism of action involved in lipid lowering activity of BC and most likely more than one will be identified confirming a synergy of biochemical events.

Glucose tolerance enhancing activity of BC

Some papers recently published report that BC is effective in animals and in vitro models to approach diabetes mellitus[11,12] and convincing clinical evidences on humans begin to be collected[13,14,15,16].

In a recent clinical investigation, human adults with a recent diagnosis for type 2 mellitus diabetes received daily and randomly BC and Metformin for a period of 90 days and the authors' conclusions were that both the molecules are similarly effective to reduce blood glucose. Particularly, authors registered and emphasized remarkable reduction in Hemoglobin A1c, fasting blood glucose, post-prandial blood glucose and plasma triglycerides in patients who received BC[16].The most plausible mechanisms of action involved in the hypoglycemic activity of BC seem concern aldose reductase inhibition,[17] glycolysis induction[18] and insulin resistance prevention through increasing gene expression of insulin receptors[19,20]. Another convincing hypothesis

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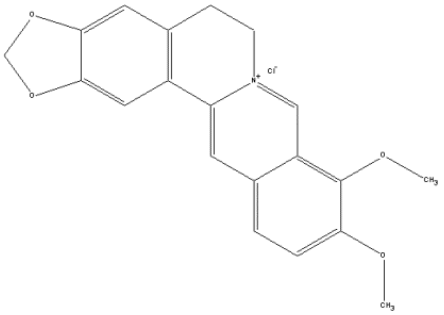

Fig 1: Structure of Berberine Chloride

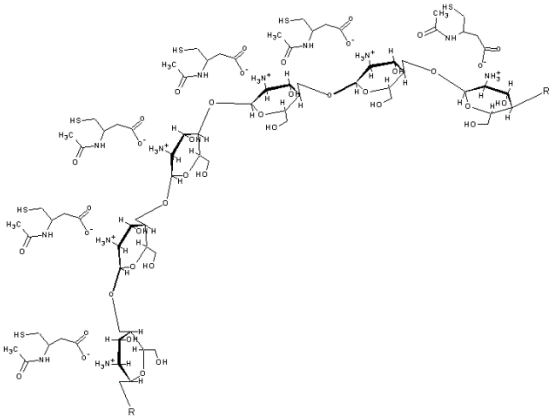

Fig 2: Chitosan cationized by NAC. Carboxylic group of NAC protonates aminic group of Glucosamine so producing a polycation.

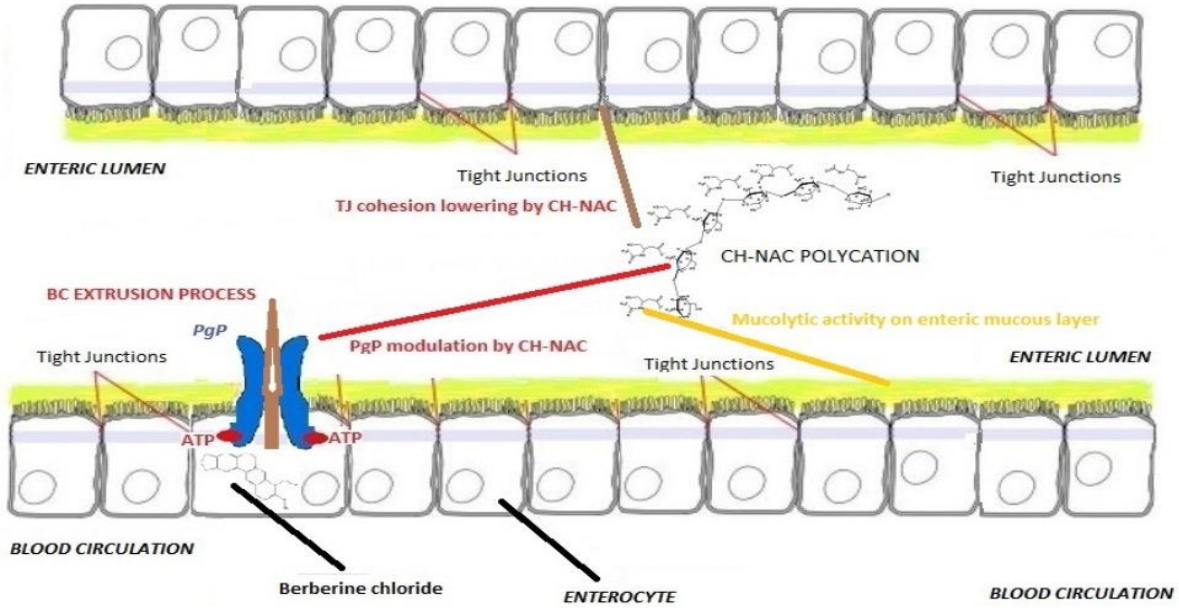

Fig. 3. Postulated mechanism of action of Enterosoma™ in modulating PgP activity and TJ cohesion lowering.

