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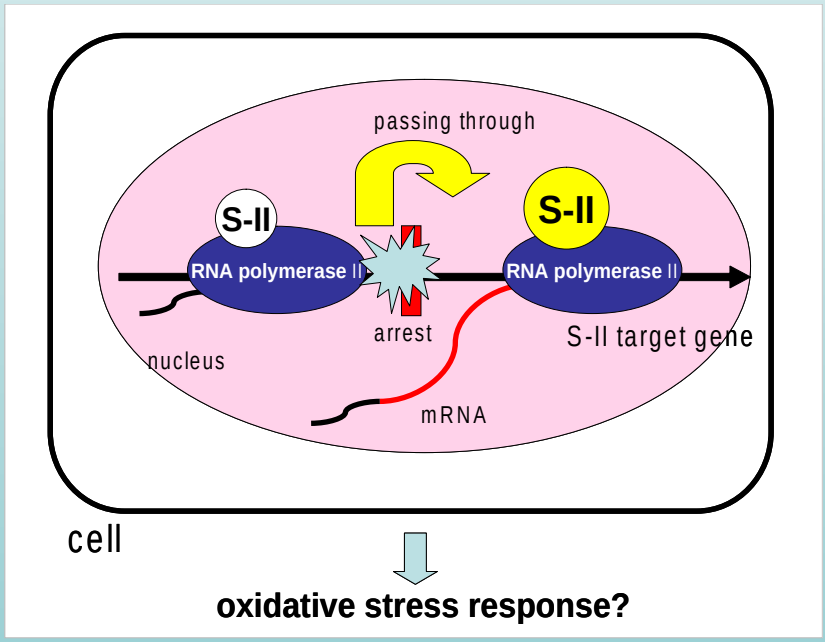

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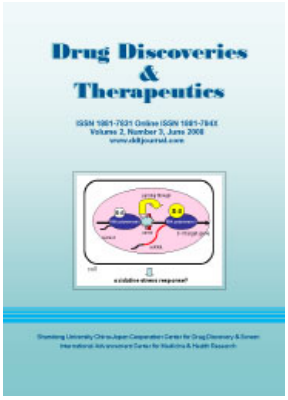
Drug Discoveries & Therapeutics



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Review

S-II mediated gene regulation

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ABSTRACT: S-II, also designated as TFIIS, was the first identified transcription elongation factor and is widely found in eukaryotes. Among known elongation factors, S-II has a characteristic biochemical activity: it facilitates the elongation process by allowing RNA polymerase II (RNAPII) to read through transcriptional blocks *in vitro*. While the biochemical and molecular mechanism of stimulating elongation *in vitro* has been shown in detail, the *in vivo* role of S-II long remained unclear. Recent research has revealed that S-II has a crucial role in the activation of expression of a set of genes *in vivo*. In this review, we summarize recent studies focusing on the regulation of gene expression by S-II and discuss the biological functions of S-II.

Keywords: Transcription, Elongation, Proofreading

1. Introduction

Regulation of gene expression has to be carried out precisely in order to maintain cellular functions for development and differentiation. Because gene expression is highly regulated at the level of transcription, this step has a great impact on cellular functions. The transcription process contains multiple steps designated as pre-initiation, initiation, elongation, and finally termination. Among these, the elongation step is one of the key steps in the activation of gene expression (*1*). Research has shown that expression of certain cellular or viral genes is regulated at this transcriptional step (*2-4*).

S-II was originally identified as a stimulation factor of RNAPII from an extract of Ehrlich ascites tumor cells in 1973 (*5*) and then purified in 1979 (*6*). Its activity was later found to be due to the ability to suppress pausing and to increase the yield of long transcripts *in vitro* (*7-10*). During mRNA elongation, RNAPII can encounter DNA sequences that cause transcriptional arrest (*11-13*). In some cases, RNAPII backtracks by several nucleotides along the template DNA. As a result, the 3'-end of the transcript is extruded from the catalytic center of RNAPII, leading to transcriptional arrest. In such cases, S-II helps RNAPII pass through the arrest sites by stimulating the 3'→5' nuclease activity intrinsic to RNAPII, designated as "cleavage" activity. Then, S-II induces cleavage of the nascent RNA leading to the resumption of transcription elongation. This characteristic activity of S-II *in vitro* may contribute to maintenance of mRNA quality, thus regulating gene expression. Indeed, it has been proposed that genetic mutations in S-II cause transcriptional arrest and produce truncated transcripts as mentioned below.

Another point of view concerning transcription arrest is that it occurs not only when RNAPII encounters arrest sites on template DNA but also when incorrect ribonucleotides are incorporated into the nascent transcripts. S-II relieves this arrest by stimulating cleavage of these mis-incorporated nucleotides (*14,15*). Thus, S-II potentially proofreads the nascent transcript and maintains transcriptional fidelity (*15-17*).

Moreover, a recent study demonstrated that homozygous inactivation of the S-II gene in mice leads to embryonic lethality with impaired definitive erythropoiesis in fetal liver (*18*). In addition, S-II contributes to oxidative stress resistance (*17*). These findings provide evidence that S-II has a distinctive role in biological processes.

2. Genes with expression levels that are changed by the inactivation of S-II

What follows in Table 1 is a summary of the reported genes with transcriptional levels that are regulated by S-II.

2.1. *SSM1 (suppressor of 6-azauracil sensitivity of the S-II null mutant 1)*

Although the yeast S-II null mutant is viable, loss of S-II activity leaves the mutant sensitive to a drug that inhibits IMP dehydrogenase (IMPDH), 6-azauracil (6AU) (*19*).

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Table 1. Genes regulated by S-II

Species	Gene	Ref.
Yeast	*SSM1/SDT1*	*19*
	IMD2/PUR5	*22*
	GAL1	*26*
Drosophila	*hsp70*	*29*
Mouse (fetal livers)	*Bcl-x_L*	*18*

In the course of identifying multi-copy suppressor genes of this phenotype, the *SSM1* gene, which encodes a 280-amino-acid polypeptide, was identified (*19*). Subsequently, *SSM1* was first described as a highly specific pyrimidine 5'-nucleotidase in *S. cerevisiae* (*20*). Shimoaraiso *et al*. also demonstrated that *SSM1* has two transcriptional arrest sites within the transcription unit of this gene *in vitro* that can be relieved by S-II, suggesting that S-II regulates the expression of *SSM1* through relief of the arrested RNAPII at the intrinsic arresting site of this gene.

2.2. *IMD2/PUR5*

The *IMD2/PUR5* gene, which encodes an IMPDH, is induced by IMPDH inhibitor 6AU and mycophenolate (*21-23*). The induction of the *IMD2/PUR5* gene in the yeast S-II null mutant is defective in response to 6AU (*22*,*24*). Cleavage activity of S-II is required for the induction of the *IMD2/PUR5* gene in response to 6AU (*25*). Reporter gene analysis indicates that the open reading frame of the *IMD2/PUR5* gene is responsible for S-II-mediated transcriptional activation in response to 6AU (*22*,*24*). These results suggest that S-II is involved in the transcription elongation of the *IMD2/PUR5* gene in response to nucleotide depletion treatment, although whether or not the *IMD2/PUR5* gene has intrinsic transcriptional arrest sites is still unclear.

2.3. *GAL1*

In the course of investigating S-II function using the *S. cerevisiae GAL1* gene, S-II was found to be strongly associated with the *GAL1* upstream activating sequence *in vivo* (*26*). S-II was also reported to be necessary for the optimal recruitment of the TATA-binding protein and RNA polymerase II to the *GAL1* promoter region. These results suggest that S-II acts as an activator of the transcriptional initiation step of *GAL1* or as an enhancer of the elongation step just downstream of the initiation site.

2.4. *hsp70*

The heat shock response gene 70 (*hsp70*) of *D. melanogaster* has been shown to be primed for activation in a dormant state without stimuli. In this uninduced condition, RNAPII is engaged but paused at the position just downstream of the initiation site (*27*,*28*). This RNAPII arrest within the promoter-proximal region of *hsp70* is dependent on S-II function, suggesting that S-II relieves the arrested RNAPII from the promoter region of *hsp70* (*29*).

2.5. *Bcl-x_L*

Homozygous inactivation of the S-II gene in mice leads to embryonic lethality with impaired definitive erythropoiesis in the fetal liver (FL) (*18*). A dramatic increase in apoptotic cells has been observed in S-II$^{-/-}$ FL. Consistent with this result, *Bcl-x_L* gene expression is reduced in FL. Reporter gene analysis also provides evidence that S-II participates in the transcriptional activation of the *Bcl-x_L* gene. These data provide one possible explanation that S-II stimulates the elongation step of *Bcl-x_L* transcription, although the possibility that S-II participates in certain transcriptional processes other than elongation, such as initiation, cannot be ruled out.

3. Contribution to oxidative stress resistance

In the course of transcription, nascent transcripts accidentally incorporate abnormal nucleotides. This mis-incorporation of abnormal nucleotides stops the transcriptional elongation chain, causing transcriptional arrest. S-II facilitates the intrinsic exonuclease activity of RNAPII when in arrest sites, and this activity leads to proofreading of nascent transcripts incorporating abnormal nucleotides during transcription.

Oxidized nucleotides such as 8-oxoguanine and 2-oxoadenine are generated in the cellular nucleotide pool by oxidative stress (*30*). Some are mis-incorporated into RNA during transcription (*31*,*32*). Proofreading systems have to be carried out correctly in order to prevent cells from producing aberrant proteins caused by translation using abnormal templates containing incorrect nucleotides. S-II potentially contributes to the maintenance of transcriptional fidelity in the presence of oxidative stress. Additionally, oxidative stress induces the direct oxidation of nucleotides in genomic DNA or mature transcripts. Research has shown that 8-oxoguanine in template DNA causes transcriptional arrest *in vitro* and that S-II relieves RNAPII at the arrest site (*33*). These results strongly suggest that S-II is involved in the oxidative stress response to maintain genetic information.

4. Discussion and Perspective

What follows is a summary of recent *in vivo* studies on gene regulation by S-II as was discussed above.

Research has found that there are genes which have arrest sites and that have expression regulated by the transcription elongation factor S-II *in vivo*.

This supports the idea that S-II makes RNAPII read through the arrest sites present in the gene, promoting transcription elongation *in vivo*. In addition, S-II is also reported to be involved in the initiation complex (*34*,*35*). In this regard, some of the genes mentioned above might be regulated by S-II participating in the initiation process. Additionally, S-II is reported to synergize with p300 (histone acetylation) in productive activator-dependent transcription *in vitro* (*36*). These S-II related findings may expand the view of gene regulation.

Those genes with expression controlled by S-II tend to be regulated in response to external stimuli, which might be a feature they share in common. *SSM1* and *IMD2* are induced by drugs that inhibit nucleotide metabolism. *GAL1* and *hsp70* are induced by external nutrients and heat shock, respectively. Additionally, *Bcl-x*$_L$ participates in anti-apoptotic responses. Therefore, one could speculate that S-II is a regulator for stress response genes when cells are exposed to stress, and this may be the biological function of S-II. S-II may be needed for regulation of genes specific to stress responses to avoid cellular dysfunction.

In terms of stress response, the reduction of *Bcl-x*$_L$ gene expression levels in S-II$^{-/-}$FL may be due to oxidative stress. Reactive oxygen species (ROS) are involved in Erythropoietin-mediated erythroid differentiation (*37*). This situation increases the possibility of generating oxidized nucleotides, leading to mis-incorporation of these nucleotides into DNA or RNA. S-II is assumed to promote read-through at the arrest sites made by oxidized DNA or to eliminate RNA containing mis-incorporated nucleotides *via* its cleavage activity. This may result in the maintenance of transcriptional fidelity, RNA quality, and oxidative stress resistance. The possible involvement of S-II in the oxidative stress response requires further study.

All cellular biochemical processes rely on the accuracy of expression of genetic information. To ensure the fidelity of genetic information flow, organisms have evolved monitoring systems to assess the quality of the units of heredity. In this sense, S-II has an important role in biological processes by contributing to the proofreading of nascent transcripts. If S-II is inactivated, genes will produce frequently aberrant transcripts such as nonstop mRNAs lacking a termination codon (*38-40*). Moreover, the inactivation of S-II results in reduced expression of genes containing arrest sites. Therefore, S-II plays significant roles in regulating gene expression and maintaining cellular functions in living cells. More information about S-II mediated gene regulation *in vivo* and *in vitro* may expand our knowledge of gene expression.

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Review

Microfabrication-derived DDS: From batch to individual production

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ABSTRACT: As a result of recent advances in microfabrication technology (MFT), microparticles including microcapsules and microspheres can be prepared individually and the disadvantages of the conventional microparticles produced by batch production, *i.e.* (i) low loading efficiency, (ii) large size variation, and (iii) initial burst release, have been remedied. In addition, all conventional microparticles have the same structure, a spherical shape, so they have only one function, sustained release. Three-layer microcapsules (TLMCs) have been designed to address these issues. TLMCs consist of a surface layer, a drug carrying layer, and a basement layer. TLMCs have sustained release as well as adhesiveness and targeting functions. TLMCs are prepared using ink-jet printer nozzle technology. The obtained TLMCs are used for the oral delivery of peptide/protein drugs and long-term sustained-release injection preparation. In addition, self-dissolving micropiles (SDMPs) can be individually produced by MFT as a percutaneous preparation. MFT allows biopharmaceutical drugs like insulin, erythropoietin, and growth hormone to be absorbed through the skin. Thus, advances in MFT have accelerated the development of pharmaceutical technology.

Keywords: Microfabrication, DDS, Three-layer microcapsules, Micropiles, Individual production

1. Introduction

Many scientists are interested in nanotechnology, and governments are supporting scientific research on nanotechnologies. In the field of pharmaceutics, nanotechnology is an attractive technology, and research on nanocarriers like liposomes and nanospheres has been widely performed (*1-3*). Nanospheres are a form of nanoparticles. However, nanospheres differ from nanocapsules as shown in Figure 1. As microcapsules, nanocapsules have a capsule structure. Preparing nanocapsules is very difficult and they are 1-2 orders smaller in size. Therefore, nanocapsules are not popular in pharmaceutics. Nanoparticles are fabricated by the same method as used for microparticles, *i.e.* they are produced by a batch system as shown in the figure. Batch systems have a long history; conventional methods of preparing microcapsules and microspheres are classified into two categories: dispersion of preformed polymers and polymerization of monomers. In addition, dispersion is further classified into three methods: (i) emulsion solvent extraction/evaporation, (ii) phase separation (coacervation), and (iii) spray-drying. Scientists have developed many modified methods to remedy the disadvantages of the three methods, *i.e.* low drug-loading efficiency and wide variation in particle size. Many scientists have struggled for three decades to resolve the problem of low drug-loading efficiency. In addition, conventional microparticles have only one function, controlled-release, due to their spherical shape. However, the batch production method has failed to provide clues to resolving these problems. With the advance of microfabrication technology, in contrast, microparticles including microcapsules and microspheres can be prepared individually. When microfabrication technology is used to produce microcapsules, microcapsules can be produced individually. In such instances, a high drug loading efficiency (100% theoretically) can be attained with microparticles of almost the same shape and size. Therefore, pharmaceutical technology is enjoying a renaissance. This review studies advances in microfabrication technology in pharmaceutics and describes the outcomes of microfabrication technology resulting from research by the author.

