EXPERT REVIEW

Meta-Analysis of Drug Delivery Approaches for Treating Intracellular Infections

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Abstract

This meta-analysis aims to evaluate the trend, methodological quality and completeness of studies on intracellular delivery of antimicrobial agents. PubMed, Embase, and reference lists of related reviews were searched to identify original articles that evaluated carrier-mediated intracellular delivery and pharmacodynamics (PD) of antimicrobial therapeutics against intracellular pathogens *in vitro* and/or *in vivo*. A total of 99 studies were included in the analysis. The most commonly targeted intracellular pathogens were bacteria (62.6%), followed by viruses (16.2%) and parasites (15.2%). Twenty-one out of 99 (21.2%) studies performed neither microscopic imaging nor flow cytometric analysis to verify that the carrier particles are present in the infected cells. Only 31.3% of studies provided comparative inhibitory concentrations against a free drug control. Approximately 8% of studies, albeit claimed for intracellular delivery of antimicrobial therapeutics, did not provide any experimental data such as microscopic imaging, flow cytometry, and *in vitro* PD. Future research on intracellular delivery of antimicrobial agents needs to improve the methodological quality and completeness of supporting data in order to facilitate clinical translation of intracellular delivery platforms for antimicrobial therapeutics.

Abbreviations

MDR	Multidrug resistant
XDR	Extensively drug resistant
mRNA	Messenger ribonucleic acid
COVID	Coronavirus disease
siRNA	Small interfering ribonucleic acid
PD	Pharmacodynamic
CFUs	Colony forming units
MIC	Minimum inhibitory concentration
IC$_{50}$	Half maximal inhibitory concentration
EC$_{50}$	Half maximal effective concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
PEG-PLGA	Poly(ethylene glycol)-block-poly(lactide-co-glycolide)
%ID	Percentage injected dose
RES	Reticuloendothelial system

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Introduction

Infectious disease and antimicrobial resistance remain a major threat to the public health (1). Of many causative organisms, intracellular pathogens have become a source of great concern worldwide. Intracellular pathogens have developed immune evasion strategies over evolution (2). For example, some pathogens infect macrophages, which are responsible for the eradication of invading pathogens from the host, and rather turn them to their sanctuaries to evade the host immunity (3, 4). Consequently, intracellular infection caused by these organisms can often incur chronic, recurrent, or disseminated infections (5). Repeated and prolonged exposure to antimicrobials also increases the risk of

turning these pathogens into multidrug resistant (MDR) or extensively drug resistant (XDR) organisms (6). Given the limited therapeutic options available and the paucity of novel antimicrobial agents in the pipeline, we face dire prospects in the global fight against intracellular infections.

To kill these pathogens, it is necessary to develop new strategies to deliver antimicrobial agents intracellularly. Ideally, antimicrobial therapeutics should selectively enter the infected cells, traffic in the cells to the desired intracellular niche (e.g., cytoplasm or vacuole) harboring pathogens, and kill the pathogens in a timely manner. The drug delivery field has a unique opportunity here, based on the knowledge obtained from the intracellular delivery of anti-cancer drugs or gene therapeutics (e.g., lipid nanoparticles of messenger ribonucleic acid (mRNA) encoding spike proteins for Coronavirus disease (COVID) vaccines (7)). In particular, gene therapeutics can contribute significantly to the therapy of intracellular infections. For example, viral infections pose further challenges to public health due to the capacity of viruses to spread and rapidly mutate rendering existing antiviral agents ineffective (7, 8). Small interfering ribonucleic acids (siRNAs) are potentially useful for the development of antiviral therapy as they can be designed to adapt to genetic changes in microorganisms and address the affected populations in a timely manner (9). With the approval of mRNA COVID vaccines, we foresee accelerated development of novel products based on advanced antimicrobial agents such as siRNA for therapy of intracellular infections. Therefore, it is a prime time to review current drug carriers targeted to intracellular pathogens and understand the achievements, limitations, and opportunities.

A wide variety of carrier systems have been investigated in the literature, ranging from nanoparticles, microparticles, live cell derivatives, prodrugs, or drug conjugates. Many studies employ particles as a carrier of antimicrobial agents, exploiting the ability of host cells (mostly macrophages) to endocytose them. The particles are made of organic or inorganic compounds that can encapsulate antimicrobial agents, in a size conducive to cellular uptake. Since mammalian cells take up particles via dedicated endocytosis pathways, the encapsulated drugs may bypass diffusional barrier imposed by the cell membrane (10) (Fig. 1). Ninety-nine papers on this topic were published in the past decade, with 89.9% of them in the last 10 years. However, there are only a limited number of commercial products based on the intracellular drug delivery approach, with silver nanoparticles being the most widely studied nanoparticles (11).
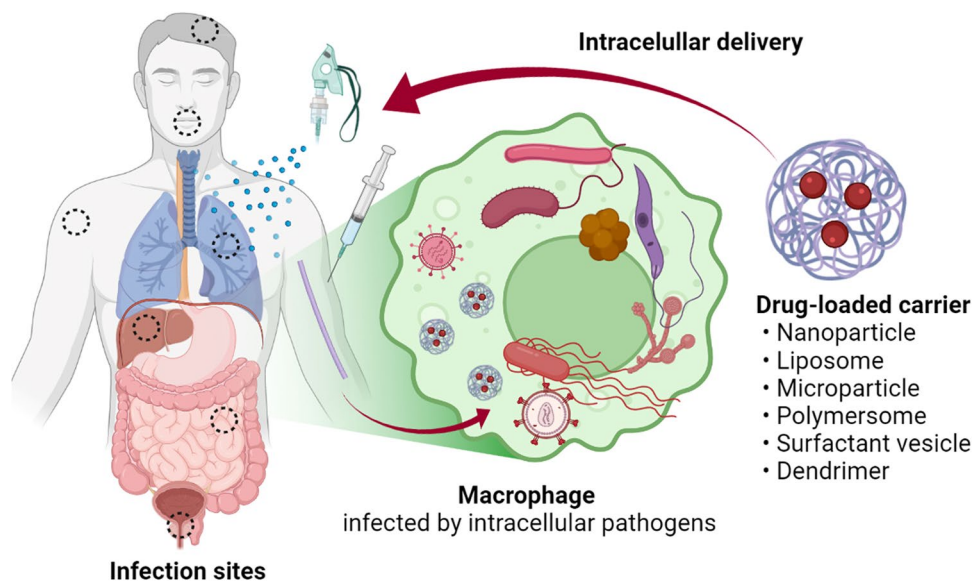
In this study, we aim to better understand the current landscape of drug delivery systems targeted to intracellular pathogens and potential barriers in their clinical translation. We performed a meta-analysis of literature concerning drug-loaded platforms targeting intracellular pathogens and reviewed study methodologies for evaluating their intracellular delivery and antimicrobial efficacy. Previous meta-analyses on drug-loaded platforms have primarily focused on anticancer therapy (12, 13). To the best of our knowledge, the present study is the first meta-analysis on carrier-mediated delivery of antimicrobial therapeutics against intracellular pathogens.

Materials and Methods

Data Source and Search Strategy

A literature search of PubMed and Embase was performed to identify original articles that evaluated carrier-mediated intracellular delivery and/or antimicrobial efficacy of

Fig. 1 Intracellular delivery of antimicrobial-loaded carriers. Created with BioRender.com

Intracellular delivery

Drug-loaded carrier
· Nanoparticle
· Liposome
· Microparticle
· Polymersome
· Surfactant vesicle
· Dendrimer

Macrophage
infected by intracellular pathogens

Infection sites

antimicrobial therapeutics against intracellular pathogens *in vitro* or *in vivo*. Here, antimicrobial therapeutics are defined broadly to include antiviral, antibacterial, antifungal, and antiparasitic agents. The initial database search strategies were prespecified as follows: search terms consisted of a combination of key words, (intracellular) in title/abstract AND (infection) in title/abstract AND (delivery) in title/abstract; no publication date restrictions were applied; the search filter for language was set as "English." Reference lists of related research were additionally screened to identify eligible studies. Ethics committee review was waived as this study conducted by pooling existing data extracted from published primary research.

Study Selection and Data Collection

Those studies identified though initial search were screened for eligibility using the following inclusion criteria: (i) experimental data are provided to demonstrate the efficacy of intracellular delivery of antimicrobial therapeutics and/or their antimicrobial efficacy against intracellular pathogens *in vitro* or *in vivo*; (ii) carrier systems are used to deliver active drugs; (iii) sufficient raw data can be obtained from primary research. Review articles and duplicate studies were excluded from the meta-analysis. Studies that applied metals or microorganisms for preparation of intracellular delivery platforms were included. Study attributes, including the first author and year of publication, host cells, etiologic organisms, characteristics of carrier, types of materials (organic, inorganic, live cell derivative), active drug, intracellular delivery study methodology, *in vitro* and/or *in vivo* pharmacodynamic (PD) markers, antimicrobial efficacy results, and free drug control were extracted utilizing a predefined summary format. Authors resolved difference in opinion or study interpretation through discussion.