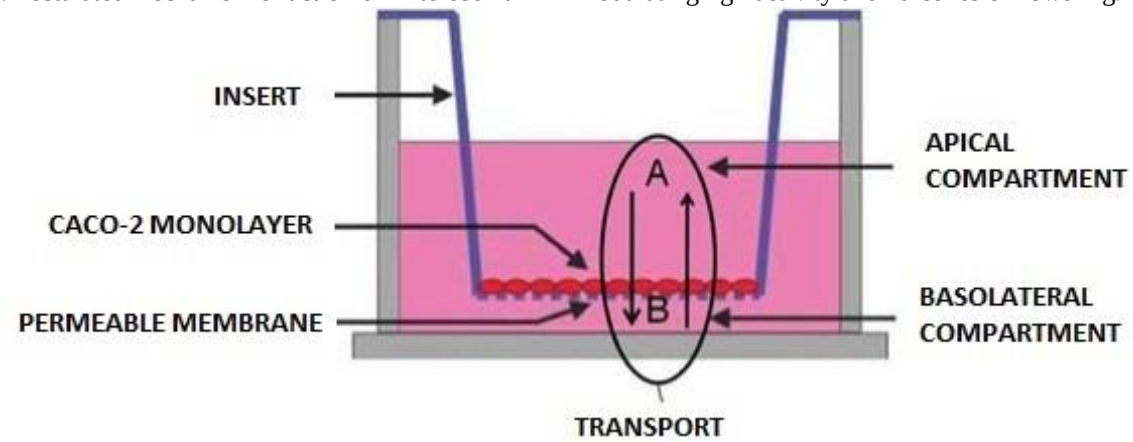

Fig. 4. Scheme of the device employed to assess CACO-2 intestinal absorption.

substantiated by scientific proof of evidence is that BC may prevent insulin resistance via modulating the release of key molecules involved in insulin signaling pathway, leading to increased glucose uptake in insulin-resistant cells[21]. A further mechanism behind the hypoglycemic activity of BC might be the up-regulation of Hepatocyte Nuclear Factor 4 Alpha (HNF4A) expression, which probably acts modulating glucokinase activity and rendering Langerhans pancreatic cells more responsive to glucose fluctuation[22]. For last, a recent biochemical investigation based on advanced metabonomic method and performed on plasma of 60 type 2 diabetics, evidenced a

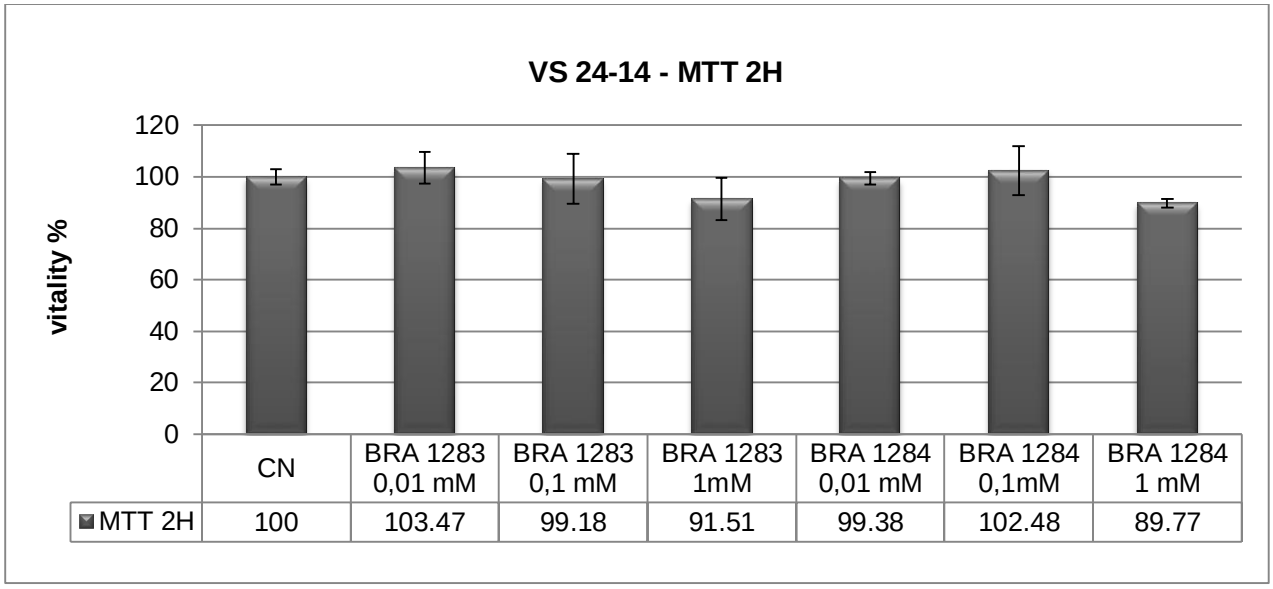

Fig. 5. MTT test after 2 hours of incubation in presence of BC at three different concentrations: 0,01mM / 0,1mM / 1mM