2. Oral preparation

The main purpose of oral microparticles, *i.e.* microcapsules and microspheres, is for oral

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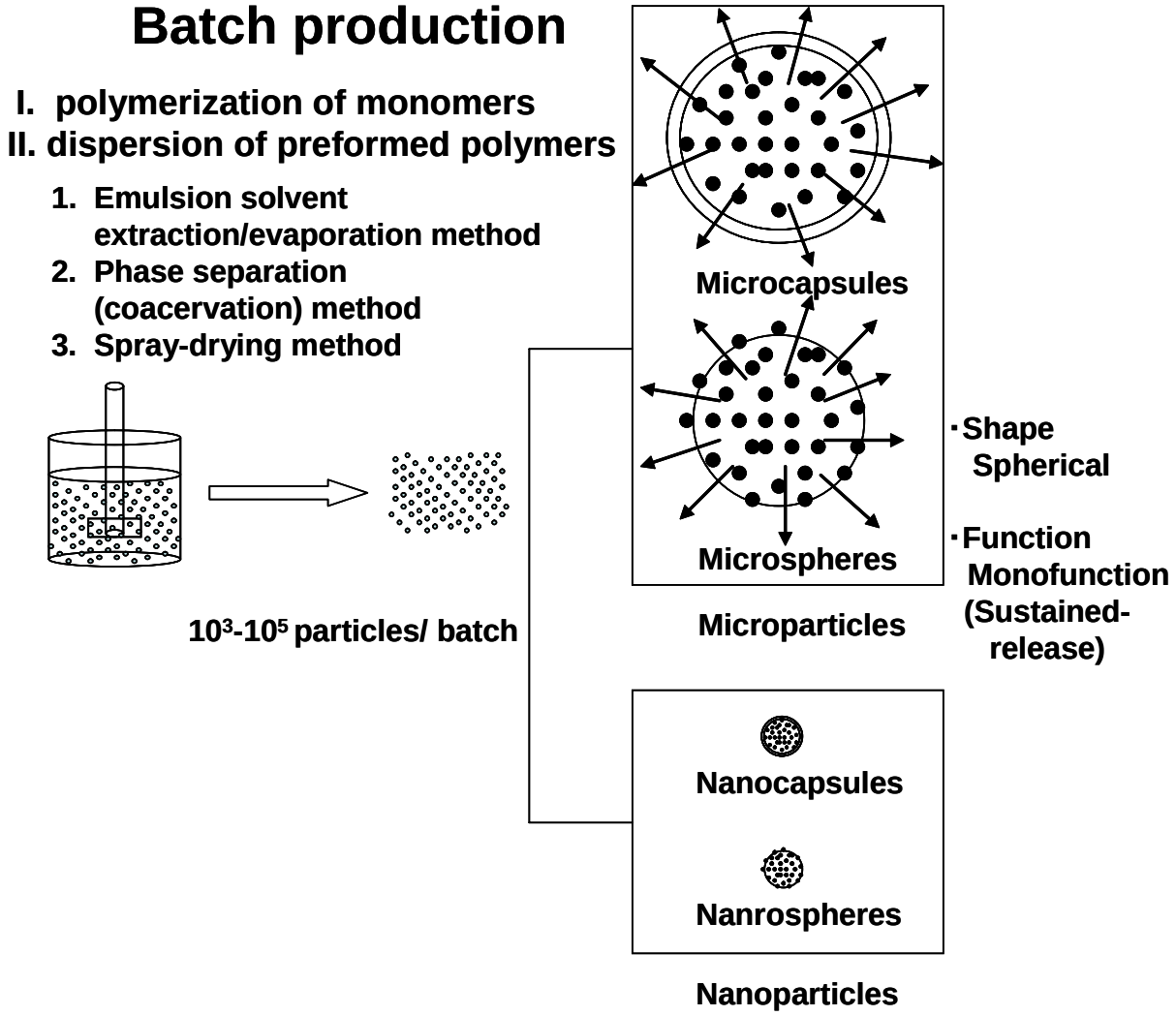

Figure 1. Conventional micro- and nano-particles prepared by batch production method.

sustained-release preparation. Both chemical and natural polymers are used as a wall-forming material. Cellulose and cellulose derivatives such as hydroxypropylmethyl cellulose (HPMC), ethyl cellulose (EC), cellulose acetate (CA), cellulose acetate trimellitate (CAT), cellulose acetate butyrate (CAB), cellulose acetate phthalate, cellulose acetate propionate, hydroxypropylmethyl cellulose phthalate (HPMCP), hydroxypropylmethyl cellulose acetate succinate (HPMCAS), carboxymethyl cellulose (CMC), methyl cellulose (MC), sodium cellulose sulfate, and sodium carboxymethyl cellulose are used as chemical materials. Natural polymers that are used as a wall-forming material include chitosan, gelatin, and alginate.

The polymerization method has not been used for oral pharmaceutical preparations because of the safety problem of the polymers obtained. In pharmaceuticals, the safety problem is crucial. When the polymerization method is used, polymerized substances with different degrees of polymerization are formed. If safety studies are performed with each polymerized substance, the production cost of the microparticles will increase tremendously. Therefore, the dispersion method is generally used.

Using these polymers as wall-forming materials, microcapsules and microspheres were prepared by both of the methods mentioned above. However, each method has both advantages and disadvantages.

The coacervation method can be performed under room temperature. However, a coacervating agent is needed. Therefore, the coacervation method often suffers from residual solvents and coacervating agents detected in the final microcapsules. In addition, each polymer requires its own coacervating agent, so there is no universal rule for the combination of a wall-forming polymer material and coacervating agent.

The solvent extraction/evaporation method has been widely used in pharmaceutics to prepare microcapsules. This requires the evaporation of the solvent by increasing the temperature during the preparation process. Therefore, the possibility of degradation increases when drugs that are susceptible to heating,

like biopharmaceuticals including peptide/protein drugs, are used as the active pharmaceutical ingredients (API). In addition, the microcapsules obtained have a substantial variation in size. The review by Freitas *et al*. provides useful information on microencapsulation by the solvent evaporation/extraction method (*4*).

The spray-drying method is simple and microspheres are easily obtained. This method cannot produce authentic microcapsules. However, microspheres can be converted to microcapsules by modifying the surface of the microspheres during the formation process. The disadvantage of this method is the difficulty in limiting the size of microspheres. Li *et al*. adequately described the conventional large-scale production of these microparticles by spray-drying method (*5*).

Table 1 summarizes the research on microparticles, indicating the core substance, API, wall-forming material, and method of preparation. These microparticles were prepared with either of the aforementioned methods or a modified form of one of those methods. In all cases, the obtained microparticles are spherical and have only one function, sustained-release of the formulated API.

In the last two decades, research focused on the wall-forming material, and natural polymers like sodium alginate were introduced in microcapsules and microspheres. Reports mentioned, for example, diclofenac sodium microspheres prepared by emulsification (*46*), L-lactate dehydrogenase microparticles prepared by spray-drying (*47*), and indomethacin microspheres prepared by precipitation (*48*). Egg albumin microspheres containing nitrofurantoin were prepared by phase separation (*49*). In addition, chitosan was used as a wall-forming material and ketoprofen was used as the core drug for preparation by emulsification/solvent evaporation (*50*). In addition, melatonin was loaded onto chitosan microcapsules by ionotropic gelation (*51*). Chitosan microspheres and nanoparticles were applied to insulin (*52,53*). The review by Kas provides useful information on microparticles made of chitosan (*54*). The purpose of those microparticles was to provide oral sustained-release preparations. On the other hand, mucoadhesive chitosan microspheres were prepared by spray-drying, and the interaction between the obtained microspheres and rat small intestinal mucosal tissue was investigated (*55*). In addition, chitosan microspheres and

Table 1. Microcapsules and microspheres as oral sustained-release preparation

Drug	Wall-forming material		Method	Reference
Acetylsalicylic acid Eudragit	RS	MS	solvent evaporation	(*6,7*)
	CMEC	MC	neutralization reaction	(*8*)
	Eudragit RS	MS	solvent partition	(*9*)
Allopurinol	EC	MC	solvent evaporation	(*10*)
Bacampicillin	Eudragit E	MS	solvent evaporation	(*11*)
Bitolterol	EC	MC	phase separation	(*12*)
Chlorothiazide	whey protein	MC	cross-link	(*13*)
Dexamethasone	Eudragit S100	NS	spray-dry	(*14*)
Diclofenac Na	CAB, PVA	MS	solvent evaporation	(*15*)
	CMC	MS	crosslink	(*16*)
Fenoterol	EC	MS	solvent evaporation	(*17*)
5-fluorouracil	EC	MS	solvent evaporation	(*18*)
Furosemide	EC	MS	spherical crystallization	(*19*)
Ibuprofen	EC	MS	solvent evaporation	(*20*)
	CAB	MS	solvent evaporation	(*21*)
	PHBV	MC	solvent evaporation	(*22*)
	Eudragit RS	MS	emulsion solvent diffusion	(*23*)
Indomethacin	EC, Eudragit RL	MS	solvent evaporation	(*24,25*)
	Polyesters	NS	spray-dry	(*26*)
Isosorbide dinitrate	EC/HPC	MC	oil-in-oil emulsion evaporation	(*27*)
Ketoprofen	CAT, EC	MS	spray dry	(*28*)
	CAB, HPMCP	MS	spray-dry	(*29*)
	Eudragit RS	MS	coacervation/spray-dry	(*30*)
Nifedipine	cetearyl alcohol/poloxamer	MP	hot air coating	(*31*)
Nitrofurantoin	CMC	MC	coacervation	(*32,33*)
Pantoprazole	Eudragit S100/HPMC	MP	spray-dry	(*34*)
Piroxicam	Hyaluronate	MS	spray-dry	(*35*)
Propranolol HCl	CAB	MC	emulsion non-solvent addition method	(*36*)
Quercetin	PMMA	MC	solvent evaporation	(*37*)
Sulfamethoxazole	CAP	MC	spray-dry	(*38*)
Theophylline	HPMC	MO	spray-drying	(*39,40*)
	CMC-Na, HPMC	MC	spray-drying	(*41*)
	EC	MC	emulsion non-solvent addition	(*42*)
	Polyglycerol esters of fatty acids	MS	spray-chilling	(*43*)
	Eudragit RL, Eudragit RS	MC	phase separation	(*44*)
Verapamil HCl	CA, cellulose acetate propionate, CAB	MS	emulsion-solvent evaporation	(*45*)

CA, cellulose acetate; CAB, cellulose acetate butyrate; CAP, cellulose acetate phthalate; CAT, cellulose acetate trimellitate; CMC, carboxymethylcellulose; CMEC, carboxymethylethylcellulose; EC, ethylcellulose; HPC, hydroxypropylcellulose; HPMC, hydroxypropylmethylcellulose; HPMCP, hydroxypropylmethylcellulose phthalate; MC, microcapsules; MP, microparticles; MS, microspheres; NS, nanospheres; PHBV, poly(3-hydroxybutyrate-co-3-hydroxyvalerate); PMMA, polymethyl methacrylate; PVA, poly(vinyl alcohol).

nanoparticles were prepared for the colon delivery of prednisolone (*56*) and oral delivery of protein (*57*). Pargaonkar *et al*. (*58*) used a new method, electrostatic layer-by-layer (LbL) self-assembling, to make core-shell structures for encapsulation of dexamethasone microcrystals with a polyelectrolyte shell. The LbL self-assembly process was used to encapsulate dexamethasone particles with up to five double layers formed by alternating the adsorption of positively charged poly(dimethyldiallyl ammonium chloride), negatively charged sodium poly(styrenesulfonate), and, depending on the pH, positively or negatively charged gelatins of type A (acid pretreated/porcine gelatin) or type B (alkali processed/bovine gelatin) onto the surface of the negatively charged dexamethasone particles. Direct surface modification of dexamethasone microcrystals *via* the LbL process produced monodispersed suspensions with diffusion-controlled sustained drug release *via* the polyelectrolyte multilayer shell. Although many studies have been performed with oral microparticle preparations, a high drug loading efficiency independent on the method of preparation was not attained. All of the methods of preparing these microparticles are batch production methods.

Since the primary goal of oral microparticles, *i.e.*, providing an oral sustained-release preparation, has been attained, scientists are now working to develop an oral delivery system for peptide/protein drugs with microparticle technology. Peptide/protein drugs undergo hydrolysis before being absorbed by the gastrointestinal (GI) tract. Microparticles are a solid preparation and can protect peptide/protein drugs from attack by hydrolytic enzymes. Cui *et al*. prepared insulin loaded copoly(lactic/glycolic) acid (PLGA)-hydroxypropylmethyl cellulose (HP55) nanoparticles as an oral DDS (*59*). The nanoparticles were prepared by diffusion of a modified emulsion solvent in water, and their physicochemical characteristics, drug release *in vitro*, and hypoglycemic effects in diabetic rats were evaluated. The particle sizes of the PLGA nanoparticles (PNP) and PLGA-HP55 nanoparticles (PHNP) were 150-169 nm, and the drug loading rates were 50.3-65.4%. The initial burst release of insulin from the nanoparticles in simulated gastric fluid over 1 h was 50.5-19.8%. In diabetic rats, the relative bioavailability (BA) of insulin from PNP and PHNP was, in comparison to subcutaneous (s.c.) injection (1.0 IU/kg) of insulin, 3.68-6.27%. Ye *et al*. (*60*) prepared chitosan and sodium alginate microcapsules containing insulin by LbL self-assembly.

In contrast, the current author designed three-layer microcapsules (TLMCs). TLMCs were prepared individually. Figure 2 shows the basic method of preparing TLMCs by discharge as is widely used in printing technology, where it is known as the ink-jet method. As each TLMC is prepared individually, the obtained microcapsules have far less variation in shape and size than conventional microcapsules. In addition, TLMCs do not have only one function, *i.e*. controlled release but other functions like targeting and adhesiveness. TLMCs were used in an oral preparation as a gastrointestinal (GI) mucoadhesive patch system known as GI-MAPS. GI-MAPS was designed to surmount the two hurdles for oral peptide/protein preparations, *i.e*. hydrolytic degradation by digestive enzymes and poor membrane permeability of peptide/protein drugs due to their 3D structures. As many conventional oral drug delivery systems (DDS) including absorption enhancers, emulsions, liposomes, and micro- and nano-capsules, protein unfolding technology, protein conjugates, and colon delivery technology have been examined to develop oral peptide/protein drugs. However, trials of all of these drugs have all faced the hurdle of a low BA because the dilution and spread of an absorption enhancer in the GI tract reduces the effectiveness of the absorption enhancer on peptide/protein drugs. GI-MAPS is designed to solve these problems. GI-MAPS consists of three layers: (i) a water-insoluble basement membrane, (ii) a drug-carrying layer, and (iii) a pH sensitive bioadhesive surface membrane. After oral administration, the surface layer dissolves at the targeted intestinal site and adheres to the small intestinal wall, where a closed space is created at the target site of the GI mucosa by adherence to the mucosal membrane. As a result, both drug and absorption enhancer coexist in the closed space and a high drug concentration gradient is formed between the system and enterocytes, contributing to the enhanced absorption of peptide/protein drugs because peptide/protein drugs are absorbed by a passive diffusion mechanism. As a result, the absorption enhancer is used to full advantage.

Microfabrication technology has been developed to prepare micron-sized GI-MAPS with a diameter of 500-1,000 μm. Figure 2 shows a manufacturing process using this method; a large-scale GI-MAPS-producing machine was developed in 2008. This machine has three to four nozzles that are modified to discharge a polymer solution prepared with an organic solvent. Three kinds of solutions are discharged in series; for example, an enteric polymer solution is first discharged onto the surface of a glass plate and then a drug solution with an absorption enhancer and adhesive polymer is discharged onto the dried enteric polymer layer with a smaller diameter of the drug layer than that of the first enteric layer. Finally, a water-insoluble polymer solution is discharged onto the drug layer with a diameter larger than that of drug layer. Figure 2 also shows the GI-MAPS obtained by this method. TLMCs are made individually *via* this method. A previous review by the current author provides useful information on the biopharmaceutical evaluation of GI-MAPS (*61*).