Data Analysis and Statistical Methods

The efficacy of intracellular carriers for antimicrobial therapeutics was evaluated qualitatively and quantitatively by pooling data from individual studies, and the results were presented with descriptive statistics. The parameters of intracellular delivery investigation assessed in this meta-analysis included what type of study methodologies were used for evaluation of intracellular delivery (microscopy, flow cytometry), whether *in vitro* and/or *in vivo* PD responses were evaluated, and whether the effects were compared against those of free drug counterparts. The primary *in vitro* PD markers investigated are: intracellular microbial burdens such as colony forming units (CFUs), viral plagues or titers, optical density, and microbial growth or inhibition rates; inhibitory concentrations including minimum inhibitory concentration (MIC), half

maximal inhibitory concentration (IC_{50}), and half maximal effective concentration (EC_{50}). As for *in vivo* PD markers, survival rates and pathogen burden at specific times were assessed in animal models of related infection.

Results

Selection of Relevant Original Studies

The process of searching and identifying relevant studies is presented as Fig. 2. The initial search from PubMed and Embase yielded 1,891 citations and 67 additional articles were identified via reference lists in reviews of related research (14–17). Of those, 700 duplicates were excluded. Of the remaining 1,258 articles that underwent further screening of titles and abstracts, a total of 1,131 articles were excluded due to the reasons listed in Fig. 2. After full-text review, 28 additional articles were further removed, resulting in 99 studies satisfying the inclusion criteria for qualitative analysis: Bacterial 62 (62.6%), viral 16 (16.2%), fungal 8 (8.1%), and parasitic 15 (15.2%; 2 articles on both bacterial and parasitic pathogens).

Characteristics of Included Studies

The yearly publication patterns of intracellular delivery of antimicrobial therapeutics show near-zero publications until the late 2000s and a sharp uptake in publication volume in the 2010s and thereafter (Fig. 3). The most commonly targeted intracellular pathogens were bacterial organisms throughout the study screening period, with the first study on *Salmonella* published in 1990 followed by scarce research until a sharp rise in publication volume in the late 2000s. None of the studies targeted parasitic, viral, or fungal intracellular pathogens until 2009, 2010, and 2015, respectively, with the first studied organism being *Leishmania*, *Ebola*, and *Cryptococcus*, respectively. The most dominant type of materials for delivery platforms was organic compounds, of which 54.5% of studies (54/99) employed synthetic polymers and 33.3% (33/99) lipid particles (Fig. 4a). The most-commonly used carrier type was nanoparticles (in 51 out of 99 studies), followed by liposomes (in 15 out of 99 studies) (Fig. 4b). The particle size in the majority of studies (63.6%) falls in the range of 100 to < 1000 nm (Fig. 4c). We have further analyzed the included studies by host cell types, carrier types, particle size ranges, and administration routes. Detailed results are summarized in Tables S1 to S8 in supplementary materials.
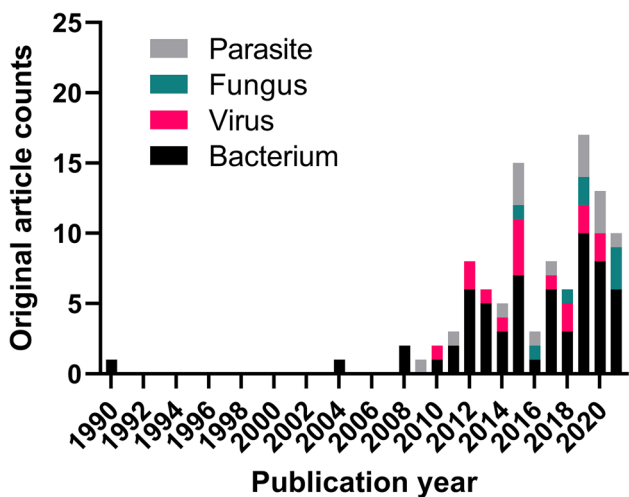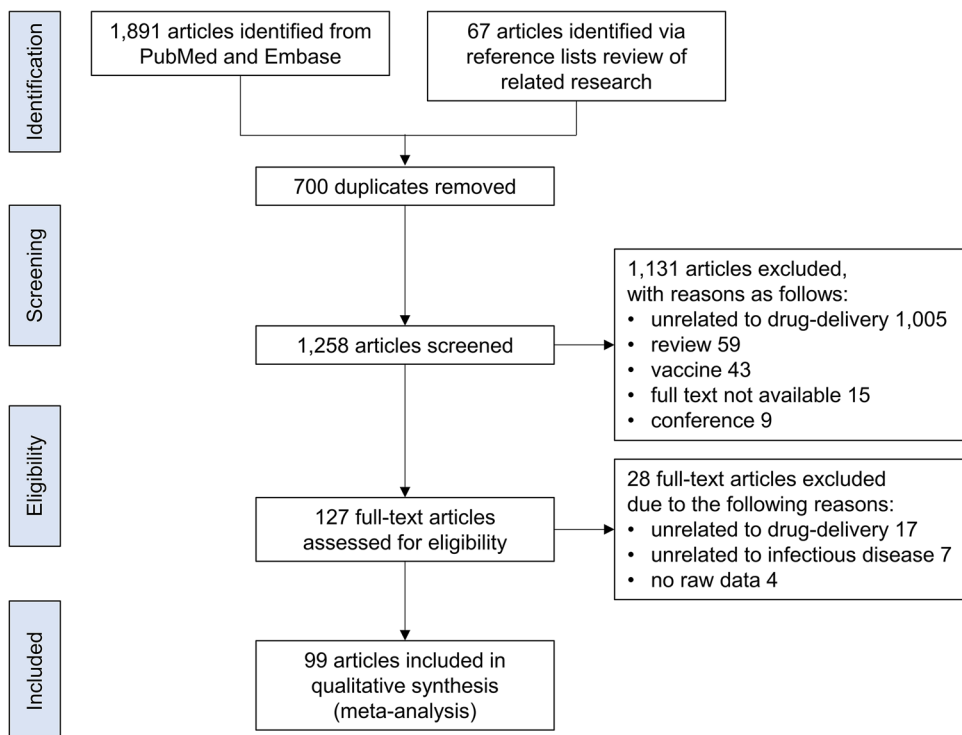
Fig. 2 Study selection flow diagram

Identification

1,891 articles identified from PubMed and Embase

67 articles identified via reference lists review of related research

Screening

700 duplicates removed

1,258 articles screened

1,131 articles excluded, with reasons as follows:
- unrelated to drug-delivery 1,005
- review 59
- vaccine 43
- full text not available 15
- conference 9

Eligibility

127 full-text articles assessed for eligibility

28 full-text articles excluded due to the following reasons:
- unrelated to drug-delivery 17
- unrelated to infectious disease 7
- no raw data 4

Included

99 articles included in qualitative synthesis (meta-analysis)

Fig. 3 Publication trends of the literature focusing on drug delivery systems for the treatment of intracellular infections, subdivided by etiologic organisms

Bacterial Intracellular Pathogens

The 62 eligible studies on bacterial intracellular pathogens are summarized in Table I. The most commonly studied intracellular bacteria were *Staphylococcus aureus* (n = 20), followed by *Mycobacterium tuberculosis* (n = 17) and *Salmonella* (n = 9) (Fig. 5). There were two studies by Lueth (2019) (18) and by Omolo (2021) (19), which provided the most comprehensive data supporting intracellular delivery and antimicrobial efficacy of drug-loaded carriers both *in*
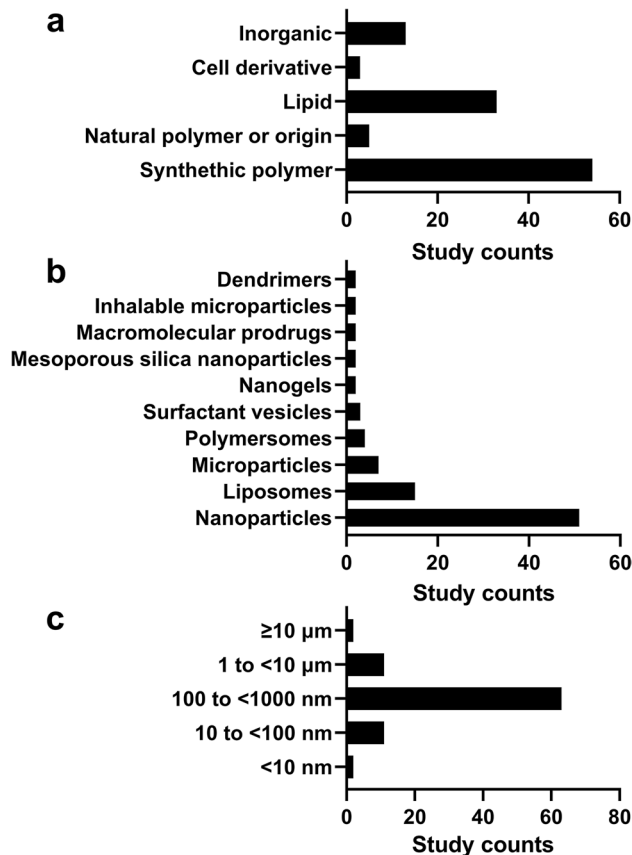