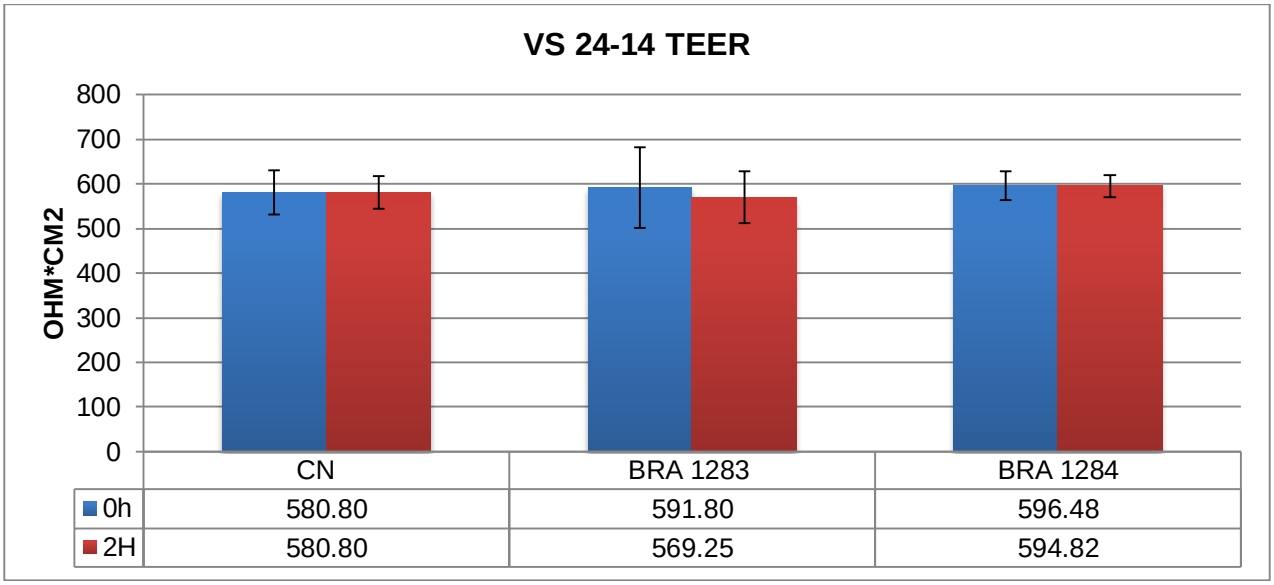

Fig.6. Measure of TEER after 2 H treatment with the samples BRA 1283 and BRA 1284 (1 Mm)

remarkable reduction in the high level of free fatty acids in patients receiving BC respect to those receiving placebo[23]. As notorious, free fatty acids are known to be toxic to pancreas tissue and to trigger insulin resistance[24]. Summarizing, accordingly to the most recent clinical and biochemical investigations, BC can be plausibly considered a shiny promise regarding Diabetes Mellitus and Metabolic Syndrome approach and could play an eminent role in the management of these chronic and socially relevant pathologies in the next future.

BC oral bioavailability in humans

Beside these encouraging facts, a big open challenge remains to engage regarding BC: the very low oral bioavailability. It is notorious indeed that BC is a water soluble, but poorly absorbable molecule through the enteric tract[25]. This is most likely due to the presence in BC structure of an ammonic quaternary function that makes it poorly absorbable by the enterocyte (Fig. 1).

Among the numerous speculations surrounding the chemical and biochemical mechanisms involved in BC poor bioavailability, the role of Pg-P is believed to be essential. There is evidence, indeed, that the major mechanism involved in BC poor bioavailability relates to the Pg-P pumping activity and reasonably, a significant amount of BC oral dosage assumed is extruded from inside the enteric cells into the lumen so missing the entry into the blood circulation[26,27]. Pg-P protein is a cell-membrane ATP-dependent efflux protein, broadly expressed in biological organisms and particularly in animals. This protein is extensively expressed in the human intestinal epithelium where it works pumping xenobiotics back from the enteric cell into the lumen. This pump protein plays a pivotal role in reducing bioavailability of several

molecules employed in clinical therapy and particularly antineoplastic, corticosteroids, antiretroviral drugs[28,29,30]: for this reason, it takes the name of Multidrug Resistance Protein (MDRP).