The advantages of TLMCs are: (i) high drug loading

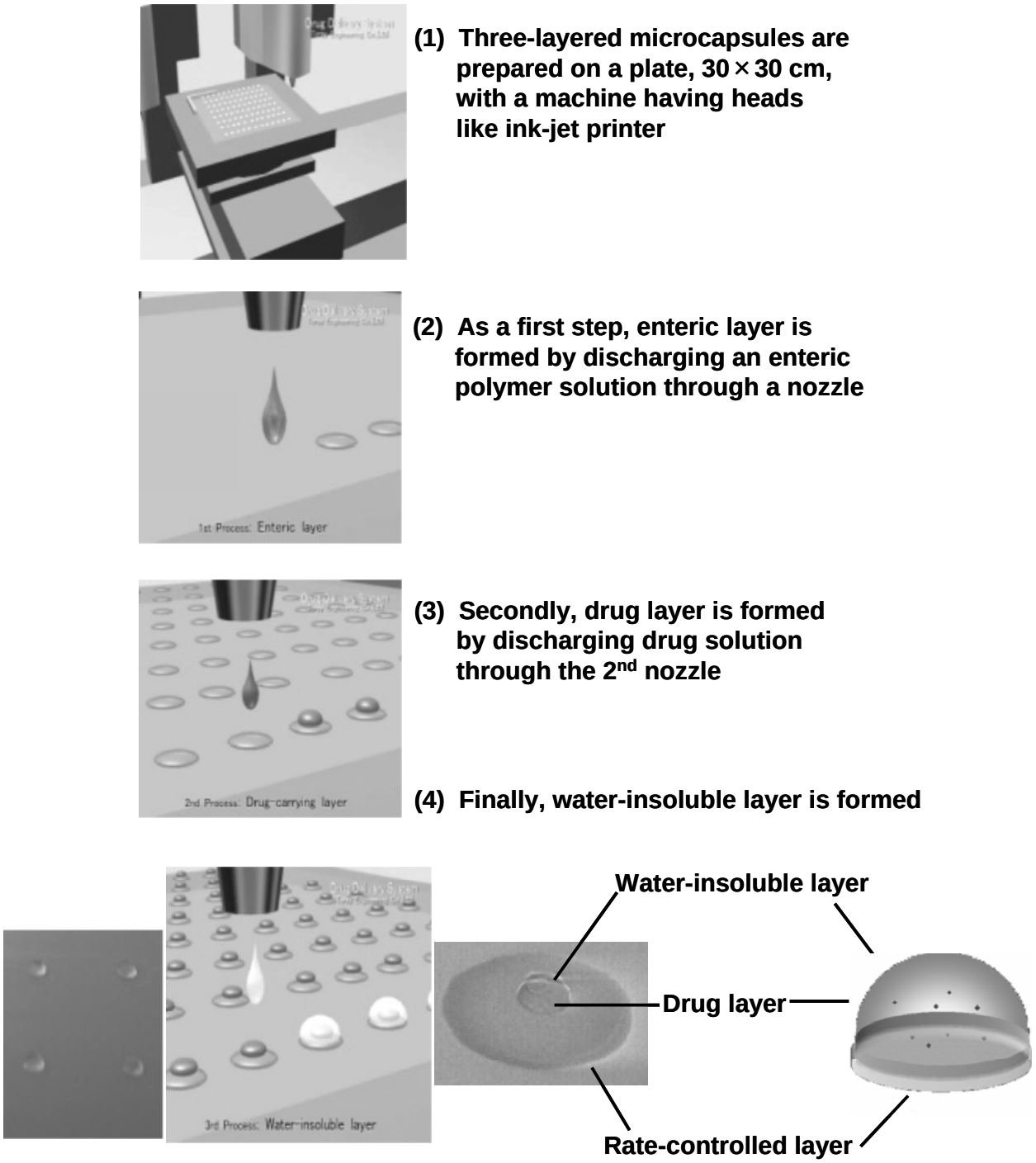

Figure 2. Three-layered microcapsules (TLMC) for oral delivery of drugs.

efficiency, *i.e.*, theoretically 100%, (ii) little size variation for the microcapsules obtained, and (iii) the obtained microcapsules have not only sustained-release but also other functions like site selectivity, targeting, and adhesiveness. The areas for use of TLMCs as an oral DDS are: (i) oral peptide/protein delivery, (ii) sustained-release preparation, and (iii) colon delivery of drugs. With oral delivery of peptide/protein drugs, their BAs improved dramatically, reaching 23% for G-CSF in dogs (*62*) and 12.3% for erythropoietin (EPO) in rats (*63*).

3. Injection preparation

Microparticles for injection preparation are also prepared by the methods described in the previous section. They are: (i) emulsion solvent extraction/evaporation, (ii) phase separation (coacervation), and (iii) spray-drying. The most clinically significant microparticle preparation is leuprolide acetate microspheres (Lupron^{TM}), created by Takeda Chemical Industries and registered with the FDA in 1989. PLGA microspheres containing leuprolide acetate

were prepared by an emulsion solvent evaporation method. A W/O emulsion was first prepared with an inner water phase containing leuprolide acetate and gelatin and an oil phase containing PLGA plus additives such as glyceryl monocaprate and D-lactide in dichloromethane. Then, a W/O/W emulsion was formed with a cooled aqueous 0.1% polyvinyl alcohol solution. Hardened microspheres were obtained by evaporating dichloromethane (*64*). Other researchers used phase separation to prepare polylactic acid (PLA) microspheres and PLGA microspheres. However, they did not succeed as fast as the Takeda group did. In such instances, a great deal of heptane was needed as an organic solvent (*65*). However, completely removing heptane proved difficult because some heptane remained as a contaminant inside microspheres. In addition, the great deal of heptane required to prepare microspheres resulted in difficulties with large-scale production of microspheres. The two water phases were prevented from mixing by increasing the viscosity of the W/O emulsion. As a result, an extremely high loading rate of leuprolide acetate was obtained. However, an initial burst release of leuprolide after injection into experimental animals occurred because the microspheres used did not have a microcapsule structure (*66*). During the initial period of development, monomers of lactic acid and glycolic acid connected by ester bonds were believed to undergo ester hydrolysis, resulting in the release of leuprolide (*67*). However, later research revealed that the basic amino acid residue of leuprolide acetate interacted with the terminal carboxyl group of PLGA inside the microspheres. This structure is very stable and leuprolide acetate was not easily released even when the dissolution medium, water, entered the microspheres. Therefore, leuprolide was released from the microspheres in a sustained manner (*68*). PLGA microspheres released leuprolide over a period of 1 to 3 months, alleviating the pain caused by daily injections and improving patient compliance (*69*). Thus, leuproleide acetate microspheres were an epoch-making preparation in the history of microparticle technology. A review by Andersen *et al*. provides useful information on the biological fate of PLA and PLGA (*70*).

Many reports have previously been published on both microcapsules and microspheres, including nanospheres, for use as injection preparations. Table 2 summarizes the reports on micro and nanoparticles as injection preparations.

As shown in this table, solvent extraction/evaporation is the most popular method for preparing PLA and PLGA microspheres. The review by O'Donnell *et al*. (*134*) provides useful information on the advantages of this method. The review by Soppimath *et al*. (*135*) also focuses on the preparation of microcapsules and microspheres by polymerization.

Microchannel emulsification was introduced to decrease the variation in size of gelatin microspheres (*136*). In such instances, the core material solution/suspension must continuously flow into the wall-forming material solution. A new trend offered by microtechnology in mechanics has allowed a continuous low flow rate, resulting in the decreased size of the obtained microcapsules with decreased variation in size (*137,138*). The basic concept of producing microcapsules is the same as that used in an encapsulator provided by Inotech AG (Switzerland) with a large size nozzle. Recent advances in microfabrication technology have allowed smaller double nozzles. PLGA in dichloromethane solution, 5 w/v%, was discharged through the outer nozzle as a wall-forming material and core material was discharged through the inner nozzle. The discharged particles were collected in a 1 w/v% polyvinyl alcohol solution. Then, microcapsules were obtained by the solvent/evaporation method. By changing the nozzle size, microcapsule size was controlled from 45 to 500 μm with less variation in size. Yeo *et al*. (*139*) described a new method for making reservoir-type microcapsules by an interfacial phase separation principle using ink-jet nozzles. Two ink-jet nozzles that comprise a dual microdispenser system continuously produce two streams of liquid droplets and are aligned so that droplets from one stream collide with droplets from the other stream. After a pair of droplets collides, the polymer droplet spreads over the aqueous droplet to cover the surface of the aqueous droplet. Mass transfer between the two liquids, *i.e.*, solvent exchange, results in the formation of a polymer membrane on the surface of the aqueous droplet. The formation of the polymer membrane depends largely on the favorable spread of the polymer solution on the aqueous droplets and fast solvent exchange and requires judicious selection of the organic solvent. Simple and fast screening methods were developed for selection of a proper solvent. Screening procedures identified ethyl acetate as one of the most desirable solvents. Ethyl acetate and the dual microdispenser system were used to form microcapsules that were subsequently examined by microscopic methods to demonstrate their unique geometry. Details are available in the review by Freitas *et al*. (*140*).

Thus microencapsulation technology has made advances in the past few decades. However, the microcapsules and microspheres obtained with either method have spherical shape. For an oral sustained-release preparation, initial burst release is not a critical factor because the rate of BA of the drug after oral administration is low. However, initial burst release is a critical factor for a sustained-release injection preparation where the rate of BA of a drug is fast after sc injection of microparticles. The conventional microparticles described above cannot solve the initial burst release of the core drug. On the other hand, TLMCs as designed by the current author do not

Table 2. Microcapsules and microspheres as sustained-release injection preparations

Drug	Wall-forming material		Method	Reference
Acetaminophen	PLA	MC	solvent evaporation	(*71*)
Amphotericin-B	albumin	NS	pH-coacervation	(*72*)
Ascorbic acid	pea protein	MP	spray dry	(*73*)
Bovine serum albumin	PLGA	NP	solvent evaporation	(*74*)
Bupivacaine	PLA	MS	solvent evaporation	(*75*)
Camptothecin	PCL	MS	solvent evaporation	(*76*)
Captopril	PLG	MS	solvent evaporation	(*77*)
Cisplatin	PLA	MS	solvent evaporation	(*78*)
	Chitosan	MS	emulsion-chemical crosslink	(*79*)
Ciprofloxacin	BSA	MS	spray dry	(*80*)
Cyclosporine A	PLGA	MS	solvent evaporation	(*81*)
	PLGA, PLA	MP, NP	solvent evaporation	(*82*)
Cytosine arabinoside	PGA/PGA derivative	NP	interfacial deposition	(*83*)
Dexamethasone	PLGA	MP	solid-oil-oil-oil	(*84*)
Diclofenac	PCL	NS	spray-dry	(*85*)
Enkephalin	PGA	MC	solvent evaporation	(*86*)
Ethyliopanoate	poly(benzyl L-glutamate)	MS	solvent evaporation	(*87*)
Finasteride	PPCM	MS	solvent evaporation	(*88*)
5-fluorouracil (5-FU)	PLA	MS	solvent evaporation	(*89*)
	poly(ortho ester)	MC	solvent evaporation	(*90*)
	poly(methylidene malonate)	MS	emulsion/extraction	(*91*)
	chitosan coated PLA	MS	solvent evaporation	(*92*)
	poly(methylidene malonate)	MS	emulsion/extraction	(*93*)
5-FU, indomethacin	PLGA	NS	emulsification solvent diffusion	(*94*)
Ganciclovir	albumin	NP	coacervation	(*95*)
	PLGA (Intraocular)	MS	solvent evaporation	(*96*)
G-CSF	PLGA	NP	emulsion/solvent diffusion	(*97*)
Gemcitabine	polycyanoacrylate	NS, NC	nanoprecipitation	(*98*)
Gentamicin	coralline hydroxyapatite	MS	dispersion polymerization	(*99*)
	BSA	MS	spray dry	(*100*)
	PLA/PLGA	MS	spray dry	(*101*)
human growth hormone	PLGA	MS	atomizer freezedry	(*102*)
	PLGA	MC	solvent evaporation	(*103*)
	dex-HEMA	MS	emulsion/polymerization	(*104*)
Glycine homopeptides	PLA	MS	solvent evaporation	(*105*)
Griseofulvin	PLA	MS	solvent evaporation	(*106*)
Heparin	gelatin	MC	coacervation	(*107*)
Indomethacin	polyesters	NS	spray-dry	(*108*)
Insulin	PLGA/agarose	MS	phase separation	(*109*)
	chitosan	MC	emulsion interfacial cross link	(*110,111*)
	PLGA	MS	solvent evaporation	(*112*)
Interferon	gelatin	MS	coacervation	(*113*)
	PLGA	MS	solvent evaporation	(*114*)
Levodopa	gelatin (Nasal)	MS	solvent evaporation	(*115*)
Methotrexate	gelatin	MS	azide coupling-grafting	(*116*)
Paclitaxel	poly(methylidene malonate	MS	solvent evaporation	(*117*)
	PLGA	NP	spray dry	(*118*)
Pentamidine	PLGA	MC	solvent evaporation	(*119,120*)
Peptides	PLGA	MS	multiple emulsion	(*121*)
	HSA/alginate	MS	emulsion transacylation	(*122*)
Prednisone	star oligo/poly(DL-lactide)	MS	ultrasonic-dispersion	(*123*)
Protein	gelatin	NS	coacervation	(*124,125*)
Ribonuclease, lysozyme	PLA	MP	supercritical carbon dioxide	(*126*)
Rifampicin	PLA	MS	solvent evaporation	(*127*)
Steroids	albumin	MS	solvent evaporation	(*128*)
Streptomycin	albumin and gelatin	MS	coacervation	(*129*)
Testosterone	PLA	MS	solvent evaporation	(*130*)
Tetanus toxoid	Poloxamer/PLGA	MS	solvent extraction	(*131*)
Timolol	PLG	MS	solvent evaporation	(*132*)
Vancomycin	PCL	MP	solvent evaporation	(*133*)

BSA, bovine serum albumin; CA, cellulose acetate; CAB, cellulose acetate butyrate; CAP, cellulose acetate phthalate; CAT, cellulose acetate trimellitate; CMEC, carboxymethylethylcellulose; dex-HEMA, hydroxyethyl methacrylated dextran; HPMCP, hydroxypropylmethylcellulose phthalate; HSA, human serum albumin; MP, microparticles; NC, nanocapsules; NS, nanospheres; NP, nanoparticles; PCL, poly(epsilon-caprolactone); PGA, poly(glycerol adipate); PLA, polylactic acid; PLG, polyglycolic acid; PLGA, copoly(lactic/glycolic) acid; Poly(THPMA), poly(2-tetrahydropranyl methacrylate); PPCM, poly(propylene carbonate maleate).

have an initial burst release of the encapsulated drug. TLMCs are unique and also applicable to a sustained-release sc injection preparation. In accordance with the preparation of TLMCs as GI-MAPS, as was described in the previous section, FITC-dextran was encapsulated into TLMCs and a proof-of-concept (POC) experiment was performed in which poly-ε-caprolactone (PCL) was used as the wall-forming material. The capsule size was less than 1,000 μm. A rate-control layer with a thickness of approximately 10 μm was first formed by discharging a PCL solution containing different amounts of plasticizer including a surfactant. Second,

FITC-dextran was discharged. Finally, the PCL solution was discharged and a water-insoluble basement layer was formed. The *in vitro* release experiment showed long term sustained-release characteristics as shown in Figure 3, and an initial burst release of FITC-dextran was not observed. TLMCs are also applicable to a wide variety of peptide/protein drugs. Therefore, TLMCs containing leuprolide acetate were prepared and sustained-release characteristics were ascertained from the serum leuprolide concentration *vs*. time profile for more than 10 days after sc administration of the TLMC preparation in rats. The advantages of TLMCs are: (i) a high drug loading efficiency, theoretically 100%, (ii) no initial burst release, and (iii) little variation in particle size. A GI-MAPS-producing machine can also be used to prepare TLMCs, although its nozzle size must be decreased.

4. Percutaneous preparation

Thanks to advances in biotechnology, several important biopharmaceuticals such as insulin, erythropoietin (EPO), granulocyte colony stimulating factor (G-CSF), growth hormone (GH), and interferon (IFN) have been developed (*141*). The demand for the delivery of macromolecular biopharmaceuticals like peptide/protein drugs is increasing (*142*). Although the most preferable form of dosage is an oral preparation, the BAs of these drugs are almost 10-23% even when strong absorption enhancers are formulated into GI-MAPS. As a result, no oral preparation of these drugs has entered the pharmaceutical market. Even today, these drugs are administered by iv and/or sc injections. Percutaneous administration is an attractive alternative for the delivery of these drugs because of its many advantages: (i) no or less degradation by hydrolytic enzymes than in the GI tract, (ii) no first-pass effects of the liver associated with oral delivery, (iii) no or less pain than sc injection, (iv) convenience of administration over iv injection, (v) a better and continuously controlled-delivery rate than oral and sc sustained-release preparations, and (vi) easy removal when side-effects appear. Despite their many potential advantages, transdermal drug delivery systems (TDDS) are severely limited by the poor permeability of drugs through the human skin, *i.e*. most drugs do not permeate through the skin at therapeutically relevant rates. Many DDSs have been examined in order to increase the rate of drug permeation through the skin, including chemical enhancers and physical methods. Among them, chemical enhancers have contributed most to the development of TDDS. Table 3 shows TDDS products launched on the American market prior to 2007. The permeability of small molecules through the skin can be enhanced by chemical enhancers (*143*). However, their use is limited because they trigger skin irritation or cause other safety concerns. Iontophoresis, electroporation, and ultrasound have been studied as methods of enhancing physical absorption (*144*). Iontophoresis uses an electric field to drive ionized molecules across the skin by electrophoresis and nonionized molecules by electroosmosis. Despite concerns about skin irritation, iontophoresis may be useful in delivering some peptides and small proteins (*145*). As shown in Table 3, a TDDS with lidocaine by iontophoresis was launched on the American market in 2004. Electroporation and ultrasound also provided temporary enhancement of skin permeability of both small drugs and macromolecules (*146,147*).