Fig. 4 Drug carriers used for intracellular delivery of antimicrobials by (**a**) material types, (**b**) formulation types, and (**c**) particle sizes

Table 1 Characteristics of Intracellular Delivery Studies on Bacterial Pathogens (n=62)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy vs free drug control	Route	Examined organs	Efficacy vs control
Afinjuomo 2019 [20]	alveolar macrophages	Mycobacterium tuberculosis	pyrazinamide	microparticles	semicrystalline delta inulin	1–2 μm	-	-	-	-	-
Akbari 2013 [21]	macrophages	Staphylococcus aureus	ciprofloxacin	surfactant vesicles	Span 40, Tween 40, cholesterol	300–600 nm	+	MIC, 2- to eightfold reduction; intracellular CFU, 3 log reduction	-	-	-
Ardekani 2019 [22]	epithelial cell line derived from a human oral squamous cell carcinoma	Porphyromonas gingivalis	metronidazole	nanoparticles	carbon quantum dot derived from chlorophyll	1–5 nm	+	IC_{50}, 0.33 μM vs 1.02 μM; intracellular CFU, 3 times more reduction	-	-	-
Arshad 2021 [23]	macrophages	Salmonella typhi	ciprofloxacin	nanoemulsions	Ciprofloxacin-hyaluronic acid conjugate, oil, surfactant	40–50 nm	+	sterilization rate based on free CFU, 99.55±0.5% vs 9±3.25%	oral	-	survival, 100% vs 40%
Brockman 2017 [24]	cystic fibrosis lung epithelial cells	Pseudomonas aeruginosa	cysteamine	dendrimers	PAMAM-DEN	4 nm	+	bacterial growth in free culture, decrease by 15%	-	-	-
Chokshi 2021 [25]	alveolar macrophages	Mycobacterium tuberculosis	rifampicin	lipid nanoparticles	Mannose coating, compritol, stearylamine	479±13 nm	-	-	oral	lung, spleen, liver	-
Chono 2008a [26]	alveolar macrophages	Chlamydia pneumoniae, Legionella pneumophila Pseudomonas aeruginosa Haemophilus influenza	ciprofloxacin	liposomes	HSPC, cholesterol, DCP	989.1±94.4 nm	-	-	pulmonary	-	PK/PD analysis for antibacterial effects, increases in AUC/MIC, C_{max}/MIC

Table I (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy vs free drug control	Route	Examined organs	Efficacy vs control
Chono 2008b (27)	alveolar macrophages	Mycobacterium tuberculsosis, Mycobacterium avium, Mycobacterium intracellulare, Chlamydia pneumoniae, Listeria monocytogenes, Legionella pneumophila, Franciscella tularensis	ciprofloxacin	liposomes	Mannose coating, HSPC, DOPC, cholesterol, DCP	1,000 nm	-	-	pulmonary	-	PK/PD analysis for antibacterial effects, increases in AUC/MIC, C_{max}/MIC; reduced risk of appearance of resistant bacteria
Chuan 2013 (28)	alveolar macrophages	Mycobacterium tuberculosis	rifampicin	lipid nanoparticles	soybean lecithin, stearic acid, palmic acid	829.6±16.1 nm	-	-	endotracheal aerosolization	lung	-
Clemens 2012 (29)	alveolar macrophages	Mycobacterium tuberculosis	isoniazid, rifampin, moxifloxacin	mesoporous silica nanoparticles	PEG-PEI-coated mesoporous silica nanoparticles	100 nm	+	free CFU, reduction by 1.5–1.7 log	-	-	-
Croitoru 2015 (30)	HeLa cells	Listeria, Pseudomonas aeruginosa, Escherichia coli	gentamicin	microcapsules	exopolysaccharidic fraction from K. pneumoniae and P. aeruginosa strains	≤30 μm	+	intracellular CFU, from increase by 10 logs to reduction by 2–4 logs	-	-	-
Das 2017 (31)	alveolar macrophages	Franciscella tularensis	ciprofloxacin	macromolecular polymeric prodrugs	PEG methacrylate	-	-	-	endotracheal aerosolization	lung, blood	survival, 75% vs 0% of free drug in lethal aerosol infection model

Table I (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy vs free drug control	Route	Examined organs	Efficacy vs control
de Faria 2012 (32)	alveolar macrophages	*Mycobacterium tuberculosis*	isoniazid	nanoparticles	PLGA	180 nm	+	intracellular CFU, reduction by >1 log	-	-	-
Dube 2014 (33)	alveolar macrophages	*Mycobacterium tuberculosis*	rifampicin	nanoparticles	1,3-β-glucan functionalized chitosan shell, PLGA	280 nm	+	fourfold increase in intracellular rifampicin; stimulation of ROS/RNS, pro-inflammatory cytokine secretion (ligand effect)	-	-	-
Ellis 2018 (34)	alveolar macrophages	*Mycobacterium tuberculosis*	rifampicin	multimetallic microparticles (MMPs)	silver, zinc oxide, PLGA	<4 μm	-	Intracellular CFU, MMP(Zn), MMP(Ag), or MMP(Ag+Zn) led to a 68 to 76% increase in rifampicin potency (vs blank MMP)	-	-	-
Elnaggar 2020 (35)	macrophages	*Shigella flexneri, Salmonella typhimurium, Listeria monocytogenes, Staphylococcus aureus*	pexiganan	nanoparticles	silver, PLGA	603.2±37.3 nm	+	intracellular CFU reduction by <3 logs	IV	liver, spleen	-

Table I (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		Efficacy vs control
							Free drug control	Efficacy vs free drug control	Route	Examined organs	
Fahimmu-nisha 2020 (36)	urothelial cells	Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumonia, Escherichia coli urinary tract infection	zinc oxide, Aloe socotrina	nanoparticles	zinc oxide, Aloe socotrina	15–50 nm	+	zone of inhibition, increase by 10–56%	-	-	-
Fenaroli 2020 (37)	macrophages	Mycobacterium tuberculosis, Mycobacterium bovis-BCG, Staphylococcus aureus	vancomycin, gentamicin, lysostaphin, rifampicin, isoniazid	polymersomes	PMPC-PDPA	100 nm	+	intracellular CFU, reduction by 1 log or complete eradication	IV	Zebrafish macrophage, granuloma	CFU, reduction by 1 log;
Fierer 1990 (38)	macrophages	Salmonella dublin	gentamicin	liposomes	partially hydrogenated egg PC, egg PG, cholesterol, α-tocopherol	<1 μm	-	-	IV	spleen	survival, 80% vs 0% (free drug control)
Franch 2020 (39)	macrophages	Mycobacterium tuberculosis	-	nanoparticles	DNA	300 nm	-	-	-	-	-
Gao 2019 (40)	macrophages	Staphylococcus aureus	vancomycin, rifampicin	nanoparticles	PLGA, membrane of S. aureus extracellular vesicle coat	104.2 nm	+	intracellular CFU, with vancomycin, comparable reduction; with rifampicin, >1 log reduction	IV	liver, spleen, lung, kidney	CFU, with vancomycin, >1 log reduction in kidney and lung; with rifampicin, >1 log reduction in kidney and lung

Table I (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD — Free drug control	In vitro PD — Efficacy vs free drug control	In vivo PD — Route	In vivo PD — Examined organs	In vivo PD — Efficacy vs control
Gaspar 2015 (41)	macrophages	*Mycobacterium avium*	paromomycin	liposomes	PC, PEG, DMPC, DMPG, DPPC, DPPG, SA	0.09–0.25 µm	-	-	IV	liver, spleen, lung	bacterial growth index, reduction by 0.5 to 1.75 in lung, liver, spleen
Gnanadhas 2013 (42)	macrophages	*Salmonella typhimurium, Salmonella typhi*	ciprofloxacin, ceftriaxone	nanocapsules	chitosan-dextran sulphate	180 ± 20 nm	+	intracellular bacterial counts, comparable reduction	IV	liver, spleen	survival, with ceftriaxone 100% vs 50%; with ciprofloxacin 100% vs 40%
Heck 2018 (43)	macrophages	*Staphylococcus aureus*	clindamycin	nanoparticles	zirconyl clindamycin phosphate inorganic–organic hybrid	73 ± 14 nm	+	intracellular bacterial counts, reduction by 50% in 4 h	-	-	-
Hlaka 2017 (44)	macrophages	*Mycobacterium tuberculosis*	minor groove binders	surfactant vesicles	distamycin template	-	+	intracellular CFU, 1.6- and 2.1- fold increase in anti-mycobacterial activity	-	-	-
Horsley 2019 (45)	urothelial cells	*Enterococcus faecalis* urinary tract infection	gentamicin	lipid microbubbles	ultrasound-activated	5.79 ± 1.53 µm	+	intracellular CFU, reduction by 20% at 15–380 fold lower doses	-	-	-
Hsu 2018 (46)	alveolar macrophages	*Staphylococcus aureus*	ciprofloxacin	nanovesicles	Span 60, cholesterol, soybean PC, DSPE-PEG	114.4 ± 0.9 nm	+	intracellular CFU, reduction by <1 log	IV	lung	bacterial burden in the lung, decrease by eightfold
Imbuluzqueta 2012 (47)	macrophages	*Listeria monocytogenes, Staphylococcus aureus*	gentamicin as bis(2-ethylhexyl) sulfosuccinate sodium salt	nanoparticles	PLGA	263 + 10 nm	+	intracellular CFU, reduction by <1 log with *s. aureus*; 1–1.5 log with *l. monocytogenes*	-	-	-