Beside this mechanism, considered the most relevant to explain poor enteric absorption of BC, the blockage by the side of enteric Tight Junctions (TJ) must be considered. TJ are the closely linked areas of two enteric cells whose membranes merge forming a virtually impermeable barrier. These structures reduce or even block the absorption of several molecules intended to human health[31, 32].

Enterosoma™: novel technology to improve BC absorption

A novel oral delivery system has been projected aiming to improve BC absorption along enteric tract. The main concept surrounding this new technological system is the evidence described in many papers speaking about property of Chitosan (CH) to interact with both enteric TJ[33,34] and PgP[35,36]. These phenomena are thought to be directly connected to the capability of CH and particularly cationized and quaternized chemical derivatives of CH, to improve absorption of poorly absorbable molecules. Particularly, quaternized derivatives of CH such as trimethyl Chitosan (TMC) showed to be an effective enteric absorption enhancer for several drugs, on Caco-2 cells in vitro model[37,38,39]. The mechanism of action appears to regard the interaction of positive charges expressed by quaternized or cationized CH with the negative charges expressed by carboxylic groups of TJ proteins. This interaction is believed to change the steric tertiary structure of the aforementioned proteins leading to an impaired TJ seal.

Starting from these evidences and premises a novel oral pharmaceutical form has been developed. The mentioned technology (Enterosoma™, Labomar Research Patent Pending) consists of a gastro-resistant tablet containing, in the inner core, besides BC, a CH polymer and an organic acid that once dispersed into the enteric fluids produce CH cationization. CH is a polyaminosugar not soluble in water except for acidic water solution (pH<5). Acidic groups, indeed, protonate amino groups of glucosamine units present in the CH structure, taking place to a poly-cationic ammonic molecule. Particularly, the acidic molecule selected to project this technology is N-Acetylcystein (NAC) that is a sulphureted N-Acetylated aminoacid very notorious as mucolytic agent (Fig. 2).

NAC has been chosen because of its double chemical behavior both acidic and mucolytic. Mentioning this well documented mucolytic activity, some published papers refer about the enteric absorption enhancing properties of NAC strictly connected to its own capability to reduce enteric mucous viscosity[40, 41]. NAC reduces mucous viscosity cleaving the S-S bounds of muco-proteins and according to this mechanism, it works efficiently in reducing viscosity of the enteric covering mucous layer though to prevent active compounds to be absorbed. This polycationic structure is thought to be the keystone to achieve both Pg-P activity modulation and TJ cohesion lowering as described (Fig. 3).

The powder core of the tablet comprising BC, CH and NAC is further moistened with Polysorbate 80 (PS 80), a notorious high HLB surfacting agent with ascertained activity of enteric and mucosal absorption enhancer, synergistic with NAC[40,41,42]. The mechanism involved seems regard the deterging action of PS 80 onto the cell membrane lipids. For last, the described tablet nucleus is coated with a gastro-resistant film to avoid premature cationization of CH occurring in the stomach by the side of gastric chloride acid that would compromise the enteric absorption enhancing effect. Summarizing, according to the aforementioned considerations, a synergy between interaction with Pg-P and TJ exerted by cationized CH and mucolytic activity on enteric mucous layer exerted by NAC are the main deliverables of this new technology

MATERIAL AND METHODS

Lucifer Yellow was purchased from Sigma Aldrich (L0144). All the other reagents were from Lonza. BC was purchased from, Nutraceutica, Italy CH was purchased from DKSH, India. NAC was purchased from Polichimica, Italy. PS 80 was purchased from Heingmann-Veronelli, Italy

Instruments and analytical devices

NAME	SUPPLIER
ANALYTICAL SCALE XS 204	METTLER -TOLEDO
INCUBATOR CO_2 HERA CELL	HERAEUS
Microplate Autoreader M200-INFINITE	TECAN
ERS - Millicell	MILLIPORE
BUERKER CHAMBER	MARIENFELD

Reagents

NAME	BATCH	SUPPLIER
DMEM	RNBC8863	LONZA
GLUTAMMINE	2MB189	LONZA
PEN/STREP	2MB251	LONZA
NEAA SOLUTION 100X	1MB268	LONZA
FBS	EU50132207	LONZA
HBSS	RNBC5907	LONZA
HEPES 1M	1690	LONZA
MES	M2933	SIGMA
LUCIFER YELLOW	MKBH0593	SIGMA
HBSS – 1% MES	APICAL	Prepared in laboratory and immediately used
HBSS – 1% HEPES	BASOL ATERAL	

Cell culture for preliminary cytotoxicity assay

Caco-2 cell line were used for cytotoxicity experiment (MTT assay).