However, recent advances in microfabrication technology have allowed preparation of microneedles, which may represent a novel TDDS. Since their first description by Henry *et al*. in 1998 (*148*), microfabrication techniques for the production of silicon, metal, glass, and polymer microneedle arrays with micrometer dimensions have been reported (*149-152*). The microneedles are either solid or hollow and posses a geometrical shape. A microneedle TDDS is roughly defined by a micron-sized needle preparation through and by which a drug is percutaneously administered. Microneedle TDDSs are classified as follows: (i) extremely small needles through which a drug solution can be injected into the skin, (ii) metallic

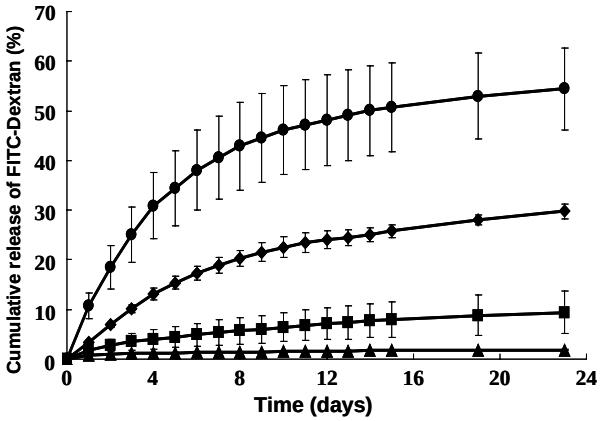

Figure 3. *In vitro* release profiles of FITC-Dextran from three-layered microcapsules (TLMC) made of poly-ε-caprolactone. Release rate was controlled by the addition of plasticizer, PEG 400, (●) 30%, (♦) 25%, (■) 20% and (▲) 5%. Each point shows the mean ± SE of 3-4 experiments.

Table 3. Transdermal DDS products in USA

1980	scopolamine patch
1981	nitroglycerin patch
1983	clonidine patch
1985	estradiol patch
1991	fentanyl patch
1992	estradiol/norethindrone patch
1993	nicotine patch
1994	testosterone patch
1999	lidocine patch
2002	norelgestromin/ethynyl estradiol patch
2003	oxybutynin patch
2004	lidocaine iontophoresis
2005	selegilene patch
2006	methylphenidate patch
2007	rotigitine patch, rivastigmine patch

and/or silastic microneedles onto which a surface drug is coated, and (iii) metallic and/or silastic microneedles by which conduits known as micropores are made on the skin and a drug solution is applied once the microneedles are removed. The physiology of the skin must be understood in order to fully appreciate the function of microneedles.

Human skin consists of three layers, *i.e*. stratum corneum (SC), epidermis, and dermis. The SC is the outer layer of the skin with a thickness of 10-15 μm and is dead tissue. The SC is a strong primary barrier against exogenous compounds including drugs. The second barrier is the viable epidermis (50-100 μm), which contains tissue-like living cells. However, there are no blood vessels in the epidermis. Deeper still, there are blood capillaries in the dermis, which accounts for the bulk of skin volume and contains living cells in the form of nerves. When microneedle arrays are inserted into the skin, conduits are created for the penetration of a drug across the SC. Once a drug penetrates the SC, it can diffuse rapidly through the deeper tissue and permeate the underlying capillaries for systemic absorption. As microneedles do not penetrate to the dermis, where the nerve system exists, pain does not result. Based on this understanding of the skin anatomy, microneedles were designed to penetrate the SC without stimulating the pain receptors found in deeper tissue (*153*).

Silicon microneedles are produced with a standard microelectromechanical system, *i.e*. microfabrication techniques. Chabri *et al*. (*154*) prepared arrays of microconduits for direct and controlled access of molecules across the SC; when inserted into the skin, the arrays enabled drugs to diffuse into the underlying viable epidermis and dermis. Although microneedle arrays originally utilized solid projections for delivery of materials, microfabricated microneedle arrays combined with fluidic microchannels for transdermal extraction of extracellular fluid and blood have also been investigated. Chabri *et al*. prepared microneedles using a modified form of the BOSCH deep reactive ion etching process, which consists of a combination of an isotropic etch and BOSCH reaction. The microfabrication of microneedles involves the use of tools developed by the microelectronic industry to make integrated circuits. Although these tools offer the potential for mass production of microneedles, production is often highly specialized and includes complex multi-step processes (*155*,*156*). For example, 450-μm-thick silicon wafers were spun-coated with photoresist and baked pre-exposure. The wafers were then exposed with the test mask and developed. The wafers were baked postexposure; the thickness of the resist obtained was approximately 8 μm. A standard lithographic mask bearing the appropriate dot array pattern was used during UV exposure to produce a photoresist etch mask. The surface was subsequently etched using a reactive blend of fluorinated and oxygen gases, with those regions directly underlying the photoresist mask being resistant to the etching process. The waters were loaded and subjected to an SF_6 etch to provide an isotropic etch profile. Subsequently, ASETM etch was used to define the length of the microneedle. Finally, the resist was removed in oxygen plasma. Thus, the method of Chabri *et al*. falls under technology used in the field of semiconductors.

In addition to silicon-based microneedles, metallic microneedles were also proposed. They are classified into two categories, hollow microneedles (*149*,*151*) and a microneedle array made of stainless steel (*157*) and titanium (*158*). Silastic and metallic microarrays are used in two ways. One is the application of a drug solution to the skin after physical conduits are made by inserting a metallic and/or silastic microarray. The second way is to use a microarray with the drug coating its surface. After the insertion of the microarray into the skin, the drug is dissolved and absorbed into the skin. Hollow microneedles have also been developed in which a drug solution is injected into the skin through hollow microneedles. As is readily apparent, these hollow microneedles are quite distinct from pharmaceuticals. Furthermore, silicon microneedle arrays are fragile, the use of silicon is relatively expensive, and silicon has yet to be proven to be a biocompatible material. Therefore, these microneedles fall under the category of medical devices.

After Prausnitz *et al*. showed that the absorption of a protein antigen, ovalbumin, was extensively increased by microneedle technology (*158*), the absorption-enhancing effects of microneedle arrays on the following drugs have been reported: (i) small compounds with a MW of less than 1 kDa like diclofenac (*159*), methyl nicotinate (*160*), and bischloroethyl nitrosourea (*161*), (ii) intermediate compounds (MW between 1 and 10 kDa) like FITC-Dextran (*162*), desmopressin (*163*), and insulin (*149*,*151*,*157*,*159*), and (iii) macromolecules (MW larger than 10 kDa) like FITC-Dextran (*162*), bovine serum albumin (*164*), ovalbumin (*158*), antisense oligonucleotides (*165*), plasmid DNA (*166*), and nanospheres (*167*).

Another area of study has been self-dissolving micropiles (SDMPs). Miyano *et al*. (*168*) proposed SDMPs made of maltose for the percutaneous application of dye for tattoos and cosmetics. In their system, maltose was used as a base to make SDMPs. To make maltose SDMPs, maltose was melted by heating it to its melting point, 103°C, and SDMPs were made by introducing maltose into a metallic mold. As a high temperature is needed to make SDMPs, insulin may easily degrade and lose its pharmacological activity. In addition, maltose is a disaccharide, so it causes difficulties in obtaining SDMPs with a hard, steep top because under high humidity in particular it absorbs

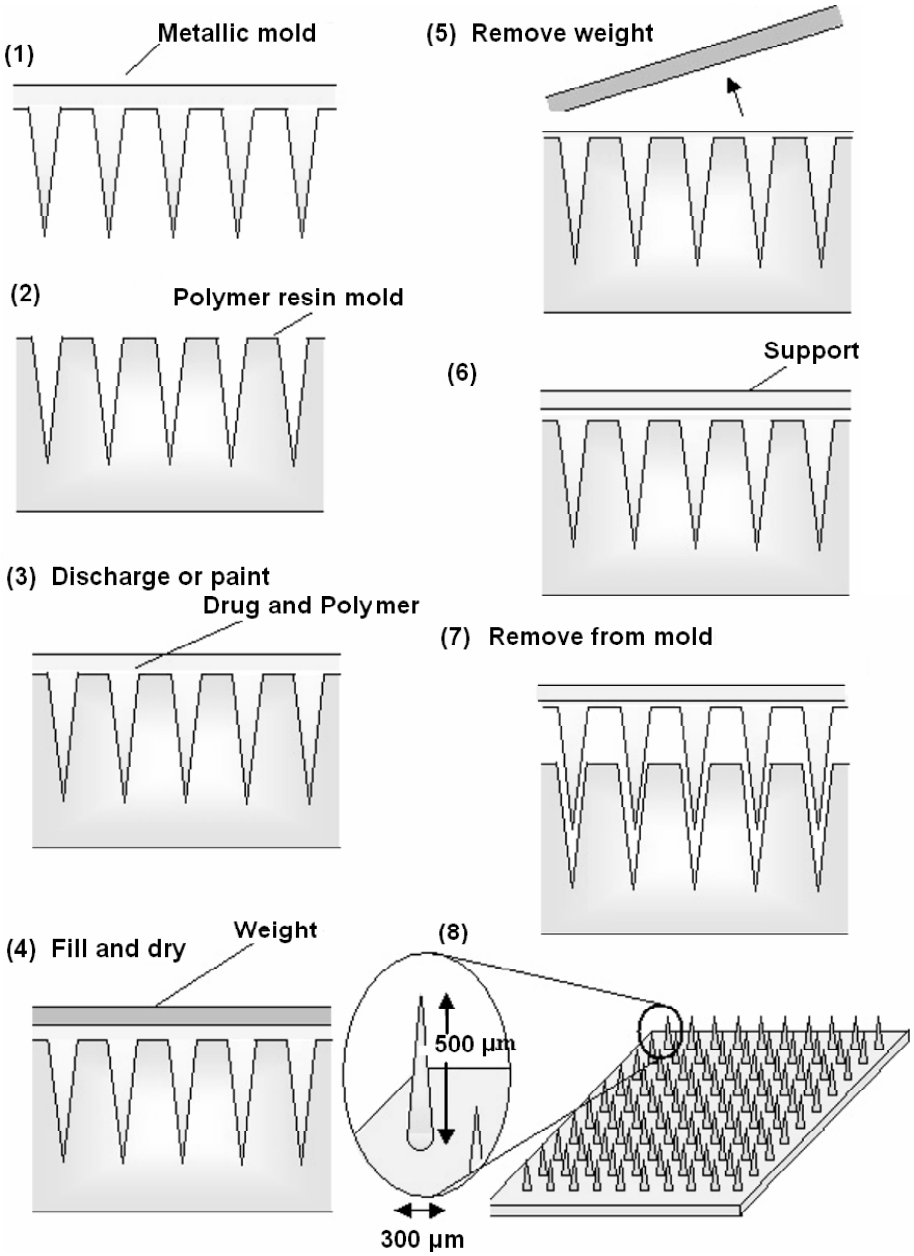

Figure 4. Fabrication process of self-dissolving micropile (SDMP) array.

water from the air; the top of micropile then bends, resulting in difficulty inserting the micropile into the skin.

To overcome these pitfalls, SDMPs made of water-soluble thread-forming polymer were developed. Polysaccharides like chondroitin sulfate, dextran, and hyaluronic acid, proteins like albumin, and synthetic polymers like polyvinyl alcohol were used as a water-soluble thread-forming polymer. A drug solution or drug powder was added to a dense solution of a polymer or combination of polymers. SDMPs were originally formed by withdrawing the top of the micropile tip after the drug and polymer were mixed under low or room temperature. However, as shown in Figure 4, microfabrication technology allows SDMPs to be made individually by means of a mold with micron-sized pores, for example, 500 µm in length and 300 µm in diameter, in the opening base. Their size can be changed from 500 µm to 100 µm in length and from 500 µm to 100 µm in diameter. The method of preparing SDMPs is simple in comparison to preparation by microelectromechanical technology. Namely, a mixture of polymer and drug solution is dispensed into a mold made of polymer resin and dried under pressure. After they fully dry, SDMPs are removed from the mold. A pressing system is useful in accelerating the polymer and drug mixture's insertion into the mold and drying. Metallic microneedles can be formed with MEMS, for example, to make a polymer resin mold. In research by the current author, 100 microneedles with a length of 500 µm and base diameter of 300 µm were formed in a 1.0-cm^2 area on a base plate. A polymer resin mold with 100 microwells was obtained wth these master micropiles. A mixture of polymer and drug was obtained by kneading water-soluble thread-forming polymer, chondroitin sulfate, and a small amount of

Figure 5. Micromissile capsules, 100 capsules, are formed on 1.0 cm^2 patch.

insulin solution; the resultant mixture was introduced into the wells of the polymer resin mold. Finally, the mold was covered with a stainless steel plate and stored in a cold room to dry overnight. The next day, the plate was removed and the hardened mixture was detaching onto supporting material to obtain SDMPs. Figure 5 shows SDMPs containing insulin and Evans blue (EB) obtained by this method. EB was used to stain SDMPs because SDMPs made of chondroitin sulfate and insulin are transparent. The mold can be changed to make SDMPs of different sizes, *e.g.* 200 μm in length and 100 μm in diameter. SDMPs can easily be prepared under low or room temperature. Therefore, SDMPs can be used with drugs that are sensitive to high temperature such as peptide/protein drugs, *i.e.* insulin, EPO, and GH. As water-insoluble drugs can be formulated into SDMN as suspensions, SDMPs can also be used with genetic materials, *i.e.* oligonucleotide delivery, and vaccines including both protein and DNA vaccines. As the shape of the obtained SDMPs is similar to that of a missile, this TDDS is often known as a "micromissile capsule". POC studies on the percutaneous absorption of peptide/protein drugs were performed using SDMPs and showed that high BAs were obtained in mice, rats, and dogs, specifically yielding 90% for insulin (*169*), 80% for EPO (*170*), 88% for IFN, and 95% for human GH. After insulin SDMPs were applied to the skin, the base polymer started to dissolve and the drug was immediately released and then absorbed into systemic circulation. In a dog experiment, the plasma glucose level decreased after insulin SDMPs were applied to the skin at 1.0 IU/kg. The same degree of hypoglycemic effect was observed after sc injection of an insulin solution in the same dogs at the same dose.

5. Conclusion

Thanks to advances in microfabrication technology, novel microparticles such as three-layer microcapsules (TLMC) and self-dissolving micropiles (SDMP) can be prepared on a large scale. As these microparticles are prepared individually, the loading efficiency of a drug is theoretically 100% and a low variation in microparticle size can be attained. These microparticles overcome the disadvantages of conventional microparticles and also have multiple functions. These microparticles have led to a renaissance in pharmaceutical technology.

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Original Article

Cholecystokinin antagonists (part 1): Antinociceptive, anxiolytic and antidepressant effects of *N*-(5-methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N*'-phenylureas and carboxamides

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ABSTRACT: The SAR optimization of the pyrazoline template resulted in novel 3-oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-indole carboxamides and novel 3-oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N*'-phenylureas. These non-peptidal heterocyclic compounds have shown to bind as potent CCK_1 selective and mixed CCK antagonists in a [^{125}I]CCK-8 receptor binding assay. The best amides 3c and 3d of this series displayed an IC_{50} of 20 and 25 nM for the CCK_1 receptor, respectively. The best ureido-pyrazoline 4b and 4e of this series displayed an IC_{50} of 20 and 25 nM, as a mixed CCK receptor antagonist. In the elevated x-maze an anxiolytic effect of the urea 4e was found from 10 µg/kg upwards for the mixed antagonist. In the despair swimming test, a model for testing antidepressants, both mixed and CCK_1 selective antagonists were found active as a modulator over a big range from from 10-500 µg/kg and the magnitude of the effects were comparable to desimipramine. The amides and the phenylureas enhanced significantly the analgesic effect of morphine over a wide dose range in mice.