Table I (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD Free drug control	In vitro PD Efficacy vs free drug control	In vivo PD Route	In vivo PD Examined organs	Efficacy vs control
Imbuluzqueta 2013 (48)	macrophages	*Brucella melitensis*	gentamicin as bis(2-ethylhexyl)sulfosuccinate sodium salt	nanoparticles	PLGA	289–299 nm	+	intracellular CFU, reduction by > 1 log	IV	liver, spleen, kidney	CFU, reduction in splenic infection by 3.13 logs
Jankie 2015 (49)	-	*Pseudomonas aeruginosa*	levofloxacin	surfactant vesicles	cholesterol, sorbitan monostearate, dicetylphosphate	8–15 µm	-	-	IV	liver, spleen, kidney	CFU/1 µL tissue, reduction by 21.8–4.47; survival, 100% vs 83.3%
Kiruthika 2015 (50)	macrophages	*Salmonella paratyphi A*	chloramphenicol	nanoparticles	Chitosan, dextran sulfate	100–200 nm	+	MIC, 80 µg/mL vs 3 µg/mL; intracellular CFU, 1 log reduction	-	-	-
Labbaf 2013 (51)	urothelial cells	*Enterococcus faecalis* urinary tract infection	gentamicin	polymeric capsules	polymethylsilsesquioxane	850±100 nm	+	free CFU, comparable reduction	-	-	-
Labouta 2015 (52)	epithelial cells	*Yersinia pseudotuberculosis*	gentamicin	liposomes	invasin fragment coating, DPPC, cholesterol, other lipid	143.0±0.4 nm	-	intracellular infection load, reduction by 30% (vs untreated control)	-	-	-
Lacoma 2020 (53)	macrophages	*Staphylococcus aureus*	cloxacillin	nanoparticles	PLGA	106.5±24 nm	+	MIC, 2- to four-fold reduction; intracellular CFU, reduction by < 1 log	-	-	-
Lau 2020 (54)	urothelial cells	Urinary tract infection-related bacteria	nitrofurantoin	microparticles	PLGA	2.8 µm	+	intracellular CFU, reduction by < 1 log	-	-	-

Table I (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy vs free drug control	Route	Examined organs	Efficacy vs control
Lee 2016 (55)	macrophages	*Francisella tularensis* pneumonia	moxifloxacin	nanoparticles	mesoporous silica nanoparticles with β-cyclodextrin-adamantyl snap tops	90 nm	+	intracellular CFU, comparable antibacterial effects	IV	liver, lung, spleen	CFU, reduction by 1 log
Lueth 2019 (18)	macrophages	*Brucella melitensis, Escherichia coli*	doxycycline, rifampicin	nanoparticles	polyanhydrides	160–330 nm	+	MIC, comparable; intracellular CFU, reduction by > 4 logs	IP	liver, spleen	CFU, reduction by 1–3 logs in spleen
Lunn 2021 (56)	alveolar macrophages	*Mycobacterium tuberculosis, Mycobacterium bovis-BCG*	isoniazid	nanoparticles	P(ManAm-co-DAAm-hydrazone-INH-co-DPAEMA)	131–280 nm	+	intracellular CFU, reduction by < 1 log	-	-	-
Maji 2019 (57)	human embryonic kidney 293 cells as a model	*Staphylococcus aureus*	vancomycin	nanoparticles	oleylamine, PAMAM-DEN	124.4±2.01 nm	+	MIC, eightfold reduction; intracellular CFU, reduction by 6 logs	-	-	-
Maya 2012 (58)	macrophages, epithelial cells	*Staphylococcus aureus*	tetracycline	nanoparticles	O-carboxymethyl chitosan	200 nm	+	MIC, 0.3–0.6 µg/mL vs 0.2–0.4 µg/m; intracellular bacterial killing, sixfold increase in antibacterial effects	-	-	-
Mishra 2011 (59)	Hep-2 as model epithelial cells	*Chlamydia trachomatis* genital infection	azithromycin	nanodevices	PAMAM-DEN	-	+	bacterial inclusion size in cells, 50% reduction vs no measurable reduction	-	-	-

Table I (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy vs free drug control	Route	Examined organs	Efficacy vs control
Monsel 2015 (60)	monocytes, alveolar epithelial type 2 cells	Escherichia coli pneumonia	cell components	microvesicles	mesenchymal stem cell microvesicles	50–200 nm	-	intracellular CFU, reduction by 25% compared to phosphate-buffered saline	IV	lung	survival, 88% versus 40% with phosphate-buffered saline
Montanari 2014 (61)	epithelial cells	Staphylococcus aureus, Pseudomonas aeruginosa	levofloxacin	nanoparticles	hyaluronic acid-cholesterol conjugate	170 ± 10 nm	+	MIC, 1.7-fold reduction; intracellular CFU, reduction by 80–90%	-	-	-
Omolo 2021 (19)	Macrophages, model epithelial cells	Staphylococcus aureus	vancomycin	liposomes	phospholipid, cholesterol, oleic acid, quaternary lipid	98.88 ± 1.92 nm	+	MIC, 4- to 16-fold reduction; intracellular CFU, reduction by 1–2 logs	IV	-	CFU, 6.33-fold decrease
Pei 2017 (62)	macrophages	Staphylococcus aureus, Listeria monocytogenes, Enterococcus fecalis, Enterococcus faecium, Streptococcus pneumoniae	vancomycin	nanoparticles	PEG-PLGA, chitosan derivative	837 ± 103 nm	+	intracellular CFU, reduction by 0.5–1.5 log	IV	liver, spleen	-
Pi 2019 (63)	alveolar macrophages	Mycobacterium tuberculosis, Mycobacterium bovis-BCG	rifampin	nanoparticles	mannosylated and PEGylated graphene oxide	120 nm	+	intracellular CFU, reduction by 10–50%	-	-	-

Table I (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy vs free drug control	Route	Examined organs	Efficacy vs control
Prabhu 2021 (64)	macrophages	Mycobacterium tuberculosis osteoarticular tuberculosis	rifampicin	nanoparticles	mannose-conjugated chitosan	130–140 nm	+	MIC, 0.009 µg/mL vs 0.0078 µg/mL	-	-	-
Pumerantz 2011 (65)	alveolar macrophages	Staphylococcus aureus	vancomycin	liposomes	DSPC, cholesterol	254 nm	+	intracellular CFU, reduction by 65%	-	-	-
Ranjan 2010 (66)	macrophages	Salmonella typhimurium	gentamicin	nanoparticles	PEO-b-PAA$^-$ Na$^+$, PEO-b-PMA$^-$ Na$^+$	90–120 nm	-	-	IP	liver, spleen	CFU, reduction by -0.15–0.87 log (free drug control)
Rodrigues 2021 (67)	alveolar macrophages	Mycobacterium tuberculosis	isoniazid, rifabutin	inhalable polymeric microparticles	chondroitin sulfate	3.9 µm	-	-	-	-	-
Sava Gallis 2019 (68)	macrophages, lung epithelial cells	Escherichia coli	ceftazidime	metal–organic framework particles	zeolitic imidazolate framework-8	-	-	bacterial growth (optical density), 0 vs 0.4 (untreated control)	-	-	-
Smitha 2015 (69)	polymorphonuclear leukocytes	Escherichia coli, Staphylococcus aureus, Salmonella paratyphi A, Enterobacter sp., Klebsiella pneumoniae, Shigella sonnei, Enterococcus faecalis	rifampicin	nanoparticles	amorphous chitin	350±50 nm	+	MIC, decrease by 0–43%	-	-	-