Table 1*:* LY paracellular flux values after 2h treatment with BC are reported

	NC	BC ENTEROSOMA (BRA 1283)	BC PLACEBO (BRA 1284)	ACCEPTABILITY VALUES
LY FLUX %	0,363% ± 0,023	3,294% ± 0,231	0,77% ± 0,030	≤ 0,7% (negative control) ≤ 10 %

Table 2: PASSAGGE A-B (Papp)

	µM applied on the surface	µM 30 min BL	µM 2h BL	µM 2h AP	µM HMG	µM TOTAL	AVERAGE MB%
BRA 1283	911,38	0,11	4,03	164,11	23,01	191,26	20,99±*0,50*
BRA 1284	966,83	0,07	0,39	186,95	20,30	207,70	21,48±*0,07*
	% RECOVERY RESPECT TO THE THEORETICAL VALUE (1000µM)	PAPP (cm/sec)					
BRA 1283	91,13%	$1,518*10^{-6}$ ±0,168					
BRA 1284	96,68%	$0,139*10^{-6}$ ±0,072					

Table 3: Data collected after active transport process (B-A) simulation test.

	µM applied at baso-lateral side	µM 30 min AP	µM 2h AP	µM 2h BL	µM HMG	µM TOTALE	AVERAGE MB%
BRA 1283	935,37	0,36	2,61	590,64	0,93	594,54	63,56±*0,40*
BRA 1284	970,02	0,66	4,81	601,67	2,00	609,14	62,80±*1,53*
	% RECOVERY RESPECT TO THEORETICAL VALUE (1000µM)	PAPP (cm/sec)					
BRA 1283	93,53%	$0,328*10^{-6}$ ±0,032					
BRA 1284	97%	$0,571*10^{-6}$ ±0,085					

Caco-2 cellular line were purchased from the American Type Culture Collection (ATCC HTB37). Caco-2 are intestinal cells derived from human colon-rectal adeno-carcinoma. The cell monolayer has a spontaneous differentiation: the differentiated cells polarize, form microvilli, tight junctions and secrete enzymes associated with the enterocyte brush border. Caco-2 monolayers can display electrical properties typical of either small intestinal or colonic enterocytes: the model is accepted as a reliable *in vitro* model system for the study of human intestinal permeability. This model is therefore useful to evaluate a large number of drug candidates for their intestinal absorption and represent an appropriate model intended to the study of transport mechanisms related to the intestinal barrier and for investigating nutrients bioavailability and absorption.

These cells were grown in Dulbecco's modified Eagle's medium (D6546, Sigma) supplemented with 10% fetal bovine serum (EU50132207 Euroclone), 1% nonessential amino acids (13-114E, Lonza), Hepes 10mM (17-737E Lonza) and 4 mM glutamine (17-605E Lonza) with 1% Pen/Strep solution (Lonza). The monolayer cultures were grown in a CO2 incubator (5% CO2) at 37°C (Artursson, Pet al., K., 2001)

Cytotoxicity Assay

The MTT assay is a colorimetric assay for measuring cytotoxicity by using the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. Caco-2 cells were seeded then in 96-well culture plates at a density of 120.000 cell/well. The cells were treated with three different concentration of BC from BRA 1283 (Enterosoma^{TM}) and BC from BRA 1284 (placebo): 0,01-0,1-1 mM in triplicate for 2 hours. After incubation the medium was replaced with 200 µl of MTT (M2128, Sigma) 0,5 mg/ml. After 3 hours at 37 °C, the medium was aspirated and replaced with 200 µl of isopropanol,

incubated for 10 minutes under agitation and absorbance was read at 570 nm.

Intestinal passage assay on CacoReady™

CacoReady™ is a ready-to-use model for in vitro intestinal absorption evaluation from Advancell.

The kit provides a 21-days cell barrier in integrated HTS Transwell plates with an exclusive and proprietary shipping medium that is stable at room temperature. Prior to each experiment, CacoReady™ inserts were washed with transport buffer. Trans-Epithelial Electrical Resistance (TEER) values were measured to check monolayer integrity. A TEER value higher than 1000 Ω indicates that the barrier system is acceptable for an absorption assay. In this experiment TEER value higher than 500 Ω cm^2 have been found before and after 2h treatment. CacoReady cell line was shipped on day 13 of differentiation and provides 14-day polarized cultures of Caco-2 cells. CacoReady™ can be used up to 9 days after receiving by changing complete medium and by measuring TEER according to the internal procedure.