Keywords: CCK-antagonists, *N*-(3-Oxo-2,3-dihydro-1*H*-pyrazol-4-yl)-indole-carboxamides, 3-Oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N*'-phenyl ureas, Elevated plus-maze, Forced swim test, Tail immersion test

1. Introduction

Cholecystokinin, which act as a neuromodulator/gut hormone and CCK-ligands, agonists as well as antagonists (*1*), have been extensively investigated as potential drug targets (*2*). CCK-antagonists were studied as growth inhibitors in certain forms of cancer (*3*), as anxiolytics (*4*), in the treatment of schizophrenia (*5*), satiety (*6*) and as anti-panic agents (*7*). An agonist, the shortened CCK tetrapeptide, was found to induce panic in patients (*8*). A phase II trial of Devazepide, a potent and CCK_1 selective antagonist (*9*), has been recently completed (*10*) showing a significant enhancement of the effect of morphine in the treatment of chronic and severe pain (*11*).

Asperlicin, a microbial metabolite, was the first non-peptidal cholecystokinin antagonist and analogues thereof, were studies as CCK ligands (*12*). Simplification of the lead structure from nature led to Devazepide, a potent CCK_1 selective cholecystokinin antagonist, containing a 1,4-benzodiazepine template and an indole moiety. The 1,4-benzodiazepine template was varied by a combinatorial solid phase synthesis (*13*) and was optimized in terms of CCK binding affinity (*14*).

In the search for new CCK ligands, in which the 1,4-benzodiazepine structure was replaced by an achiral template, the diphenyl pyrazolone template was selected as starting point.

The combination of indole carboxylic acids (*15*) and phenyl ureas (*16*) with amino-pyrazolines resulted in the discovery of potent lead structures and the results are reported in this publication.

Traditionally, the pyrazoline template had been used for anti-pyretic, anti-rheumatic and analgesic drugs (*17*). Having realized the relevance of the CCK_1 receptor in the treatment of pain (*18*) and depression (*19*), indole amides and phenyl ureas of the pyrazoline template were prepared by a short synthetic approach and evaluated in receptor binding assays. The aim of our study was to convert a template, usually linked with non-steroidal anti-inflammatory agents into a CNS drug, thus creating a non-benzodiazepine template (*20*)

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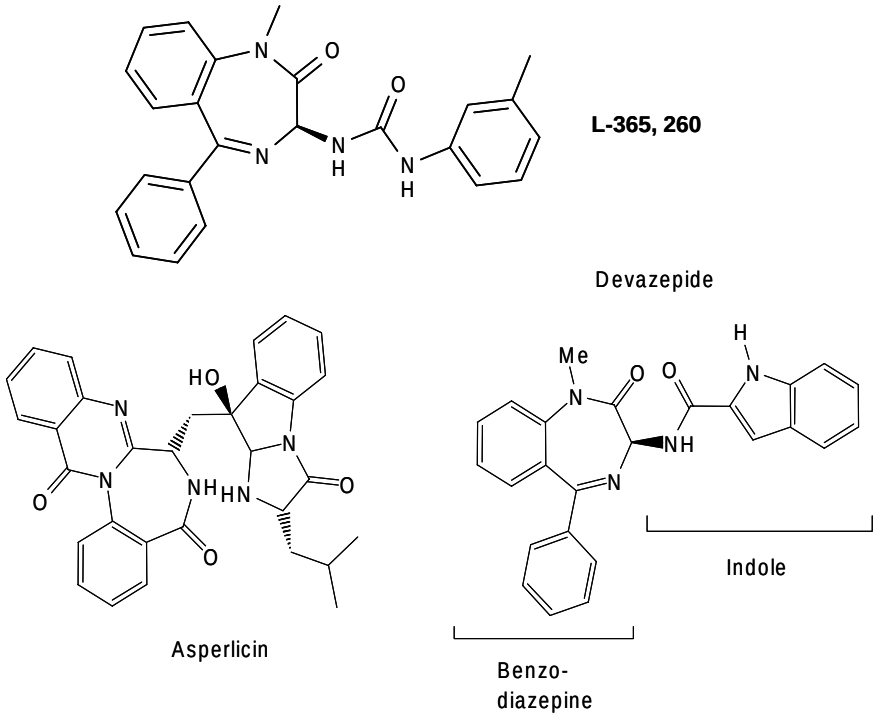


Figure 1. Development of cholecystokinin ligands.

based cholecystokinin antagonist.

The synthesis of novel diphenyl pyrazolinyl amides and ureas, the receptor binding properties on the CCK receptor subtypes and the evaluation of the most potent ligands in various animal models is reported. It is supposed that the 1,5-diphenylpyrazoline moiety is able to mimic the receptor interactions of the 1,4-benzodiazepine scaffold.

2. Materials and Methods

2.1. *Synthesis*

The chemicals were obtained from Aldrich (Gillingham, UK) and Lancaster (Lancaster, UK). Atmospheric pressure chemical ionization mass spectroscopy (APCI), negative or positive mode, was carried out using a Hewlett-Packard 5989b quadrupole instrument (Vienna, Austria). Proton and Carbon NMR spectra were obtained on a Bruker AC 250 instrument (Follanden, Switzerland), operating at 250 MHz, calibrated with the solvent reference peak or TMS. IR spectra were plotted from KBr discs on a Mattson 300 FTIR Spectrometer. Melting points were recorded from a Stuart Scientific (Coventry, UK) Melting Point and are uncorrected.

2.1.1. *Synthesis of 5-methyl-1,2-diphenyl-1,2-dihydro-3H-pyrazol-3-one* ***1***

Diphenyl hydrazine (50.0 g, 0.27 mol) and aceto ethyl acetate (2 Eq. 69.0 mL, 0.52 mol) were heated at 130-150°C for 2 h, with a Dean stark trap. The mixture was then heated for an additional 1.5 h to 180°C, to remove water and ethanol. The remaining solution was distilled at 230-250°C/2mm Hg. This removed any unreacted diphenyl hydrazine to give a viscous black liquid. The mixture was allowed to cool to RT and then ether was added to precipitate out crude black crystals. These were subsequently recrystallized twice from toluene. Yield: 22.1 g, 32.8%. Mol. Weight: 250.3. Mol. Formula: $C_{16}H_{14}N_2O$. MS (APCI(+)): 251 (M+1) m/z. IR (KBr disc): 3465, 3090, 1671, 1590, 1490, 1380, 1349, 1241, 971, 753 and 688 cm^{-1}. ^1H NMR ($CDCl_3$) 300K: 2.07 (s, CH_3), 5.55 (s, CH), 7.05-7.37 (m, Ar-10H) p.p.m. ^{13}C NMR ($CDCl_3$) 300K: 13.7 (CH_3), 99.2 (CH), 123.6, 125.5, 125.9, 128.0, 128.6, 129.3, 135.7, 139.0, 156.3 (C-N), 166.5 (C=O) p.p.m.

2.1.2. *Synthesis of 4-amino-5-methyl-1,2-diphenyl-1,2-dihydro-3H-pyrazol-3-one* ***2*** *via the synthesis of 4-nitroso-5-methyl-1,2-diphenyl-1,2-dihydro-3H-pyrazol-3-one*

5-Methyl-1,2-diphenyl-1,2-dihydro-3*H*-pyrazol-3-one (10.0 g, 0.04 mol) was warmed in HCl (conc) (60.0 mL), when dissolved the solution was diluted with water (up to 400 mL). Sodium nitrite (2.8 g, 0.041 mol) in water (50.0 mL) was added in drops to the mixture at 0°C, whilst stirring. A green precipitate was produced, which was allowed to stand for 45 min, then was filtered, washed with cold water and was reacted further into the amine **2**.

The 4-nitroso-5-methyl-1,2-diphenyl-1,2-dihydro-3*H*-pyrazol-3-one intermediate was dissolved in ethanol (250 mL). A mixture of tin chloride (20.4 g, 0.11 mol) in 20% HCl (120 mL) was heated to 90°C. When

dissolved, this hot mixture was added to the alcoholic solution of the nitroso-intermediate and the mixture was allowed to cool to RT overnight. Ammonia solution (conc 33%) was added to the mixture until no further precipitation occurred. The mixture was filtered, dried and extracted several times with ethanol. The ethanol was removed in *vacuo* and the crude mixture was recrystallized in ethanol to give bright yellow crystals.

Yield: 3.9 g, 37.0%. Mol. Weight: 265.3. Mol. Formula: $C_{16}H_{15}N_3O$. MS (APCI(+)): 266 (M+1), 251 (M+) m/z. IR (KBr-disc): 3407, 3210, 1654, 1592, 1492, 1351, 1262, 751 and 690 cm^{-1}. ^1H NMR (DMSO-d_6) 300K: 1.88 (s, CH_3), 5.57 (s, NH), 7.05-7.12 (tt, Ar-H, J = 7.3 Hz), 7.20-7.45 (m, Ar-9H) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 11.09 (CH_3), 120.3, 122.5, 123.8, 125.5, 128.0, 129.1, 129.8, 136.4, 142.7, 156.3, 166.3 (C=O) p.p.m.

2.1.3. *Synthesis of N-(5-methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)-1H-indole-carboxamides* ***3a-3e***

General method: A solution of 4-amino-5-methyl-1,2-diphenyl-1,2-dihydro-3*H*-pyrazol-3-one (0.2 g, 0.76 mmols) was dissolved in dry acetonitrile (20 mL). The appropriate indole acid (1.25 eq) was added, with DIC (3 eq). The mixture was heated to 60°C and left overnight. The resulting precipitated crystals were filtered, washed and dried.

N-(5-Methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)-1H-indole-2-carboxamide ***3a***

Yield: 201 mg, 65%. Mol. Weight: 408.5. Mol. Formula: $C_{22}H_{20}N_4O_2$. MS (APCI(-)): 409 (M+1), 408 (M+), m/z. IR (KBr-disc): 3401, 3339, 2965, 2358, 1710, 1615, 1583, 1454, 1361, 1172 and 748 cm^{-1}.

N-(5-Methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)-1H-indole-3-carboxamide ***3b***

Yield: 241 mg, 78%. Mol. Weight: 408.5. Mol. Formula: $C_{25}H_{20}N_4O_2$. MS (APCI(+)): 409 (M+1) m/z. IR (KBr-disc): 3343, 2965, 1615, 1581, 1535, 1494, 1453, 1318, 1249, 1191 and 750 cm^{-1}. ^1H NMR (DMSO-d_6) 300K: 2.04 (s, CH_3), 7.09-7.20 (m, Ar-3H), 7.27-7.45 (m, Ar-10H), 7.44-7.47 (d, Ar-H, J = 7.0 Hz), 7.99 (s, Ar-H), 9.16 (s, NH), 11.69 (s, NH) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 12.6 (CH_3), 109.7 (C-NH), 112.4, 121.0, 121.1, 121.5, 122.6, 123.6, 126.1, 126.3, 126.9, 128.6, 129.2, 129.3, 130.1, 132.7, 136.4, 139.9, 152.8, 164.5, 171.9 (C=O) p.p.m.

N-(5-Methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)-2-(1H-indol-3-yl)acetamide ***3c***

Yield: 211 mg, 66%. Mol. Weight: 422.5. MS (APCI(+)): 423 (M+1) m/z. IR (KBr-disc) : 3337, 2965, 1679, 1648, 1629, 1592, 1525, 1488, 1312, 1243 and 749 cm^{-1}. ^1H NMR (DMSO-d_6) 300K: 1.86 (s, CH_3), 3.73 (s, CH_2), 6.94-7.00 (t, Ar-H, J = 8.0, 7.9 Hz), 7.03-7.09 (t, Ar-H, J = 8.2, 8.1 Hz), 7.10-7.17 (t, Ar-H, J = 6.8, 6.7 Hz), 7.27-7.38 (m, Ar-10H), 7.61-7.64 (d, Ar-H, J = 7.7 Hz), 9.38 (s, NH), 10.88 (s, NH) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 12.5 (CH_3), 23.8 (CH_2), 109.1 (C-NH), 109.4, 111.8, 118.4, 119.2, 121.5, 123.7, 124.4, 126.1, 126.4, 127.7, 128.6, 129.3, 130.0, 136.1, 136.6, 139.7, 151.9, 162.7, 170.8 (C=O) p.p.m. Anal. cal. for $C_{26}H_{22}N_4O_2$: C, 73.92; H, 5.25; N, 13.26; O, 7.57. Found C, 73.90; H, 5.22; N, 13.27; O, 7.61.

N-(5-Methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)-3-(1H-indol-3-yl)propanamide ***3d***

Yield: 261 mg, 79%. Mol. Weight: 436.5. MS (APCI(+)): 375 (M+1) m/z. IR (KBr-disc): 3436, 3284, 1640, 1590, 1548, 1490, 1459, 1317 and 753 cm^{-1}. ^1H NMR (DMSO-d_6) 300K: 1.84 (s, CH_3), 2.65-2.71 (t, CH_2, J = 7.2, 7.1 Hz), 2.98-3.04 (t, CH_2, J = 7.3, 7.4 Hz), 6.94-7.00 (t, Ar-H, J = 6.8, 6.8 Hz), 7.03-7.09 (t, Ar-H, J = 6.9, 6.9 Hz), 7.11-7.17 (m, Ar-2H), 7.27-7.41 (m, Ar-11H), 7.55-7.58 (d, Ar-H, J = 7.7 Hz), 9.27 (s, NH), 10.77 (s, NH) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 12.5 (CH_3), 21.4, 23.8 (CH_2), 109.3 (C-NH), 111.8, 114.1, 118.6, 118.8, 121.4, 122.8, 123.7, 126.1, 126.4, 127.6, 128.6, 129.3, 130.1, 136.2, 136.7, 139.9, 151.9, 162.7, 172.1 (C=O) p.p.m. Anal. cal. for $C_{27}H_{24}N_4O_2$: C, 74.29; H, 5.54; N, 12.84; O, 7.33. Found: C, 74.30; H, 5.53; N, 12.82; O, 7.35.

N-(5-Methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)-4-(1H-indol-3-yl)butanamide ***3e***

Yield: 273 mg, 80%. Mol. Weight: 450.5. Mol. Formula: $C_{28}H_{26}N_4O_2$. MS (APCI(+)): 450 (M+1) m/z. IR (KBr-disc): 3235, 3046, 1656, 1635, 1590, 1544, 1494, 1432, 1276 and 699 cm^{-1}. ^1H NMR (DMSO-d_6) 300K: 1.92-1.98 (m, CH_3, CH_2 (overlapping), 2.35-2.40 (t, CH_2, J = 7.3, 7.3 Hz), 2.71-2.77 (t, CH_2, J = 7.4, 7.5 Hz), 6.93-6.99 (t, Ar-H, J = 6.9, 7.2 Hz), 7.02-7.10 (t, Ar-H, J = 6.9, 6.9 Hz), 7.13-7.16 (t, Ar-2H, J = 7.3, 7.1 Hz), 7.24-7.42 (m, Ar-11H), 7.51-7.54 (d, Ar-H, J = 7.7 Hz), 9.20 (s, NH), 10.75 (s, NH) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 12.5 (CH_3), 23.8, 24.8, 26.7 (CH_2), 109.4 (C-NH), 111.8, 114.6, 118.4, 118.8, 121.3, 122.8, 123.7, 126.1, 126.4, 127.7, 128.6, 129.5, 130.1, 136.2, 136.8, 139.8, 152.0, 162.8, 172.4 (C=O) p.p.m.

2.1.4. *General experimental for the formation of diphenylpyrazolinylureas* ***4a-4o***

4-Amino-5-methyl-1,2-diphenyl-1,2-dihydro-3*H*-pyrazol-3-one **3** (0.1 g, 3.8×10^{-4} mol) in dry acetonitrile (10-15 mL) was stirred at room temperature. The

appropriate substituted isocyanate (1.3 eq) in dry acetonitrile was added slowly over 5 min, allowed to stir at room temperature or heated to 60°C and was left overnight. The precipitate that formed was filtered, washed (twice) with cold acetonitril and dried, to give the corresponding urea as a pure product.

N-(5-Methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)N'-3-methoxy-phenylurea ***4b***

Yield: 36%. Mol. Weight: 414.6. Mol. Formula: $C_{24}H_{22}N_4O_3$. MS (APCI(+)): 415 (M+1), 266 (M+) m/z. IR (KBr-disc): 3207, 1708, 1646, 1619, 1594, 1540, 1488, 1453, 1282, 761 and 697 cm^{-1}. ^1H NMR (DMSO-d_6) 300K: 2.02 (s, C-CH_3), 3.72 (s, OCH_3), 6.50-6.55 (dd, Ar-H, J = 8.2 Hz), 6.88-6.92 (Ar-H, J = 8.1 Hz), 7.12-7.18 (m, Ar-3H), 7.26-7.44 (m, Ar-9H), 7.57 (s, NH), 8.88 (s, NH) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 12.6 (C-CH_3), 55.4 (OCH_3), 99.7 (C-CH_3), 104.2, 107.7, 109.7, 110.8, 123.6 (2×C), 125.6 (2×C), 126.2, 128.6, (2×C), 130.0 (2×C), 136.1, 139.2, 141.6, 143.0, 151.2, 153.8 (Ar-C), 160.2, 163.0 (C=O) p.p.m.