Table I (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy vs free drug control	Route	Examined organs	Efficacy vs control
Subramaniam 2019 (70)	macrophages	*Staphylococcus aureus*	rifampicin	mesoporous silica nanoparticles	mesoporous silica nanoparticles	40 or 100 nm	+	intracellular CFU, reduction by 30%	-	-	-
Uskoković 2014 (71)	osteoblasts	*Staphylococcus aureus* osteomyelitis	clindamycin	nanoparticles	calcium phosphate	61.8–83.8 nm	+	intracellular CFU, reduction by 70–100%	-	-	-
Vaghasiya 2019 (72)	macrophages	*Escherichia coli*	no drug	inhalable microspheres	sodium alginate	5.64–31.51 μm	-	intracellular CFU, reduction by 1 log (vs untreated control)	-	-	-
Vieira 2017 (73)	alveolar macrophages	*Mycobacterium tuberculosis*	rifampicin	lipid nanoparticles	stearylamine, Aerosil, D-(+)-mannose	302–327 nm	+	intracellular CFU, reduction by <1 log	-	-	-
Vyas 2004 (74)	alveolar macrophages	*Mycobacterium smegmatis*	rifampicin	liposomes	egg PC, cholesterol, dicetylphosphate, maleylated BSA, O-steroyl amylopectin	2.32–3.85 μm	+	intracellular bacterial viability, reduction by 31–84%	pulmonary	lung	-
Xiong 2021 (75)	macrophages	*Staphylococcus aureus*	vancomycin	nanogels	PEG, PCL, polyphosphoester	429±31 nm	+	intracellular CFU, reduction by 1 log	-	-	-
Yang 2015 (76)	macrophages	*Staphylococcus aureus*	rifapentine	hen egg lipoprotein conjugates	hen egg LDL	33±6.8 nm	+	intracellular CFU, reduction by 0.5 log	-	-	-
Yu 2020 (77)	lung epithelial cells	-	ciprofloxacin, colistin	liposomal powder formulations	HSPC, DSPG, DSPE-PEG-OMe, cholesterol	97.1–102.8 nm	-	-	-	-	-
Zaki 2012 (78)	enterocytes, macrophages	*Salmonella typhimurium*	ceftriaxone	nanoparticles	chitosan tripolyphosphate	202–221 nm	+	intracellular bacterial count, reduction by 99% vs 33–49%	-	-	-

Table I (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy vs free drug control	Route	Examined organs	Efficacy vs control
Zhang 2017 (79)	osteoblasts	Staphylococcus aureus osteomyelitis	vancomycin	nanoparticles	N-trimethyl chitosan	200–325 nm	+	intracellular bacterial count, reduction by > 50%	implanted	-	relative colony count, reduction by 75% (free drug control)

Intracellular CFU: CFU of intracellular bacteria; free CFU: CFU of free bacteria; free culture: free bacterial culture (no intracellular infection)

Abbreviations: MIC, minimum inhibitory concentration; IC50, 50% inhibitory concentration; PAMAM-DEN, polyamidoamine dendrimer; PEG, poly(ethylene glycol); PEI, polyethyleneimine; CFU, colony forming unit; IV, intravenous; HSPC, hydrogenated soybean phosphatidylcholine; DCP, dicetylphosphate; DOPC, dioleoyl phosphatidylcholine; PK/PD, pharmacokinetic/pharmacodynamics; AUC, area under the curve; Cmax, maximum concentration; PLGA, poly (lactic acid-co-glycolic acid); MMP, multimetallic microparticle; PMPC-PDPA, poly(2-(methacryloyloxy) ethylphosphorylcholine)-co-poly(2-(diisopropylamino)ethyl methacrylate); PC, phosphatidylcholine; PG, phosphatidylglycerol; DMPC, dimiristoyl phosphatidylcholine; DMPG, dimiristoyl phosphatidylglycerol; DPPC, dipalmitoyl phosphatidylcholine; DPPG, dipalmitoyl phosphatidylglycerol; SA, stearylamine; ManAm, mannopyran-1-oxyethyl acrylamide; DPAEMA, diisopropylamino ethyl methacrylate; PEGA, poly(ethylene glycol)methyl ether acrylate; IP, intraperitoneal; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; MPEG-2000-DSPE, methylpolyethyleneglycol–1,2-distearoyl-phosphatidyl ethanolamine conjugate; PEO-b-PMA⁻⁺Na, poly(ethylene oxide-b-sodium acrylate); PEO-b-PMA⁻⁺Na, poly(ethylene oxide-b-sodium methacrylate); CTAB, cetyltrimethylammonium bromide; TEOS, tetraethyl orthosilicate; BSA, bovine serum albumin; PCL, poly(ε-caprolactone); LDL, low-density lipoprotein; DSPG, 1,2-distearoyl-sn-glycero-3-phosphoglycerol; DSPE-PEG-OMe, N-(methylpolyyoxyethylene oxycarbonyl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine
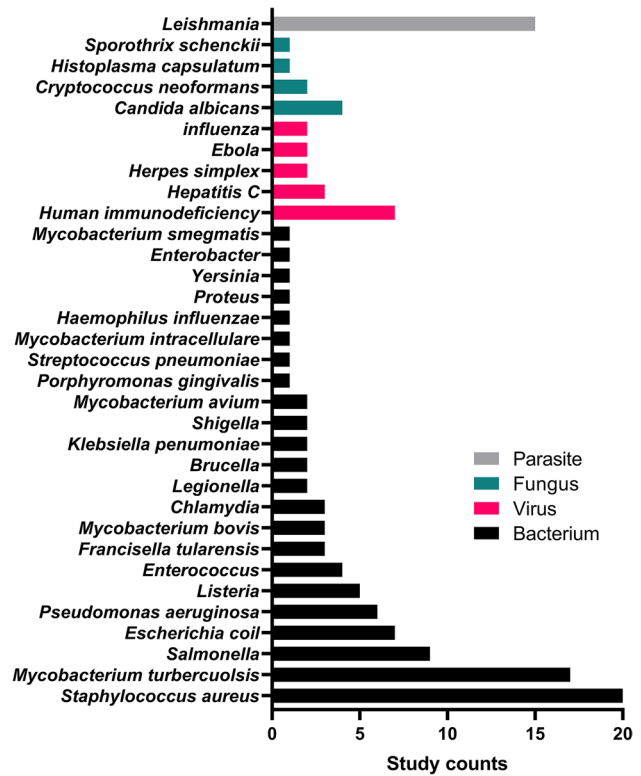

Fig. 5 Commonly studied intracellular pathogens

vitro and *in vivo*. In the former, a polymer-based nanoparticle was developed to encapsulate doxycycline and rifampicin to eradicate *Brucella melitensis* or *Escherichia coli* (18). Intracellular delivery was confirmed by microscopic imaging. In the latter, pH-responsive liposomes were produced to load vancomycin to kill methicillin-resistant *Staphylococcus aureus* (MRSA) (19). Here, intracellular delivery was confirmed by flow cytometry. In both studies, antibacterial activity (changes in MIC and CFU of intracellular bacteria) was compared against those of a free drug control *in vitro*, where the encapsulated drug showed more efficient bacterial killing than the free drug counterpart. The *in vitro* results were further validated in mouse models of systemic or topical bacterial infection (CFU changes). The distribution of particles in the main organs of interest (liver and spleen) were determined only in the former study.

Viral Intracellular Pathogens

Table II summarizes the 16 intracellular delivery studies of antimicrobial therapeutics targeting viral pathogens. The most commonly studied intracellular viruses were *human immunodeficiency virus* (n = 7), followed by *hepatitis C virus* (n = 3) (Fig. 5). A 2018 study by Hu et al. (80) showed the most comprehensive data for intracellular delivery of diphyllin or bafilomycin and their antimicrobial efficacy. The drug was encapsulated in nanoparticles prepared with

Table II Characteristics of Intracellular Delivery Studies on Viral Pathogens (n = 16)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD Free drug control	In vitro PD Efficacy study vs free drug control	In vivo PD Route	In vivo PD Examined organs	Efficacy study vs control
Chandra 2012 (81)	macrophages, liver cells	HCV	siRNA mixture	lipid nanoparticles	cholesterol, DOTAP	100 nm	-	HCV replication, reduction by 4–6 logs (vs untreated control)	intratumoral, IV	liver tumor xenograft	HCV-RNA levels, reduction by > 2 logs
Creighton 2019 (82)	macrophages, lymphocytes	HIV	raltegravir prodrug	nanoparticles	PLGA	-	+	IC_{50}, 2.9–27 nM vs 3.2 nM	-	-	-
das Neves 2012 (83)	macrophages, lymphocytes	HIV	dapivirine	nanoparticles	PCL with PEO, SLS, or CTAB as a surface modifier	182–204 nm	+	EC_{50}, comparable or 4- to 13-fold decrease	-	-	-
Donalisio 2020 (84)	Infected cells	HSV	acyclovir	nanodroplets	chitosan, sulfobutyl ether-β-cyclodextrin	395.4 ± 12.6 nm	+	IC_{50}, 0.32 μM vs 0.89 μM	-	-	-
Geisbert 2010 (85)	Infected cells	EBOV	siRNA	lipid nanoparticles	cholesterol, dipalmitoyl PC, 3-N-[(ω-methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxy-propylamine, cationic 1,2-dilinoleyloxy-3-N,Ndimethylamino propane	81–85 nm	-	-	IV	Liver (mice)	survival, 66–100% vs 0% (untreated control) (rhesus macaques)
Gong 2020 (86)	macrophages, lymphocytes	HIV	elvitegravir	nanoparticles	poloxamer-PLGA	135.7 ± 1.5 nm	+	viral replication, reduction by 80%	-	-	-
Guedj 2015 (87)	macrophages, lymphocytes	HIV	protein	nanoparticles	PLGA	126.30 ± 2.48 nm	-	-	SC	-	-
Hillaireau 2013 (88)	macrophages, lymphocytes	HIV	NRTI prodrugs	nanoassemblies	squalene-drug conjugate, cholesterol-PEG, squalene-PEG	217 ± 47 nm; 204 ± 22 nm	+	ED_{50}, 2- to threefold decrease	oral	liver, spleen, bone marrow	-