Trans-epithelial electric resistance assay (TEER)

To evaluate the integrity of the monolayer, TEER was measured using a Millicell-ERS (Millipore). TEER of the filter was subtracted from the total TEER measurements of CacoReady™ cell epithelia.

TEER is a direct measure of skin and enteric epithelium barrier function: it reflects the overall strength of the tissue due to both its structure and thickness and it measures barrier integrity at the level of TJ.

Lucifer yellow assay

Another parameter to evaluate integrity of the monolayer, Lucifer yellow (LY) permeability, has been performed. LY is a fluorescent dye impermeable to the cell membrane. It is mainly used to study the paracellular permeability of a substance. When the junctions are unbroken, LY shows a very low permeability, on the other side, if the joints are damaged, LY flow is much more higher. Therefore, this assay is used to verify the integrity of cell junctions in the presence of the substance to be evaluated. LY was applied in the apical compartment at a concentration of 100 µM after exposure to the substance (1mM of BC) to be tested (the highest non-cytotoxic MTT test concentration) dissolved in HBSS-1% MES buffer (0,25 ml). 0.75 ml of HBSS-1% Hepes buffer was added in basolateral compartment. The transport of LY was assessed as a switch from apical to the basolateral compartment after a defined incubation period of 1h at 37 ° C.

The reading was performed by mean of a spectrofluorimeter (TECAN INFINITE M200) set up at 428 nm excitation and emission 535 nm. The measurement of fluorescence (RFU) was taken at apical and baso-lateral level and flux and permeability were calculated according with the following formulas:

LY Flux= (RFU_{BL} / RFU_{AP}) x 100

RFU_{BL}=value of Fluorescence at basolateral side

RFU_{AP}=value of Fluorescence at apical side

In a monolayer, the LY flow is less than 0,7% and/or the Apparent Permeability Coefficient (Papp) is less than 1 x 10^{-6} cm /sec for not treated tissues.

CacoReady Intestinal Absorption Assay

CacoReady™ at 21th day of differentiation has been used for the test. Each product has been tested in triplicate. Test items at the highest defined non-cytotoxic concentration (1mM) in HBSS-1% MES (pH 6,5 to reproduce the first enteric tract low acidity) or HBSS-1% HEPES (pH 7,4 to reproduce small intestine) have been applied respectively on the apical compartment (A) (0,25mL) (Passive Transport: A>B) or basolateral compartment (B) (0,75mL) (Active Transport: Efflux: B>A).

Samples were taken from the Apical t=0 (A>B) or Basolateral (B>A) side at 30 and 120 min post-dosing, and the volume withdrawn was replaced with fresh transport medium, which was corrected for further calculations. Following the transport study, each Transwell filter with cells was placed in 0.5 ml of water in order to lyse the cells. All the samples were stored at 4 °C until the performing of the analysis by HPLC-UV. The steady-state flux was estimated from the slope of the linear portion of a plot of cumulative amount of the drug that appeared on the basolateral or apical side versus time. The apparent permeability coefficient (Papp, in centimeters per second) was calculated from the experimental data using the equation:

$Papp = dQ/dt*A*C_0$.

C_0 is the initial drug concentration in the donor phase and A is the surface area of the filter (1 cm^2)

dQ/dt is the change in drug concentration in the receiver phase per unit time (micrograms per milliliter per second). The B-A Papp has been compared to the A-B Papp for the same test compound. The ratio of B-A Papp to A-B Papp is designated as the efflux ratio (ER). Compounds with an ER value greater than 2 are likely to be transported by one or more of the efflux systems and, as a result, their net absorption is most likely controlled by intestinal secretion.

HPLC and chromatographic conditions

The analytical study has been performed and validated for LOQ and LOD.

Samples have been analyzed on a Liquid chromatograph AGILENT series 1200 with Chemstation. Column: Phenomenex KINETEX C18, 5 µm, 250 x 4.6 mm, according to the following chromatographic conditions:

Eluent: Water-Acetonitrile (1:1)