1-(4-Methoxy-phenyl)-3-(5-methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)-urea ***4c***

Yield: 75%, Melting Point: 244-246°C, Rf (ether) = 0.14, Mol. Weight: 414.6. Mol. Formula: $C_{24}H_{22}N_4O_3$ MS (APCI(+)): 266 (M+), 415 (M+1) m/z, IR (KBr-disc): 3300, 3261, 2929, 1708, 1642, 1618, 1552, 1512, 1420, 1250, 1207, 1018, 833, 763, 697 cm^{-1}, ^1H NMR (DMSO-d_6) 250 MHz: 8.70 (s, NH), 7.57 (s, NH), 7.24-7.49 (m, 11H, Ar-H), 7.11-7.20 (m, Ar-H), 6.79-6.90 (d, Ar-H), 3.72 (s, CH_3-O), 2.02 (s, CH_3-C) p.p.m. ^{13}C NMR (DMSO-d_6) 250 MHz: 162.5 (C-C=O), 150.6 (NH-C=O), 154.3 (ArC), 153.6 (2×ArC), 139.4, 135.6 (2×ArC), 132.9 (C-CH_3), 129.5 (2×ArC), 128.7, 128.6 (2×ArC), 128.1 (2×ArC), 125.8 (2×ArC), 123.1 (2×ArC), 119.8 (2×ArC), 113.9 (ArC), 109.5 (C-C=O), 55.1 (CH_3-O), 12.1 (CH_3-C) p.p.m.

N-(5-Methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)N'-3-methylphenylurea ***4e***

Yield: 91%. Mol. Weight: 398.5. Mol. Formula: $C_{24}H_{22}N_4O_2$. MS (APCI(+)): 399 (M+1), 266 (M+) m/z. IR (KBr-disc): 3322, 1698, 1644, 1625, 1538, 1490, 1285, 1211, 759 and 697 cm^{-1}. ^1H NMR (DMSO-d_6) 300K: 2.01 (s, CH_3), 2.25 (s, C-CH_3), 6.75-7.78 (d, Ar-H, J = 7.2 Hz), 7.10-7.44 (m, Ar-13H), 7.59 (s, NH), 8.80 (NH) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 12.7 (C-CH_3), 21.7 (CH_3), 109.9, 115.7, 119.1, 123.0, 123.7 (2×C), 126.4, 128.7, 129.1 (2×C), 130.1 (2×C), 136.1, 138.4, 139.9, 140.2, 142.9, 151.1 (Ar-C), 153.9, 163.0 (C=O) p.p.m.

1-(5-Methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)-p-tolyl-urea ***4f***

Yield: 73%, Melting Point: 250-252°C, Rf (ether) = 0.15, Mol. Weight: 414.6. Mol. Formula: $C_{24}H_{22}N_4O_2$ MS (APCI(+)): 266 (M+), 399 (M+1) m/z, IR (KBr-disc): 3299, 3058, 2924, 2857, 2358, 2334, 1711, 1648, 1624, 1601, 1540, 1506, 1416, 1292, 1202, 756, 693 cm^{-1}. ^1H NMR (DMSO-d_6) 250 MHz: 8.80 (s, NH), 7.57 (s, NH), 7.24-7.49 (m, 11H, Ar-H), 7.06-7.28 (m, Ar-H), 2.27 (s, Ar-CH_3), 2.02 (s, CH_3-C) p.p.m. ^{13}C NMR (DMSO-d_6) 250 MHz: 162.6 (C-C=O), 150.6 (NH-C=O), 153.5 (2×ArC), 139.4, 137.2, 135.5 (3×ArC), 130.5 (C-CH_3), 129.5 (2×ArC), 129.1 (2×ArC), 128.7 (ArC), 128.1 (2×ArC), 125.7 (2×ArC), 123.1 (2×ArC), 118.1 (2×ArC), 109.4 (C-C=O), 20.3 (Ar-CH_3), 12.1 (CH_3-C) p.p.m.

N-(2-Chlorophenyl)-N'-(5-methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)urea ***4g***

Yield: 73%. Mol. Weight: 418.9. Mol. Formula: $C_{23}H_{19}N_4O_2$. MS (APCI(+)): 418, 420 (M+1), 266 (M+) m/z. IR (KBr-disc): 3293, 3212, 1710, 1621, 1590, 1530, 1488, 1422, 1291, 1191, 761 and 680 cm^{-1}. ^1H NMR (DMSO-d_6) 300K: 2.01 (s, CH_3), 6.97-7.03 (tt, Ar-H, J = 6.8 Hz), 7.11-7.18 (tt, Ar-H, J = 6.9, 6.8 Hz), 7.22-7.44 (m, Ar-12H), 7.89 (s, NH), 9.09 (s, NH) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 12.6 (CH_3), 109.5, 117.4, 118.3, 122.2, 123.7 (2×C), 126.2 (2×C), 128.7, 129.3 (2×C), 130.8 (2×C), 130.9, 133.7, 139.8, 141.5, 141.9, 151.4 (Ar-C), 153.8, 162.9 (C=O) p.p.m.

N-(4-Bromophenyl)-N'-(5-methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)urea ***4i***

Yield: 92%. Mol. Weight: 463.3. Mol. Formula: $C_{23}H_{19}BrN_4O_2$. MS (APCI(+)): 464, 466 (M+1), 266 (M+) m/z. IR (KBr-disc): 3285, 3062, 1704, 1644, 1490, 1534, 1486, 1288, 1209, 757 and 705 cm^{-1}. ^1H NMR (DMSO-d_6) 300K: 2.01 (s, CH_3), 6.50-6.52 (d, Ar-H, J = 6.9 Hz), 7.01-7.17 (m, Ar-2H), 7.29-7.43 (m, Ar-11H), 7.65 (s, NH), 9.02 (s, NH) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 12.6 (CH_3), 109.6, 113.9, 116.3, 120.7, 123.7 (2×C), 125.9 (2×C), 126.2, 128.7 (2×C), 129.3 (2×C), 130.5, 131.9, 132.0 (2×C), 136.1 (2×C), 139.8, 153.8 (Ar-C), 157.8, 162.9 (C=O) p.p.m.

N-(5-Methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)N'-2-nitrophenylurea ***4j***

Yield: 65%. Mol. Weight: 430.4. Mol. Formula: $C_{23}H_{20}N_5O_4$. MS (APCI(+)): 431 (M+1), 266 (M+) m/z. IR (KBr-disc): 3318, 3181, 3010, 1712, 1658, 1635, 1588, 1502, 1432, 1344, 1272, 1201, 759 and 688 cm^{-1}. ^1H NMR (DMSO-d_6) 300K: 2.02 (s, CH_3), 7.12-7.44 (m, Ar-11H), 7.64-7.71 (t, Ar-H, J = 7.3, 7.4 Hz), 8.06-8.10 (d, Ar-H, J = 8.4 Hz), 8.28-8.32 (d, Ar-H, J = 8.5 Hz),

8.90 (s, NH), 9.71 (s, NH) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 12.4 (CH_3), 122.7, 123.9, 124.0, 125.9, 126.3 (2×C), 126.6, 127.3 (2×C), 128.8, 128.9, 129.3 (2×C), 130.1 (2×C), 133.2, 135.6, 136.0, 138.0, 139.6 (Ar-C), 153.5, 162.8 (C=O) p.p.m.

N-(5-Methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)-N'-phenylurea ***4l***

Yield: 91%. Mol. Weight: 384.4. Mol. Formula: $C_{23}H_{20}N_4O_4$. MS (APCI(+)): 385 (M+1), 266 (M+) m/z. IR (KBr-disc): 3420, 3297, 3072, 3065, 1706, 1640, 1544, 1492, 1448, 1297, 1202, 755 and 697 cm^{-1}. ^{1}H NMR (DMSO-d_6) 300K: 2.02 (s, CH_3), 6.91-6.97 (tt, Ar-H, J = 7.3 Hz), 7.11-7.17 (tt, Ar-H, J = 7.0, 7.1 Hz), 7.22-7.45 (m, Ar-13H), 7.60 (s, NH), 8.87 (s, NH) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 12.6 (CH_3), 109.8 (C-CH_3), 118.5 (2×C), 122.2, 122.4 (2×C), 123.7 (2×C), 125.9 (2×C), 126.2, 128.6 129.1 (2×C), 129.8 (2×C), 136.1, 139.9, 140.3, 151.1 (Ar-C), 153.8, 163.0 (C=O) p.p.m.

N-(5-Methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1H-pyrazol-4-yl)-N'-cyclohexylurea ***4n***

Yield: 86%. Mol. Weight: 390.5. Mol. Formula: $C_{23}H_{23}N_4O_2$. MS (APCI(+)): 391 (M+1), 266 (M+) m/z. IR (KBr-disc): 3359, 3299, 2929, 2849, 1636,1694, 1596, 1538, 1488, 1276, 1228, 763 cm^{-1}. ^{1}H NMR (DMSO-d_6) 300K: 1.10-1.88 (m, -CH, -CH_2-, 11H), 1.95 (s, CH_3), 6.27-6.30 (d, Ar-H, J = 7.9 Hz), 7.12-7.16 (tt, Ar-H, J = 6.8, 6.9 Hz), 7.24-7.42 (m, Ar-8H), 7.63 (s, NH), 8.86 (NH) p.p.m. ^{13}C NMR (DMSO-d_6) 300K: 12.9 (CH_3), 24.9 (-CH_2-×2), 25.8 (-CH_2-), 33.5 (-CH_2-×2), 48.5 (-CH-NH), 99.7 (C-CH_3), 110.0 (C-N), 123.5 (2×C), 126.1 (2×C), 128.5, 129.2 (2×C), 130.0 (2×C), 136.1, 140.3, 150.2 (Ar-C), 155.6, 163.1 (C=O) p.p.m.

2.2. *Pharmacology*

2.2.1. *Cholecystokinin binding assay, [^{125}I]CCK-8 receptor binding assay*

CCKA and CCKB receptor binding assays were performed, by using guinea pig cerebral cortex (CCKB) or rat pancreas (CCKA). Male guinea pig brain tissues were prepared according to the modified method described by Saita *et al.* (*21*). Pancreatic membranes were prepared as described by Charpentier *et al.* (*22*).

Tissues were homogenized in ice cold sucrose (0.32 M, 25 mL) for 15 strokes at 500 rpm and centrifuged at 13,000 rpm for 10 min. The supernatant was re-centrifuged at 13,000 rpm for 20 min. The resulting pellet was re-dispersed to the required volume of buffer at 500 rpm and stored in aliquots at 70°C.

Binding was achieved using radioligand ^{125}I-Bolton-Hunter labeled CCK, NEN at 25 pM. The samples were incubated with membranes (0.1 mg/mL) in 20 mM Hepes, 1 mM EGTA, 5 mM $MgCl_2$, 150 mM NaCl, at pH 6.5 for 2 h at RT and then centrifuged at 11,000 rpm for 5 min. The membrane pellets were washed twice with water and the bound radioactivity was measured in a Packard Cobra Auto-gamma counter (B5005). All binding assays were carried out with L-363, 260 as control.

2.2.2. *Animal studies*

Experiments were conducted in male standard IRC mice obtained from the animal house, Faculty of Medicine, Khon Kaen University. Each experimental group consisted of 6 animals and the treatment procedures were approved by the ethical committee, Faculty of Medicine, Khon Kaen University (BEA030699).

Mice were intraperitoneal injected with either test compound dissolved in 5% DMSO at the volume not more than 0.2 mL/animal. At 30 min after treatment, animals were tested as described in the following sections.

Anxiolytic activity tests, nociception tests and antidepression tests were performed as described in DDT 2007 (*19*).

2.2.3. *Anxiolytic activity tests*

The light/dark box: Mice were placed in the light part of the light/dark box. The box was a Plexiglass cage, 25×50×20 cm, having one-third as a dark and two-third as a light compartment. A 40-W light bulb was used and positioned 10 cm above the center of the light component. The animals could walk freely between dark and light parts through the opening. The time animals spent in light part during the 5 min interval was recorded. The mouse was considered to be in the light part when its 4 legs were in the light part.

The elevated plus-maze: The wooden elevated plus-maze consisted of two open arms (30×10 cm) without any walls, two enclosed arms of the same size with 5-cm high side walls and end wall, and the central arena (10 ×10 cm) interconnecting all the arms. The maze was elevated approximately 30 cm height from the floor. At the beginning of the experiment the mouse was placed in the central arena facing one of the enclosed arms. During a 5 min interval, the time animals spent in the open arms of plus-maze was recorded. The mouse was considered to be in the open part when it had clearly crossed the line between the central arena and the open arm with its 4 legs.

2.2.4. *Nociception tests*

The tail immersion test: The thermal response latency was measured by the tail immersion test. The animals were placed into individual restraining cages leaving

the tail hanging freely. The tail was immersed into water preset at 50°C. The response time, at which the animal reacted by withdrawing its tail from water, was recorded and the cut-off time was 10 sec in order to avoid damaging the animal's tissue.

The thermal response latency was measured by the tail immersion test. The base line withdrawal thresholds (BT) were recorded prior to the first injection. Test thresholds (TT) were measured 60 min after the second injection. The cut off time was set to 45 sec. This was to avoid any tissue damage to the paw during the course of analgesia testing. The test thresholds were expressed as a percentage of Maximal Possible Effect (% MPE) using the equation:

$$\% \text{ MPE} = \{(\text{TT-BT}) / (45\text{-BT})\} \times 100$$

The hot plate test: Mice were placed on a hot plate that was thermostatically maintained at 50°C. A Plexiglass box was used to confine the animal to the hot plate. The reaction time of each animal (either paw licking or jumping) was considered a pain response. The latency to reaction was recorded. For prevention of heat injury, the cut-off time of the test was 30 sec.

2.2.5. *Antidepression tests*

The tail suspension test: Mice were hung by their tail on the tail hanger using sticky tape for tail fixation, at approximately 1 cm from the end. The hanger was fixed in the black plastic box (20×20×45 cm) with the opening at the top front. The distance between the hangers to the floor was approximately 40 cm. The mouse was suspended in the air by its tail and the immobile time was recorded during the period of 5 min. The duration of immobility was defined as the absence of all movement except for those required for respiration.

The forced swim test: The forced swim test was carried out in a glass cylinder (20 cm diameter, 30 cm height) filled with water to the height of 20 cm. The water temperature was approximately 25-28°C. Mice were gently placed into the water and the immobility time was recorded by an observer during the period of 5 min. Immobility was defined as absence of all movement and remained floating passively in the water with its head just above the water surface.

2.2.6. *Motor activity tests*

The rota-rod test: Mouse was placed on the rotating drum with the acceleration speed (Acceler. Rota-rod, Jones & Roberts, for mice 7650, Ugo Basile, Italy). The time animal spent on the rod is recorded.

The wire mesh grasping test: The mouse was placed on a wire mesh (20×30 cm). After a few seconds, the mesh was turned 180° and the time the animal hold on the mesh was recorded.

2.2.7. *Statistical methods*

The data were expressed as mean ± SD and one-way analysis of variance (ANOVA) and supplementary Tukey test for pairwise comparison were tested to determine for any significant difference at $p < 0.05$.

3. Results and Discussion

3.1. *Synthesis*

The diphenyl-pyrazolone template **1** was synthesised (*23*) in a condensation reaction by direct heating of diphenyl-hydrazine and ethylacetoacetate (*24*). Nitrosation and subsequent reduction served the building block for the preparation of amides and ureas.

Nitrosation of **1** with sodium nitrite furnished a nitroso-intermediate, which was reduced *in situ* with a solution of $SnCl_2$ in hydrochloric acid to give amine **2**. The nitroso-intermediate was found unstable, but may be isolated as green crystals.