Table II (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy study vs free drug control	Route	Examined organs	Efficacy study vs control
Hu 2018 (80)	influenza virus	*Influenza*	diphyllin, bafilomycin	nanoparticles	PEG-PLGA	178 nm; 197 nm	+	IC_{50}, reduction by 10–43%	IV	lung	survival, 33% vs 0% (empty NP); viral RNA copies, reduction by 1 log
Jiang 2015 (89)	macrophages, lymphocytes	*HIV*	maraviroc, etravirine, raltegravir	nanoparticles	PLGA	311.2–371.4 nm	+	IC_{50} 0.40 nM vs 3.2 nM	-	-	-
Mandal 2019 (90)	macrophages, lymphocytes	*HIV*	bictegravir	nanoparticles	PLGA	189.2±3.2 nm	+	EC_{50}, 0.0038 µM vs 0.604 µM	-	-	-
Mazzaglia 2018 (91)	infected cells	*HSV*	cidofovir	carbon nanotubes	carbon nanotube, cyclodextrin, PEI	100–300 nm	+	viral plaques, comparable reduction	-	-	-
Thi 2015 (92)	infected cells	*EBOV*	siRNA	lipid nanoparticles	lipid	-	-	Viral RNA copies, reduction by <1 log (vs untreated control)	IV	-	survival, 100% vs 0% (untreated control); viral load, reduction by 8 logs
Timin 2017 (93)	infected cells	*Influenza*	siRNA	inorganic–organic hybrid capsules	PARG, DEXS, SiO_2, $CaCO_3$	-	-	virus titer, reduction by 50–85% (vs untreated control)	-	-	-
Wohl 2014 (94)	macrophages, liver cells	*HCV*	ribavirin	macromolecular prodrugs	Polymeric drug conjugated to PVP, PHPMA, PAA, or PMAA	-	+	Therapeutic index (IC_{50}/EC_{50}), 4- to eightfold increase	-	-	-

Table II (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy study vs free drug control	Route	Examined organs	Efficacy study vs control
Zhang 2015 (95)	macrophages, liver cells	HCV	cationic peptide p41	peptide-polymer complexes	Gal-terminated PEG-block-poly(L-glutamic acid) copolymer	108 nm	-	HCV RNA, reduction by 80% (vs untreated control)	IV	liver	-

Abbreviations: *HCV*, hepatitis C virus; siRNA, small interfering RNA; *DOTAP*, 1,2 dioleoyl-3-trymethylammonium-propane; SC, subcutaneous; HIV, human immunodeficiency virus; PLGA, poly (lactic acid-co-glycolic acid); IC50, 50% inhibitory concentration; PCL, poly(ε-caprolactone); PEO, poloxamer 338 NF; SLS, sodium lauryl sulfate; CTAB, cetyl trimethylammonium bromide; EC50, 50% effective concentration; *HSV*, herpes simplex virus; *EBOV, Ebola virus*; PC, phosphatidylcholine; IV, intravenous; NRTIs, nucleoside reverse transcriptase inhibitors; PEG, polyethylene glycol; ED50, 50% effective dose; Neu2en, 2,3-Didehydro-2-deoxyneuraminic acid; PARG, poly-L-arginine hydrochloride; DEXS, dextran sulfate; TEOS, tetraethyl orthosilicate; NP, nanoparticle; PEI, polyethylenimine; PVP, poly(N-vinylpyrrolidone); PHPMA, poly(N-(2-hydroxypropyl) methacrylamide); PAA, poly(acrylic acid); PMAA, poly(methacrylic acid); APN, antiviral peptide nanocomplex

poly(ethylene glycol)-block-poly(lactide-co-glycolide) (PEG-PLGA) copolymers. Intracellular delivery of the nanoparticles was confirmed microscopically. For *in vitro* antiviral effects against influenza virus, IC_{50} and viral titers were compared with those of free drug controls. In an *in vivo* mouse model of influenza infection, survival rates and viral RNA copies were measured as PD markers. The delivery of intravenously injected particles to the lungs, the organ of interest, was demonstrated based on the reduction of viral loads, but nanoparticle distribution in other organs was not examined.

Fungal Intracellular Pathogens

The 8 studies on fungal intracellular infection are summarized in Table III. The most commonly studied intracellular fungi were *Candida albicans* ($n = 4$) (Fig. 5). In an exemplary study by Batista-Duharte (96), a needle-like lipid assembly was prepared to deliver amphotericin B intracellularly to kill *Sporothrix schenckii*. Microscopic imaging data were provided to confirm intracellular delivery. The *in vitro* antifungal activity, in terms of CFU and MIC changes, of the carrier-mediated delivery was superior to that of a free drug. The antifungal effects were validated in a mouse model of systemic fungal infection with CFUs as a PD marker. The liver and spleen of the treated animals were examined to evaluate the fungal load.

Parasitic Intracellular Pathogens

Table IV summarizes the 15 intracellular drug delivery studies targeting parasitic pathogens. Here, all studies chose *Leishmania* species as their target pathogen (Fig. 5). Three studies (104–106) incorporated the most comprehensive supporting data. Intracellular delivery of particles was confirmed by microscopy and flow cytometry in all three studies. *In vitro* killing effects (parasitic burden and IC_{50} changes) were compared with those of free drug controls. All three studies demonstrated enhanced *in vivo* parasite inhibition effects of the carrier-mediated delivery *versus* a free drug. The biodistribution of intravenously injected carrier particles was investigated in two studies, based on drugs delivered to major organs, showing that the liver and spleen were common targets (104, 105).

Study Methodologies: Intracellular Delivery and Antimicrobial Efficacy

We investigated how intracellular drug delivery was evaluated in each study: imaging and flow cytometric analysis of labeled carriers, *in vitro* PD (e.g., killing of intracellular bacteria or counting viral plaques), or *in vivo* PD. Table V summarizes study methodologies employed in the analyzed

studies. Due to the lack of common readouts and the non-linear relationship of dye concentration *versus* fluorescence intensity, it was difficult to compare the studies with respect to the intracellular delivery efficiencies of different carriers. As for the intracellular delivery of carriers, 73.7% (73/99) and 37.4% (37/99) of studies performed microscopy and flow cytometry, respectively, and 78.8% (78/99) of them incorporated either of the two methodologies. Twenty-one out of 99 (21.2%) performed neither study to support the intracellular delivery. For the antimicrobial efficacy, 80.8% (80/99) of studies investigated differential effects of drug-loaded carriers on *in vitro* PD markers as compared to either untreated, empty carrier, or free drug controls. Of those, 69.7% (69/99) of studies compared antimicrobial effects against a free drug control, utilizing either microbial burdens (55/99) or inhibitory concentrations (31/99) or both (17/99) as PD markers. Thirty-three out of 99 (33.3%) studies conducted *in vivo* PD studies with pathogen burdens or survival as readouts. Eight studies (8.1%) did not show any evidence of intracellular delivery of antimicrobial therapeutics (no microscopic imaging, no flow cytometry, and no *in vitro* PD study).

Discussion

Intracellular pathogens have evolved to counterbalance the host immunity to survive or multiplicate within macrophages, otherwise hostile environments. In order to treat intracellular infections, it is important to develop an effective intracellular delivery system that helps antimicrobial therapeutics to enter the infected cells, traffic in the cells to the desired intracellular niche harboring pathogens, and kill the pathogens in a timely manner. In this meta-analysis, we performed a descriptive and qualitative evaluation of the studies focused on carrier development for the intracellular delivery of antimicrobial agents. Unlike drug delivery studies in the cancer arena, most intracellular pathogens-targeting studies do not report intracellular drug concentrations, percentage injected dose (%ID) or mg/kg from infected cells or target organs following drug administration. Therefore, not enough raw data were available to perform physiological and pharmacokinetic model-based analyses. Of all the bacterial, viral, fungal, and parasitic intracellular pathogens, bacteria were the most common microbes studied, with *Staphylococcus aureus* and *Mycobacterium tuberculosis* being the top two etiologic organisms. They are well known for their ability to acquire antibiotic resistance, and one of the mechanisms behind the resistance is to parasitize macrophages. Notably, *Leishmania* were the sole species that were chosen as a target pathogen in the parasite category. Leishmaniasis is one of the deadliest infections caused by the obligate intracellular protozoa, by which more than 1.5

million people are newly affected and about 70,000 deaths occur each year (117). It can be transmitted via sand flies, and about 70 animals including humans are known hosts of *Leishmania* (118). There are other protozoan parasites, such as *Toxoplasma gondii* and *Trypanosoma cruzi*, which take advantage of phagocytes to grow, replicate, and evade the host immune system. However, *Leishmania* species differ from other protozoan parasites in that they predominantly exploit macrophages as host cells (119). Pentavalent antimonial compounds, for example meglumine antimoniate, are the mainstay of treatment but with severe side effects, such as hepatotoxicity and cardiotoxicity (120). Due to the severity of the disease and many challenges that it poses against mammalian populations, there have been a steady and increasing number of studies targeting *Leishmania*.