Column temperature: 40°C

Detector wavelenght: 345 nm

Flow: retention time of berberine is about 10 minutes

LOQ=0,25mg/L

LOD=0,1 mg/L

NAME OF THE ACTIVE	**BERBERINE CHLORIDE**	**BERBERINE CHLORIDE**
NAME OF THE SAMPLE TESTED	**ENTEROSOMA TM**	**PLACEBO**
UNIVOQUE	BRA 1283	BRA 1284
BATCH CODE		
ASPECT OF THE SAMPLE TO BE TESTED	Clear water solution Yellowish-Orange	Clear water solution Yellowish-orange
STORAGE	25°C	25°C
pH (range)	4,5-4,9*	5,5-6,5
BERBERINE MOLECULAR WEIGHT	371,81 g/mole	371,81 g/mole
CONCENTRATION USED for MTT	0,01 / 0,1 /1 mM	0,01 / 0,1 /1 mM
CONCENTRATION USED for the PERMEABILITY TEST	1mM	1mM
QUALI-QUANTITATIVE DESCRIPTION OF THE SAMPLES TO BE TESTED (mg/100g)	Berberine HCl (220mg) Polysorbate 80 N-Acetylcysteine Chitosan Water	Berberine HCl (220mg) Water

220mg/100g=5,9mM

Main chemical and physical features of the samples tested

*Acidity is due to NAC, which is the acid necessary to cationize Chitosan

Scheme: Summarizing scheme of the chemical and physical features of the samples tested.

Sample BRA 1283 (ENTEROSOMA^{TM}) appears as a clear yellowish-orange solution with a slight sediment easy to disperse for simple agitation (most likely due to the uncompleted hydration of CH). This water dispersion simulates the dissolution of BC into the enteric fluids after tablet disaggregation containing the excipients reported in Scheme. 1. Sample BRA 1284 (PLACEBO) appears as clear yellowish-orange solution. BC is a water soluble molecule. The difference in pH of the two reported samples to be tested can be attributed to the presence of NAC in BRA 1283 (ENTEROSOMA^{TM}) which acidifies the system permitting CH cationization and dissolution.

RESULTS

Evaluation of BC citotoxicity at three concentrations – MTT

Both the samples (BRA 1283 and BRA 1284) have been tested at three different concentrations for 2 hours (Fig. 5). For all the reported concentrations BC from BRA 1283 (ENTEROSOMA^{TM}) and BC from BRA 1284 (PLACEBO) have not shown cytotoxic effect with an overall vitality data comparable to the negative control. A dose-dependant effect has not been evidenced. According to the above mentioned data, the higher not cytotoxic concentration of BC is 1 mM. Paracellular passage: evaluation of the TJ toxicity through TEER and LY flux measurements. All the inserts prepared for the study have shown initial TEER values ≥1000 Ω*cm^2 and therefore valid to evaluate BC from BRA 1283 (ENTEROSOMA^{TM}) and BC from BRA 1284 (PLACEBO) toxicity by mean of LY flux measure assesment. The inserts have been treated for 2 hours in presence of the described samples. At the end of the treatment, 0,1 mM of LY has been introduced. The insert negative control (NC) has been added with the sole LY. No significant differences in terms of TEER values have been found and registered after treatment with BC from BRA 1283 (ENTEROSOMA^{TM}) and BC from BRA 1284 (PLACEBO) in comparison with the cells not treated (NC) (Fig. 6). The results reported confirm a neutral action of the tested samples on the TJ structure with slightly different values regarding LY permeability

Permeability test for the dosage of Berberine

The results regarding BC dosage coming from BRA 1283 and BRA 1284, achieved through HPLC-UV method are reported and summarized in Table II (in triplicate). Analytical results revealed a slightly lower concentration of BC in the solution employed for the study respect to the calculated theoretical one, present in the 1 mM sample with a recovery rate not lesser than 91%. In the following tables are reported the two main transport processes (passive A-B, Tab II and active B-A, Tab III) involved in BC absorption from samples BRA 1283 and BRA 1284 considering the distribution in the different compartments.

TAB.II. Data collected after passive transport process (A-B) test.

The mass balance (MB) that is to say the percentage of BC recovered in the different compartments at the end of the intestinal passive passage A-B, is resulted to be low. No further investigation has been carried out. Nevertheless, it was possible to calculate the Apparent Permeability Coefficient (P_{app}), considering the mass values measured out in the single compartments.

Analysis of Berberin distribution in different compartments

Both the samples tested evidenced a higher BC distribution at the apical side and in the cellular in the cellular monolayers (HMG, homogenates). These data confirm the internalization of BC into the enteric cells.

Tab. III PASSAGGE B-A (Papp)

The mass balance at the end of the enteric active passage (B-A) falls within the acceptability range and even in this case the apparent permeability (P_{app}) of BC has been calculated.

Analysis of Berberin distribution in different compartments

Both the samples tested evidenced a higher BC distribution at the baso-lateral side. Approximatively double of the concentration of BC coming from BRA 1284 (PLACEBO) has been found, after 2h, at the apical side respect to BRA 1283 (ENTEROSOMA^{TM}), index of an high extruding rate of BC from the first sample.