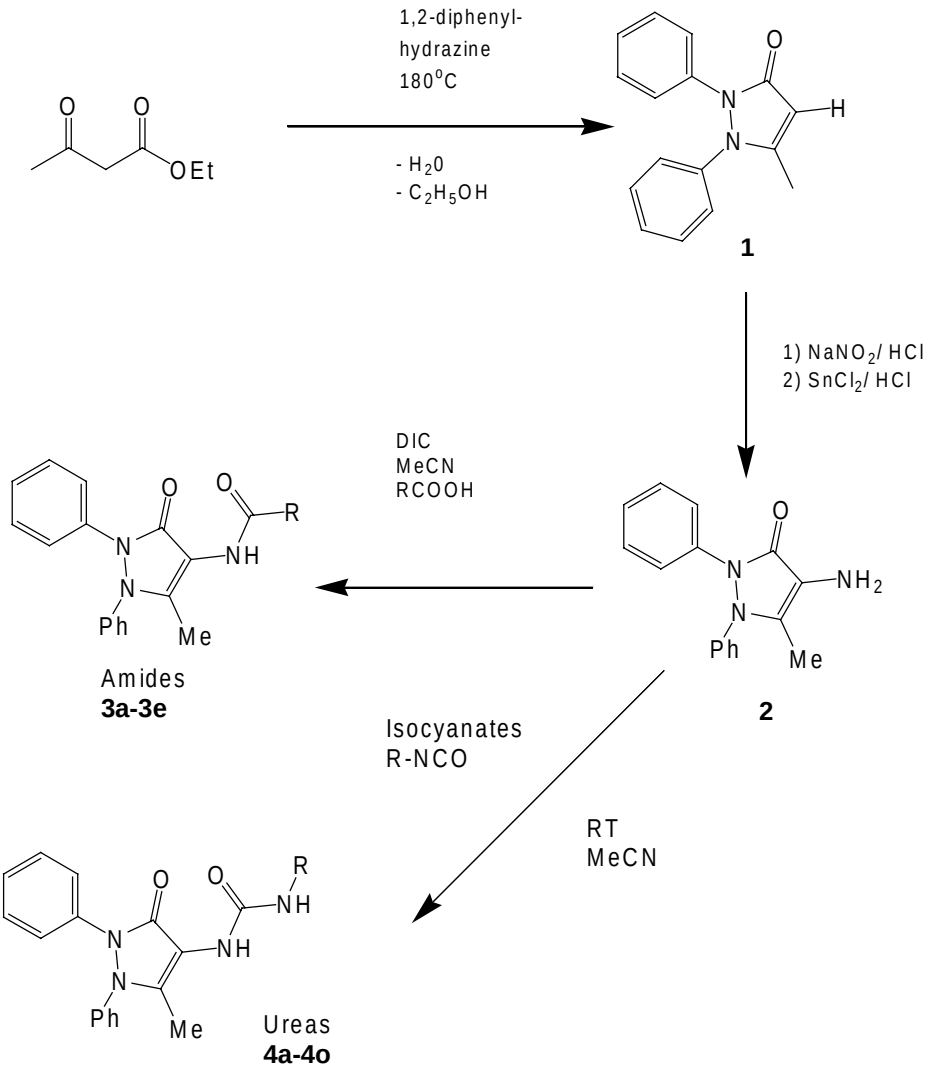
The nucleophilic amino group of the pyrazoline **2** was reacted with the DIC activated series of indole carboxylic acids giving the amido-pyrazolines **3a**-**3e**. For the preparation of diphenyl ureas **4a**-**4e** the amine **2** was reacted with the appropriate isocyanate to give the desired compounds as white solids in high yields (Scheme 1).

No purification by column chromatography was required for this chemical approach and selected spectroscopic data of diphenyl pyrazolyl indole carboxamides and ureas were reported in the experimental section. Other known intermediates and targets were reported by Farghaly (*25*).

3.2. *SAR studies / receptor binding affinity*

The diphenyl pyrazoline derivative of 2-indole carboxylic acid **3a**, in which the 1,4-benzodiazepine moiety of the known cholecystokinin ligands, was replaced by a pyrazoline template, showed a binding activity with an IC_{50} of 20 nM for the CCK_1 receptor, but a very poor solubility in water and organic solvents ($CHCl_3$, DMSO, MeCN) it could therefore, not fully characterised nor be tested *in vivo*.

Analogue **3b** of the diphenyl template, derived from 3-indolylcarboxylic acid, occurred a better solubility than **3a**, but showed a low binding affinity. A series of homologues containing a C1 (**3c**) and a C2 spacer unit (**3d**) displayed a CCK_1 selective binding affinity of 20 and 25 nM, respectively. The introduction of a C3 unit resulted in a loss of binding affinity for the derivative **3e**.



Scheme 1. Synthesis of pyrazolone based cholecystokinin-antagonists.

In a SAR optimisation of the ureido-diphenylpyrazoline template, it was found that *m*-substituted phenylureas generally displayed the highest binding affinity. The *o*-methoxyphenyl isocyanate gave poor yields for the **4a**, and was also biologically inactive. Only the *o*-derivative containing a nitro group was formed in good yield.

Compound **4e**, a meta-toluidine urea displayed an IC_{50} of 25 nM for the CCK_2 and 20 nM towards the CCK_1 receptor subtype. Approximately the same binding profile was determined for the *m*-methoxyphenylurea **4b**, which was selected for further evaluation. However, ureas containing ortho and para toluidine substituents showed generally no binding activity. Compound **4h**, containing a para-chlorine group, showed no activity, in line with the *p*-methyl and *p*-methoxy ureas **4c** and **4f**.

The cyclohexyl urea **5n** exhibited a modest binding affinity of ~1 μM. The unsubstituted phenyl urea **4l** and the aromatic naphthylurea (**4m**) analogues were found of only micromolar binding affinity (Table 1).

The first step of the evaluation was the determination of the receptor binding affinity for the CCK_1 and CCK_2 subtype, followed by an *in vivo* evaluation (*26*).

The highest MPE (maximum possible effect) value for antinociception, in conjunction with morphine, was obtained for the CCK_1 selective amide **3d** named **MPP** and it was tested further *in vivo*.

In the ureido series phenylurea **4b** and **4e**, ligands of the same binding profile, displayed the same high MPE. The mixed CCK antagonist, urea **4b**, was selected for further evaluation, due to the highest mixed binding affinity, the best clog p value and the highest chemical yield. In further investigations **4b** was named **MPM** (MPE data not shown, Figure 2).

3.3. *Antidepressant-like effects of amide **MPP** and urea **MPM***

Antidepressant drugs have the effect of reducing the duration of immobility in the despair swim test (immobility time test) (*27*). Desipramine, a tricyclic antidepressant served as positive control, which was found less potent, but has shown a similar magnitude of

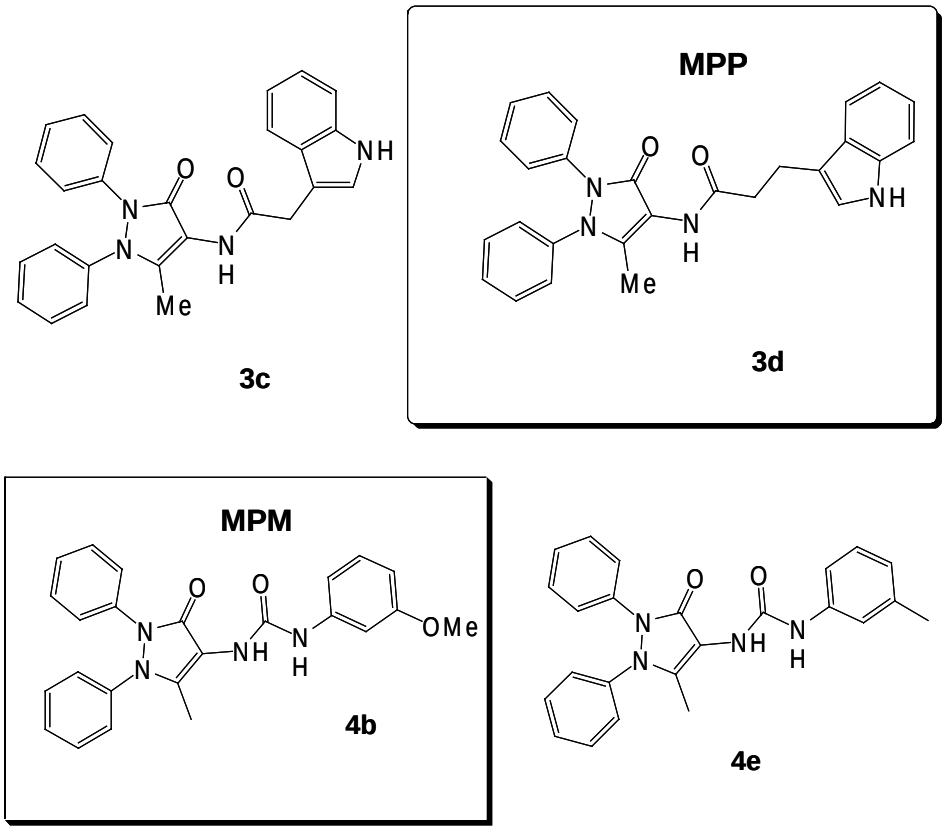


Figure 2. Selected structures of *N*-(3-oxo-2,3-dihydro-1*H*-pyrazol-4-yl)-1*H*-indole-carboxamides and ureido-pyrazolines.

the antidepressant effect.

Mice were intraperitoneally injected with 5% DMSO or antagonists **MPM** or **MPP** at various doses either 0.005, 0.01, 0.05 or 0.5 mg/kg BW or desipramine (10 mg/kg). The immobility times of the animals in each treatment group were shown in Figures 3 and 4.

MPM showed a significant antidepressant-like effect from 0.01 mg/kg BW and at higher doses (Figure 3). No dose-dependent effect of the CCK antagonists were seen and the effects of **MPM**, at all doses (except 0.005 mg/kg BW of **MPM**), were comparable to 10 mg/kg BW of desipramine.

MPP showed a significant antidepressant-like effect at all doses tested (0.005, 0.01, 0.05 and 0.5 mg/kg BW) and the antidepressant-like effect of desipramine was clearly observed (Figure 4).

In this study, the CCK_1 selective and the mixed CCK antagonist showed antidepressant-like effects supporting the roles of a CCK system in depression. The pharmacological effects seemed to be all-or-none without a dose-dependent property (*28*).

3.4. *Anxiolytic-like effects*

Animals have been evaluated in the black and white test (*29*) and the elevated plus maze test (x-maze)

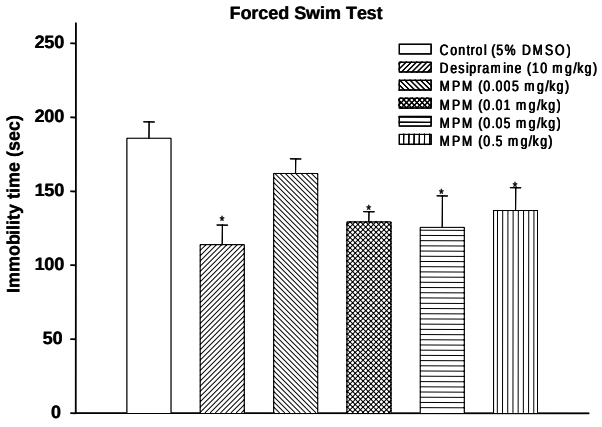


Figure 3. Dose-effect relationship of **MPM** on the immobility time of mice in the forced swim test.

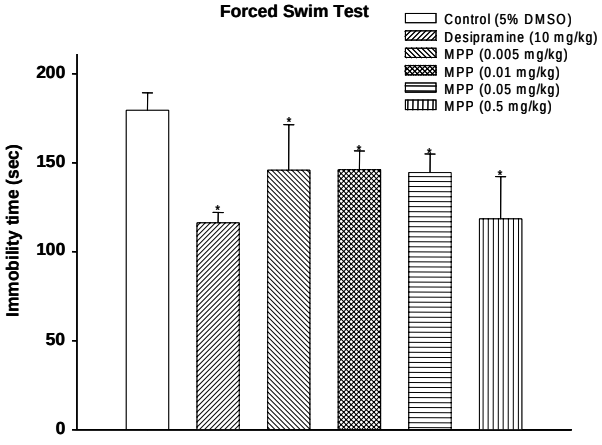
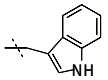
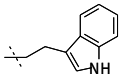
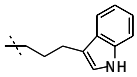


Figure 4. Dose-effect relationship of **MPP** on the immobility time of mice in the forced swim test.

Table 1. Structure and binding affinity of *N*-(5-methyl-3-oxo-1,2-diphenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N*'-phenylureas pyrazol-4-yl amides

Entry	Group R	Yield [%]	IC_{50} CCK_B [μM]	IC_{50} CCK_A [μM]
3a		65	2.1 ± 0.1	0.020 ± 0.001
3b		78	3.5 ± 0.4	2.2 ± 0.2
3c		66	2.5 ± 0.2	0.020 ± 0.002
3d		79	2.4 ± 0.2	0.025 ± 0.002
3e		80	20 ± 1	20 ± 1
4b	*m*-MeOPh	71	0.025 ± 0.002	0.010 ± 0.002
4c	*p*-MeOPh	76	> 20	9
4e	*m*-MePh	91	0.025 ± 0.002	0.020 ± 0.002
4f	*p*-MePh	89	> 20	13
4g	*m*-ClPh	73	> 20	7
4h	*p*-ClPh	81	> 20	> 20
4i	*p*-BrPh	92	> 20	> 20
4j	*o*-NO_2Ph	65	7.5	> 20
4k	*p*-NO_2Ph	77	> 20	> 20
4l	Ph	91	3	3
4m	Naphtyl	75	> 20	8
4n	Cyclohexyl	86	0.85 ± 0.2	12
4o	*t*-Bu	60	11	> 20

(*30*). In the elevated plus maze test a greatly enhanced exploration of the open arms with an increased number of total crossings was observed for the mixed antagonist **MPM**.

Mice were intraperitoneally injected with 5% DMSO or **MPM** or **MPP** at various doses either 0.005, 0.01, 0.05 or 0.5 mg/kg BW or diazepam (1 mg/kg) as positive control.

The anxiolytic-like effect of **MPM** (Figure 5) was observed at 0.05 and 0.5 mg/kg BW and no difference among the **MPM** (0.05 and 0.5 mg/kg BW) and diazepam-treated group could be seen. The 2 lower doses were not significantly different from the control. Thus, 50 μg/kg of the CCK antagonist **MPM** displayed the same anxiolytic effect as 1 mg/kg of the benzodiazepine ligand diazepam in mice. At a dose of 10 mg/kg **MPM** no impairment of coordination was found, which is associated with higher doses of the benzodiazepine anxiolytics.

MPP-treated groups were inactive in the x-maze test and the light and dark box test. No anxiolytic-like effect of **MPP**, the CCK_1 selective antagonist was obtained. Evidence indicated that CCK_1 receptors were involved in the mediation of anxiolytic-like effects in the elevated x-maze (*31*). In the same model CCK_2 antagonists also showed anxiolytic-like effect (*32*).

Thus, both CCK_1 and CCK_2 receptors antagonists

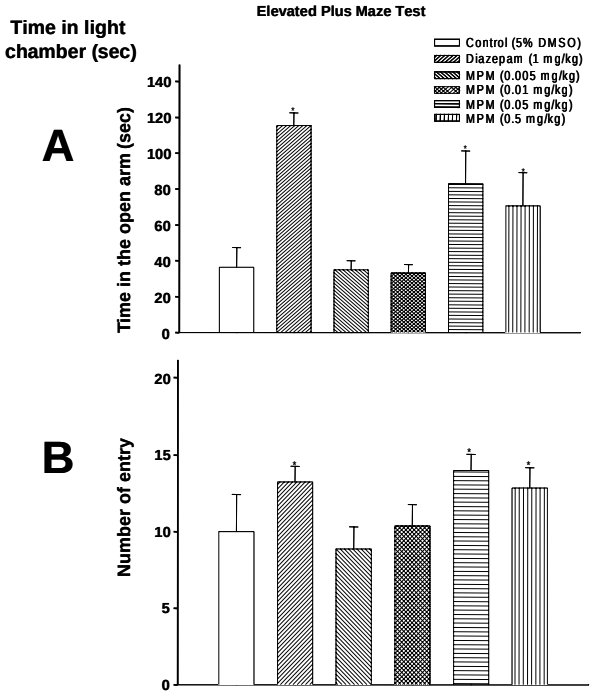


Figure 5. Dose-effect relationship of **MPM** on time in the open arms (A) and number of entry (B) in the elevated plus maze test.

in conjunction seem to have roles in the modulation of anxiety-related behaviour in animal models.