Our analysis shows that seventy-four (74.7%) out of 99 studies are done with macrophages (Table S1). The predominance of macrophages as the host cells is attributed to their role in innate immunity as the first defense to the invading bacteria (121). The most common administration route used in *in vivo* PD studies was intravenous injection (in 25 (58.1%) out of 43 studies) (Table S5). Upon administration, drug-loaded nanocarriers undergo opsonization, distribution in the reticuloendothelial system (RES) organs, and subsequent phagocytic uptake predominantly by macrophages. The particle size in the majority of studies (63.6%) falls in the range of 100 to < 1000 nm (Fig. 4c, Table S3), which is likely driven by the administration route (intravenous injection) and biodistribution in the RES organs where the most infected phagocytes are located (14, 35). The most commonly used carrier type was nanoparticles followed by liposomes, in 51 (51.5%) and 15 (15.2%) out of 99 studies, respectively (Fig. 4b, Table S7), some of which contained mannose or β-glucan ligands to target receptors (e.g., CD206 or Dectin 1) expressed on macrophages.

Most studies (78.8%) provided some proof of intracellular delivery based on microscopic imaging of labeled carriers in the cells or flow cytometric quantitation of cellular fluorescence after incubation with the carriers, but not to the organelle levels: how the carriers traffic in the cells and escape endosomes to finally reach the intracellular niche where the pathogens are hiding. We found that only 31 out of 99 studies provided comparative inhibitory concentrations (MIC, IC$_{50}$, or EC$_{50}$) against a free drug control. There were 8 studies that claimed their carrier system was for intracellular delivery of antimicrobial therapeutics but did not provide any experimental evidence supporting such claims, such as microscopic imaging, flow cytometry, and *in vitro* PD.

While microscopy and flow cytometry are dominant tools to investigate intracellular delivery of carriers, the following limitations need to be considered in interpreting the results. To enable microscopic or flow cytometric detection of carriers, fluorescent dyes are incorporated in the carriers by

Table III Characteristics of Intracellular Delivery Studies on Fungal Pathogens (*n* = 8)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy study vs free drug control	Route	Examined organs	Efficacy study vs control
Batista-Duharte 2016 (96)	macrophages	*Sporothrix schenckii*	amphotericin B	needle-like lipid assemblies	detoxified LPS-containing lipid	5–10 μm in length	+	MIC, 0.25 μg/mL vs 1 μg/mL; MFC 0.5 μg/mL vs 2 μg/mL	IP	liver, spleen	CFU, comparable or reduction by <1 log
Cheng 2021 (97)	macrophages	*Cryptococcus neoformans*	amphotericin B	micro-to-nano systems	BSA-binding MMP-3-responsive peptides, PEG	115 nm to 7 μm	+	concentration for effective inhibition, 1 μg/mL vs 2 μg/mL	IV	liver, spleen, lung	survival, 70% vs 0% (untreated control); CFU, reduction by 2–3 logs (vs untreated control)
Diez-Orejas 2018 (98)	macrophages	*Candida albicans*	-	nanosheets	PEGylated graphene oxide	200–600 nm	-	Intracellular CFU inhibition, increase by 5–25% (vs untreated control)	-	-	-
Diez-Orejas 2021 (99)	macrophages	*Candida albicans*	-	inorganic nanoparticles	mesoporous SiO_2-CaO	250 nm	-	-	-	-	-
Li 2019 (100)	oral epithelia	*Candida albicans* oral candidiasis	amphotericin B	carbon dots	carbon dots with positively charged guanidine groups	4.72 ± 1.25 nm	+	MIC, 31.25 μg/mL vs 2.50 μg/mL; biofilm CFU, comparable reduction	-	-	-
Mejía 2021 (101)	macrophages	*Histoplasma capsulatum*	itraconazole	nanoparticles	PLGA core, TPGS coating	147.3– 188.5 nm	+	MIC, 0.061 μg/mL vs 0.031 μg/mL; IC_{50}, comparable: 0.031 μg/mL	-	-	-
Shao 2015 (102)	brain capillary endothelial cells	*Cryptococcus neoformans* meningitis	itraconazole	polymeric micelles	DHA-PEG-pLys-pPhe	-	-	-	IV	brain	survival, 30% vs 0% (free drug control); CFU, reduction by 2 logs (vs free drug control)

Table III (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD		In vivo PD		
							Free drug control	Efficacy study vs free drug control	Route	Examined organs	Efficacy study vs control
Xie 2019 (103)	macrophages	Candida albicans	amphotericin B	cell membrane-coated liposomes	RBC membranes, cationic liposomes, P4.2-derived peptides	100 nm	+	Fungal growth comparable	IV	lung	survival, 75% vs 10% (LIP-AmB control); CFU, reduction by > 1 log (vs LIP-AmB control)

Abbreviations: LPS, lipopolysaccharide; MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; IP, intraperitoneal; CFU, colony forming unit; BSA, bovine serum albumin; MMP-3, matrix metalloproteinase 3; PEG, polyethylene glycol; IV, intravenous; PLGA, poly (lactic acid-co-glycolic acid); IC50, 50% inhibitory concentration; DHA, dehydroascorbic acid; RBC, red blood cell; OD630, optical density at 630 nm; LIP-AmB, liposomal amphotericin B

chemical conjugation or physical encapsulation (122). In the former, the fluorescent signals indicate the carriers (provided that the conjugation is stable throughout the experiment). In the latter, the fluorescent dye is considered a proxy of the delivered drug. Both cases have caveats. It is often assumed that the intracellular delivery of a carrier is synonymous to intracellular delivery of a drug. It is not always the case if the drug is prematurely released before the carrier enters the cells. Drug release profile measured in buffered saline does not warrant stable drug encapsulation since serum proteins can accelerate the drug release (123); therefore, one may not exclude the possibility of premature drug release *in vivo* on the basis of *in vitro* release kinetics. On the other hand, when a dye is physically encapsulated in the delivery platform, intracellular fluorescent signal only indicates that the *dye* can be delivered or released into the cell. While this result may provide a reasonable anticipation that a drug may be able to enter the cells likewise, it cannot be taken for granted since the dye does not always represent physical and chemical properties of the therapeutic agents. Moreover, some of the lipophilic dyes may leach out of carriers and partition to the cell membrane upon the carrier-to-membrane contact, without the carrier entering the cells (122). In this case, the fluorescence-based methods can produce a misleading conclusion about the carrier. Therefore, the results from both methods may not be used as sole evidence for intracellular delivery of drugs. If these methods indicate intracellular delivery of carriers and drug surrogates, additional studies need to be performed with drug-loaded carriers to confirm the intracellular delivery of a drug. For example, drug concentration can be measured *in vitro* after treating the cells with the drug-loaded carriers. Nevertheless, the entry of drug into cells via the designed carrier does not warrant intracellular trafficking to the target organelles such as endoplasmic reticulum-like vacuoles, late endosomal compartments, and macrophage phagosomes. Therefore, it is preferable to complement with *in vitro* PD tests, such as intracellular CFU.

To understand the contribution of carriers to intracellular delivery of a drug, control groups need to be selected carefully. When we surveyed the published reports, 69.7% have compared the *in vitro* antimicrobial efficacy of encapsulated therapeutic agents to the free control. Of these, only 31.3% provided comparative MIC, IC_{50}, or EC_{50} *versus* that of the free drug control. Given the potential bioactivity of carriers, it is desirable to include a drug-free carrier and a mixture of drug and drug-free carrier as additional controls. For example, chitosan or fucoidan, one of the commonly used carrier materials, have shown to induce antibacterial activity (124, 125). Without a comparison with an unformulated drug, drug-free carrier, and their mixture, it is difficult to determine whether the enhanced therapeutic efficacy of a carrier-loaded drug is due to the encapsulation and intracellular