At the end of this work, after assessment of both the rate of active passage (A-B) and the passive one (B-A), the ratio of efflux (ER) related to the samples treated has been calculated as reported in the table below:

TAB. IV. Calculation of the Efflux Ratio relative to BC containing samples for both active and passive transport.

Table 4: Efflux Ratio Calculation

	TRANSPORT	ER (Papp B-A/Papp A-B)
BRA 1283 ENTEROSOMA	PASSIVE	0,216
BRA 1284 PLACEBO	ACTIVE	4,108

DISCUSSION

The main purpose of this work was to assess the enteric absorption of BC through a new CH based absorption enhancer on CACO-2 in vitro cells. The experiments have been conducted on BC in form of ENTEROSOMA^{TM}, the mentioned technology though to enhance bioavailability of BC and on BC in form of PLACEBO (BC alone) both tested at concentration of 1mM based on preliminary cytotoxicity evaluations. The goal was to evidence, if existing, the differences of absorption between the aforementioned samples containing BC, emphasizing as much clearly as possible, the role of CH as "key molecule" to improve its bioavailability. Both the data of paracellular passage achieved by mean of LY flux and TEER measures have clearly showed that BC doesn't produce toxicity to the intercellular junctions (TJ). Pg-P, though to play the main role in the BC poor bioavailability, is particularly expressed in the cell membranes, especially in the apical side of the enteric cell (enteric epithelium cells). At this level, Pg-P promotes extrusion from inside the cell to the enteric lumen of several compounds, among which BC, previously penetrated for passive diffusion into the enteric cells after oral administration. Therefore, the evidence of reduced plasmatic concentration of BC is not the result of its poor enteric cell penetration, as postulated according to the quaternary ammonic function, but on the other side of a massive extrusion into the enteric lumen, as many authors reported. According to the results achieved in this work, intestinal absorption simulation of the sample containing BC with ENTEROSOMA^{TM}, which most likely inhibit Pg-P, evidenced an ER of 0,216. This data gives proof of evidence that this CH-based technology, improved with NAC to produce CH cationization and PS 80 working as solubilizing agent, seems to be an effective strategy to entrap BC into the enteric cell, gradually making it in condition to reach the blood circulation and preventing it to be extruded back into the lumen. The extrusion at apical level is observed, on the other hand, with the sample containing BC PLACEBO in which the sole BC was present. These data confirm, taken together, that in absence of ENTEROSOMA^{TM}, BC is massively extruded into the enteric lumen and the high value of ER (4,108) revealed for this sample, shows that this passage from inside to outside the cell is active and energy mediated (pump protein). Differently from what expected and according to the preliminary results of this work, ENTEROSOMA^{TM} technology doesn't seem to interact with TJ, as many authors referred for cationized and quaternized derivatives of CH. The TEER values, comparable with NC, registered for both samples of BC, ENTEROSOMA^{TM} and PLACEBO, clearly show that no interaction occurred between this technology and TJ. CH-NAC salt seems therefore does not interact with the intercellular junctions proteins as postulated, but on the other side has shown to work efficiently in lowering pumping ATP-based activity of P-gP. Presumably, the mucolytic activity of NAC plays a role in reducing viscosity of the mucous that covers enteric epithelium, but given the water solubility of BC, this mechanism seems not playing a pivotal role in enhancing BC enteric absorption. The final and more attractive and impressive data achieved from this work is that BC contained in the sample with ENTEROSOMA^{TM} technology is 19 times more retained into the enteric cells respect to the sample PLACEBO containing the sole BC.

CONCLUSION

To conclude, even though this must be considered a preliminary scientific evaluation to assess a new technology intended to improve oral absorption of BC, it seems suggestive that an association of CH cationized with a mucolytic agent such as NAC and with a solubilizing agent such as PS 80, could potentially represent an interesting new insight to achieve this goal. The experimental and clinical evidences available in published literature, show interesting perspectives on use of BC in the treatment of high blood LDL cholesterol and diabetes even though the poor oral bioavailability. Since this poor bioavailability is mainly due to the role of the P-gP-MDRP, placed on the apical side of the enteric cells, the use of an effective P-gP inhibitor like CH or its quaternized or cationized derivatives, could improve BC enteric kinetic after oral administration and consequently its clinical efficacy performances. Further experimental tests on animals and above all clinical evaluation on humans should be carried out to assess if the preliminary encouraging data collected in this work can be confirmed, contextually opening the status of art to a new pharmaceutical technology capable to enhance clinical expectation regarding BC.

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AUTHOR DISCLOSURE STATEMENT
We declare herewith to have not commercial interest, which could potentially create a conflict of interest with this paper contents.

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