Table IV Characteristics of Intracellular Delivery Studies on Parasitic Pathogens ($n = 15$)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD Free drug control	In vitro PD Efficacy study vs free drug control	In vivo PD Route	In vivo PD Examined organs	In vivo PD Efficacy study vs control
Borborema 2011 (107)	macrophages	Leishmania	meglumine antimoniate	liposomes	PC, cholesterol, phosphatidyl-serine	141.0–142.3 nm	+	IC$_{50}$ tenfold reduction	-	-	-
de Oliveira 2020 (108)	macrophages	Leishmania cutaneous infection	quinoxaline derivative	liposomes	hyaluronic acid coating, cholesterol, DOPC, DOPE	214.1–238.1 nm	+	IC$_{50}$ on intracellular amastigotes, comparable	IV, topical	liver, spleen	-
Fernandes Stefanello 2014 (109)	macrophages	Leishmania	pentenoate derivative	nanogels	hyaluronic acid-poly(DEGMA-co-OEGMA) conjugate	150–214 nm	-	-	IV	liver, spleen	-
Franch 2020 (39)	macrophages, myoblast cells	Leishmania	-	nanoparticles	DNA	300 nm	-	-	-	-	-
Gaspar 2015 (41)	macrophages	Leishmania	paromomycin	liposomes	PC, PEG, DMPC, DMPG, DPPC, DPPG, SA	90–250 nm	-	-	IV	liver, spleen, lung	parasite burden, 7 log reduction (liver); 2 log reduction (spleen)
Gupta 2015 (104)	macrophages	Leishmania	amphotericin B	lipo-polymerosomes	glycol chitosan-stearic acid copolymer, cholesterol, lipoteichoic acid coating	443.1 ± 17.6 nm	+	IC$_{50}$ on intracellular amastigotes, 0.082 µg/mL vs 0.295 µg/mL	IV	liver, spleen, lung	parasite inhibition 89.25% vs 56.54%
Heidari-Kharaji 2016 (110)	macrophages	Leishmania	Paromomycin	lipid nanoparticles	stearic acid	120 nm	-	-	IM	lymph node	parasite burden, reduction by > 6 logs (vs free drug control)
Jain 2015 (105)	macrophages	Leishmania	amphotericin B	dendrimeric nano-conjugates	mannose-conjugated PPI	-	+	IC$_{50}$ on intracellular amastigotes, 0.0385 µM vs 0.24 µM; inhibition of parasite, increase by 15%	IV	spleen	inhibition of parasites, 80.16% vs 43.51%

Table IV (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD Free drug control	In vitro PD Efficacy study vs free drug control	In vivo PD Route	In vivo PD Examined organs	In vivo PD Efficacy study vs control
Kumar 2019 (111)	macrophages, neutrophils	*Leishmania*	amphotericin B	nanovesicles	macrophage membrane	100 nm	+	LD_{50} on intracellular amastigotes, 3- to fourfold reduction	-	-	-
Nahar 2009 (112)	macrophages	*Leishmania*	amphotericin B	nanoparticles	mannose-PEG-PLGA	157–198 nm	+	percent inhibition of intracellular amastigotes, 87.50% vs 61.77%	-	liver, spleen, lymph nodes	-
Ortega 2021 (113)	macrophages	*Leishmania*	maglumine antimoniate	liposomes	EPC, cholesterol, POPS, α-tocopherol	339.4 ± 10.9 nm	+	infection index on intracellular amastigotes, reduction by 78% vs 43% (at 48 h); 85% vs 56% (at 96 h)	-	-	-
Rebouças-Silva 2020 (114)	macrophages	*Leishmania*	amphotericin B	nanostructured lipid carriers	lipid	242.0 ± 18.3 nm	+	IC_{50} on intracellular amastigotes, 11.7 ng/mL vs. 5.3 ng/mL	IP	-	parasite load in infected ears, reduction by 3 logs (vs. liposomal AmB control)
Romanelli 2019 (115)	macrophages	*Leishmania*	sertraline	liposomes	PS, PC, cholesterol	128 nm	+	EC_{50} on intracellular amastigotes, 2.5 µM vs 4.2 µM	SC	liver, spleen	parasite burden, reduction by 72–89% (vs untreated control)
Sousa-Batista 2019 (116)	macrophages	*Leishmania*	amphotericin B	microparticles	PLGA	5.5 µm, 10.3 µm	+	IC_{50} on intracellular amastigotes, 0.05 µg/mL vs 0.08 µg/mL	SC	-	parasite burden, reduction by 97% (vs free drug control)

Table IV (continued)

Study	Host cell	Etiologic organism	Active drug	Carrier	Carrier material	Size	In vitro PD			In vivo PD		
							Free drug control	Efficacy study vs free drug control		Route	Examined organs	Efficacy study vs control
Want 2017 (106)	macrophages	Leishmania	artemisinin	liposomes	PC, cholesterol	83 ± 16 nm	+	IC_{50} on intracellular amastigotes, 6.0 μg/mL vs 15.2 μg/mL; percent infected macrophages, reduction by 10–20%		IP	liver, spleen	percentage inhibition, 82.4% vs 68.3% (liver); 77.6% vs 62.7% (spleen)

Abbreviations: PC, phosphatidylcholine; IC50, 50% inhibitory concentration; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; IV, intravenous; DEGMA, di(ethylene glycol) methacrylate; OEGMA, oligo(ethylene glycol) methacrylate; PEG, poly(ethylene glycol); DMPC, dimiristoyl phosphatidylcholine; DMPG, dimiristoyl phosphatidylglycerol; DPPC, dipalmitoyl phosphatidylcholine; DPPG, dipalmitoyl phosphatidylglycerol; SA, stearylamine; SDS, sodium dodecyl sulphate; IM, intramuscular; PPI, poly(propylene imine); LD50, 50% lethal dose; PLGA, poly(lactide-co-glycolide acid); EPC, egg phosphatidylcholine; POPS, palmitoyl oleoyl phosphatidyl serine; IP, intraperitoneal; PS, phosphatidylserine; EC50, 50% effective concentration; SC, subcutaneous; LIP-AmB, liposomal amphotericin B

Table V Study Methodologies Used to Assess Intracellular Delivery and Antimicrobial Efficacy

Study methodologies	Bacteria (*n* = 62)	Virus (*n* = 16)	Fungus (*n* = 8)	Parasite (*n* = 15)
Intracellular delivery study				
Microscopy, n (%)	**45 (72.6)**	**11 (68.8)**	**8 (100.0)**	**11 (73.3)**
Flow cytometry, n (%)	**20 (32.3)**	**6 (37.5)**	**5 (62.5)**	**8 (53.3)**
Antimicrobial efficacy study				
In vitro **PD, n (%)**	**49 (79.0)**	**14 (87.5)**	**6 (75.0)**	**11 (73.3)**
Microbial burden *vs.* free drug control, n (%)	40 (64.5)	5 (31.3)	3 (37.5)	7 (46.7)
MIC, IC50 or EC50 *vs.* free drug control, n (%)	12 (19.4)	7 (43.8)	4 (50.0)	8 (53.3)
Both *vs.* free drug control, n (%)	9 (14.5)	2 (12.5)	2 (25.0)	
In vivo **PD, n (%)**	**18 (29.0)**	**4 (25.0)**	**4 (50.0)**	**8 (53.3)**
No evidence of intracellular delivery, n (%)	**7 (11.3)**	**0 (0)**	**0 (0)**	**1 (6.7)**

Note: No evidence of intracellular delivery is defined when a study was conducted with neither microscopy/flow cytometry nor *in vitro* PD

Abbreviations: PD, pharmacodynamics; MIC, minimum inhibitory concentration; IC50, 50% inhibitory concentration; EC50, 50% effective concentration

delivery of the drug or to the additive/synergistic activities of the drug and carrier. Although the blank delivery platform control was not employed as a quality evaluation criterion in this meta-analysis, it would be ideal to include not only free drugs but also drug-free carriers when comparing the PD in future studies.

Our analysis also reveals an access barrier that formulation scientists may face in entering the antimicrobial delivery field. Many organisms of clinical significance require a laboratory with the biosafety level 3 or higher and biocontainment facilities due to the infectious nature of microorganisms (*Mycobacterium tuberculosis*, *Francisella tularensis*, *Brucella*, *Ebola*, etc.) (126). Non-pathogenic models that share the intracellular residence and drug sensitivity as pathogens of interest will be extremely valuable to drug delivery scientists who intend to assess the effectiveness of delivery systems. For instance, *Mycobacterium smegmatis* was once considered as a non-pathogenic, fast-growing surrogate of *M. tuberculosis* (127, 128). Although *M. smegmatis* was criticized for the differential sensitivity to drugs and stresses and the inability to persist in phagocytes (129), a new strain similar to *M. tuberculosis* in drug sensitivity has been reported in recent literature (130). We expect that growing availability of such non-pathogenic model organisms will help introduce more investigators into the antimicrobial drug delivery field. Animal models are another bottleneck to the translation of new technology. *M. tuberculosis* has been studied in various preclinical models, including zebrafish, mice, rabbits, guinea pigs, and nonhuman primates, infected with model organisms (37, 131–136). The sensitivity to infection, immune responses to the pathogens, and the type of lesions vary with animal models; thus, the choice of animal models depends on the purpose of research (131). In the evaluation of new carriers, mouse models are dominantly used for locating the carriers in organ- and cell

levels. However, a recent study shows an example using zebrafish to observe *in vivo* trafficking of polymersomes to *M. marinum*-infected macrophages and granulomas with the convenience of optical transparency and fluorescent transgenic lines (37). To determine the effectiveness of new delivery technology in cost- and time-efficient manner, it will be important for drug delivery scientists to develop broader understanding of available animal models and their utility and limitations.

Conclusions

Substantial advancements have been made in nanotechnology and precision medicine over the last few decades. The present meta-analysis reviewed the quality of carrier-medicated antimicrobial delivery studies against intracellular pathogens and revealed that several studies lack essential components of PD efficacy studies. To facilitate the clinical translation of antimicrobial products for intracellular targets, future drug delivery research on intracellular pathogens need to consider the utility and limitations of dominant experimental methodologies and improve the rigor of carrier evaluation.

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