3.5. *Potentiation effect of **MPM** and **MPP** on morphine-induced analgesia in mice*

Nociception and motor activity tests: In all treated groups, no effect on nociception (*33*) was observed in the tail immersion test (*34*) and the hot plate method (*35*) up to 10 mg/kg for the test compounds as single agents. An impairment of motor activity could not be observed in all tested models up to a dose of 10 mg/kg in the wire mash grasping and the rota rod test.

MPM and **MPP** were selected to study the potentiation of morphine antinociception at a dose of 0.5 mg/kg body weight. From previous experiments fully developed biological effects were observed at this relatively high dose.

DMSO (5%), **MPM** or **MPP** (in 5% DMSO) was intraperitoneally injected as the first injection. Twenty min after the first, the second injection was subcutaneously injected with morphine at either 2, 4, 8 or 16 mg/kg BW. The thermal response latency of the animals was determined by the tail immersion test and the results were expressed as % MPE. Although no intrinsic analgesic effect of **MPM** and **MPP** could be observed, both CCK antagonists increased % MPE in response to morphine, at all tested morphine doses.

The morphine potentiation of **MPP** (0.5 mg/kg) and **MPM** (0.5 mg/kg) was determined at 60 and 90 min for a dose range of morphine and the results were outline in Figures 6A and 6B.

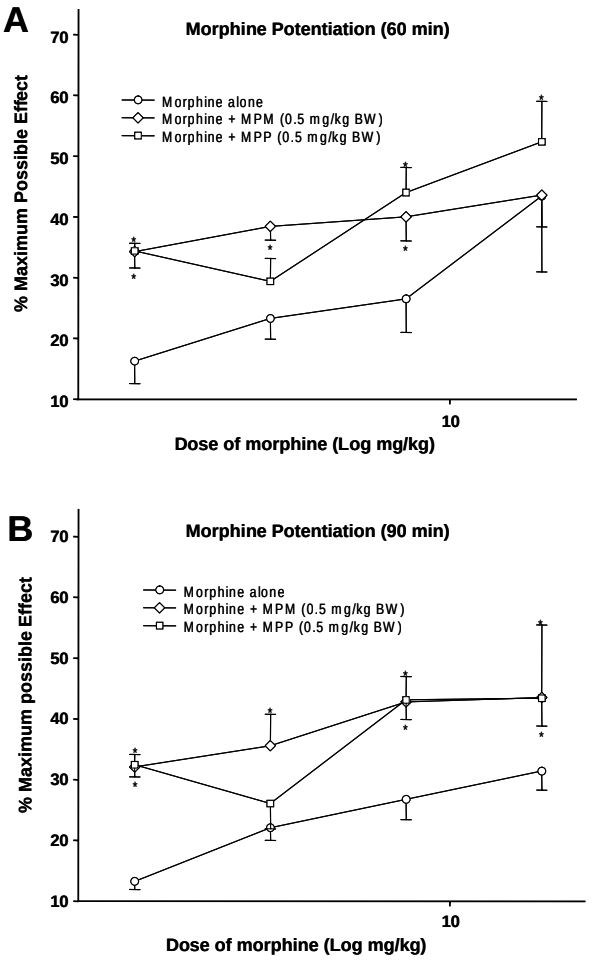


Figure 6. (A), Potentiation effect of **MPM** or **MPP** on morphine-induced analgesia at 60 min after morphine injection in mice; (B), Potentiation effect of **MPM** or **MPP** on morphine-induced analgesia at 90 min after morphine injection in mice.

In general, at a certain dose of morphine, there was no difference of MPE between the **MPM** and **MPP** groups. The potentiation was higher for low doses, such as 2 mg/kg of morphine and less for high doses, such as 16 mg/kg of morphine. In the middle range (4 mg/kg of morphine) the mixed antagonist **MPM** at a fixed dose of 0.5 mg/kg seemed to be superior to the CCK_1 selective **MPP**, but for final conclusions more data are required.

The effects of cholecystokinin on the modulation of pain transmission and the anti-opioid effects of cholecystokinin were well documented (*36*). CCK antagonists potentiated the antinociceptive effects of morphine and could also block the development of morphine tolerance (*37*).

4. Conclusions

The pyrrazol lead structure had been previously discovered in a chemically diverse library and subsequent SAR optimization was completed in the classical manner for a series of amides and phenylureas. The 2-phenyl pyrrazolone ring system and the meta-substituents on the *N*-phenylurea moiety were found essential for a potent cholecystokinin antagonism.

Our diphenyl-pyrrazoline template contained no chiral centre in the molecule. Merck's ureas had to be separated into enantiomeres, as the CCK_1/CCK_2 selectivity was dependent on the stereochemistry of the C3 center.

The importance of the removal of the stereo center was even greater, when it was found (*38*) that the one isomer of a 1,5-benzodiazepine urea acted as agonist the other as antagonist.

Lilly's diphenylpyrazolidinones had 2 chiral centres and after undergoing clinical trials, compound LY288513 was discontinued due to major adverse effects (*39*). The pyrrazoline template used here, displayed anti-inflammatory properties. From current animal experiments, there were no signs of acute toxicity and no long term toxicity (part 2).

These novel phenyl ureas represent CCK antagonists with a mixed binding profile and a potency in the nanomolar range for both receptor subtypes. Merck and other major pharmaceutical companies developed selective, mainly CCK_2 selective antagonists. The results of our animal experiments do not justify this approach, as a better pharmacological profile of our mixed antagonists was obtained.

The anxiolytic properties of the mixed antagonists were not associated with the side effects of benzodiazepines, acting on the $GABA_A$ receptor (*40*). Antidepressant effects were observed for both, the amido- and phenyl urea-pyrrazolines.

In terms of analgesia, a tenth of the morphine dose was required for the same antinociceptive effect in conjunction with a small dose of the CCK antagonist.

Acknowledgement

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Original Article

Injection of cell-wall skeleton of *Mycobacterium bovis* BCG draining to a sentinel lymph node eliminates both lymph node metastases and the primary transplanted tumor

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ABSTRACT: Based on recent developments in innate immunity, we focused on a microbial immunostimulator for cancer immunotherapy. If innate immunity is properly activated, tumor antigens distributed endogenously in cancer patients will be exploited to activate tumor immunity. We chose the cell-wall skeleton of *M. bovis* BCG (BCG-CWS) and investigated the potential of mono-therapy without exogenous tumor antigens. We used strain 2 guinea pigs bearing syngenic line 10 hepatoma, which is an excellent disease model of spontaneous lymph node metastasis, and examined the tumor-eradicating activity of highly purified BCG-CWS (SMP-105), excluding the effect of local inflammation on tumor growth. SMP-105 eliminated both established metastases and the implanted tumor, when injected into different but not distant sites from the tumor, whereas, when injected into the opposite side, neither metastases nor the primary tumor was eradicated. SMP-105 was observed in the draining lymph node engulfed by phagocytes, presumably macrophages or dendritic cells, but was not detected in distant lymph nodes or the spleen. It took about 2 weeks until the tumor-eliminating effect was observed. Taken together it is considered that macrophages or dendritic cells were activated by SMP-105 and encountered tumor cells in the sentinel lymph node to generate tumor immunity during the lag time. In conclusion, we suggested the potential of mono-therapy with a strong immunostimulator and that SMP-105 is a most promising agent for cancer immunotherapy. Separate injection from tumor draining to a sentinel lymph node using classical guinea pig models will be a useful method for investigating immunostimulators.

Keywords: Adjuvant, Cell-wall skeleton of BCG, Immunotherapy, Lymph node metastasis

1. Introduction

About 30 years ago, bacteria and polysaccharides were intensively studied as biological response modifiers (BRMs) for cancer therapy (*1-6*), but limited effects were observed in patients. In those days, little was known about the mechanisms leading to tumor suppression or eradication, and regimens and target diseases were therefore not optimized. Recently, with the development of immunology, innate immunity in particular, the activation of macrophages and dendritic cells (DCs) has been successfully bridged to elicit acquired immunity (*7,8*). We now know that a draining lymph node plays a central role in the generation of immunity against invading organisms. Furthermore, the molecular aspects of macrophage and DC activation by microbes are becoming clear (*9,10*) and agonists of toll-like receptors (TLRs), such as imiquimod and CpG oligodeoxynucleotide, have been highlighted (*11-13*). BCG-CWS, known to be one of the strongest adjuvants (*14,15*), also stimulates TLRs (*16,17*) and gene-induction profiles have been demonstrated (*18*). This prompted us to reevaluate BCG-CWS based on the recent evidence of cancer immunology. We prepared CWS from *M. bovis* BCG Tokyo 172 strain with purity of more than 97% (SMP-105) and investigated the effect on macrophages to identify TLR2/MyD88-dependent activation (*19,20*). Partial structures were chemically synthesized and the macrophage-activating activities were investigated (*21*).

Most studies of cancer immunotherapy involve exogenous tumor antigens (*22-25*), but cancer

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patients have an enormous number of tumor antigens endogenously. We think that when endogenous tumor antigens are properly exploited and presented to T cells by activating innate immunity, tumor immunity will be generated and tumor cells will be eradicated. The potential of mono-therapy with BCG-CWS was therefore studied.

To select a disease model, we focused our attention on spontaneous lymph node metastases, because we expected that tumor cells would be easily exploited as antigens for activating T cells by targeting the lymph nodes; furthermore, this is an important disease model as the prognosis of cancer treatment is almost always determined by the control of lymph node metastases. A transplantable tumor cell line, line 10 hepatocarcinoma, derived from a diethyl-nitrosamine-induced hepatoma in an inbred strain 2 guinea pig (*26*), establishes metastases in a sentinel lymph node at high frequency (*27*,*28*) and, for more than 100 years, guinea pigs have been used as important experimental animals for the study of infectious microbes. We therefore employed strain 2 guinea pigs inoculated with syngeneic line 10 hepatoma.

Many reports studied the antitumor effects of BCG or the cell wall of BCG using line 10 hepatoma from the 1970s to 1980s (*29*-*36*), but in those papers, the microbe or microbial fraction was injected into the tumor, or admixed tumor cells were inoculated. By these methods, however, antitumor activities are not properly evaluated because tumor cells may be damaged by local inflammation induced by stimulation with the microbe or the microbial fraction (*37*,*38*).

In this study, the injection route was improved to exclude the effect of local inflammation on tumor growth. SMP-105 revealed a strong tumor-eliminating effect both on primary tumor and lymph node metastases. Separate injection from tumor draining to a sentinel lymph node is an improved method in classical guinea pig models for investigating immunostimulators.

2. Materials and Methods

2.1. *Animals*

Male strain 2 guinea pigs, five weeks of age, were obtained from Japan SLC Inc., Shizuoka and used when they were six weeks old. Animals were maintained under specific pathogen-free conditions. Their maintenance and all experiments were conducted with the approval of the DSP Animal Care and Use Committee.

2.2. *Cell line*

Line 10 hepatocellular carcinoma cells were implanted intraperitoneally and a range of cell stock was prepared in liquid nitrogen. In each experiment, cells were freshly thawed before intradermal inoculation.

2.3. *Preparation of SMP-105*

SMP-105 is a product of Dainippon Sumitomo Pharma Co., Ltd. and chemical analysis data have been reported by Uenishi *et al*. (*39*). Briefly, SMP-105 contains less than 3% (w/w) of sugars and amino acids assumed not to constitute CWS. Both DNA and trehalose dimycolate are removed to less than 0.05% (w/w) and lipopolysaccharide is about 0.0015 EU/mg by gel-clot technique. An oil-in-water emulsion of SMP-105 was prepared and lyophilized on the thousand-vial scale. Each vial contained 1.2 mg of SMP-105, 32 mg of squalane, 20 mg of polysorbate 80 and 100 mg of mannitol. Vehicle preparation used the same formulation except for SMP-105. SMP-105 in emulsified form was used for inoculation, and the suspended form in saline was prepared for *in vitro* use.

2.4. *Direct cytotoxic effect on line 10 hepatoma*

Line 10 hepatoma cells were incubated with SMP-105 or mitomycin C for 48 h and viability was assayed using WST-8 (DOJINDO Laboratories, Kumamoto, Japan).

2.5. *Antitumor effects*

Line 10 hepatoma cells stored in liquid nitrogen were rapidly thawed and washed three times with Hanks' balanced salt solution (HBSS). 4×10^7 cells were injected into strain 2 guinea pigs intraperitoneally and ascites were collected from failing animals after about 10 days. Line 10 hepatoma cells were prepared by washing the ascites cells three times with HBSS and inoculated intradermally at 1×10^6 cells in 0.1 mL into the right thoracic flank region. SMP-105 or vehicle was injected intradermally into sites distal to the site of tumor inoculation on days 0, 7 and 14. In the postoperative model, the primary dermal tumor nodule was excised on day 7 under ketamine and xylasine anesthesia, and SMP-105 at a dose of 60 μg or vehicle was injected into sites dorsal and ventral to the site of tumor excision on days 7 and 14, respectively. In the no-operation group, SMP-105 was injected on days 0, 7 and 14.

The size of the primary skin tumor nodule was calculated as the squared average of the long and short diameter perpendicular to each other.

Animals were sacrificed by anesthesia with a high concentration of carbon dioxide and an axillary lymph node was collected and weighed. For pathological study, the lymph node was fixed with 10% of formaldehyde solution. It was then cut into two equal pieces in the apsis direction and a slice of the section was stained with hematoxylin and eosin. Metastasis

was scored from 0 to 4 based on the area occupied by tumor cells.

2.6. *Challenge of live tumor cells*

Line 10 hepatoma cells collected from ascites were treated with mitomycin C (MMC) (Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan) at 100 μg/mL for 20 min at 36°C in a water bath, washed three times with HBSS and stored in liquid nitrogen. 1×10^6 inactivated cells were inoculated into the right thoracic flank region, and SMP-105 or vehicle was injected into the same side distal to the site of tumor inoculation. Fourteen days after treatment, live line 10 hepatoma cells were injected into the opposite side. Animals were observed for tumor growth.

2.7. *Distribution of SMP-105 in lymph nodes and spleen*

SMP-105 was injected intradermally into the thoracic flank region of strain 2 guinea pigs at 60 μg, and the spleen and bilateral axillary lymph nodes were sampled at various time points over 7 days. Lymph nodes and spleen were fixed with 10% of formaldehyde solution, and SMP-105 was analyzed immunohistochemically using rabbit anti-*M. bovis* BCG antibody (DAKO Japan Co. Ltd., Kyoto, Japan).

2.8. *Statistical analysis*

Wet weights of lymph nodes and primary tumor sizes of each observation day were compared with the vehicle group using Steel's test, or Wilcoxon's test when comparing two groups. Statistical analysis was performed using the SAS system for Windows (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. *Antitumor effect of SMP-105 on lymph node metastasis and primary tumor*

In order to use SMP-105 as an immunostimulator, the direct cytotoxic effect was determined *in vitro*. SMP-105 showed no cytotoxic activity in line 10 hepatoma cells (Figure 1).

Line 10 hepatoma cells were inoculated and an oil-in-water emulsion of SMP-105 or vehicle was injected into sites different from the tumor inoculation site in order to avoid damage to tumor cells by local inflammation.

SMP-105 demonstrated prominent antitumor activity at very low doses such as 3.75 μg for both the primary tumor (Figure 2A) and lymph node metastases (Figure 2B), from which the effect of local inflammation on tumor cells was excluded. Growth of the primary implanted tumor began to decrease in some animals about 2 weeks after the first dose of SMP-105.

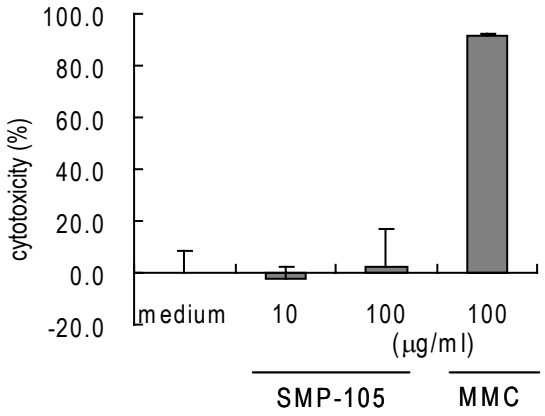

Figure 1. Cytotoxic effect of SMP-105 on line 10 hepatoma. Cell viability was assayed using WST-8, and cytotoxicity was calculated as (1-T/C) × 100 (%). Average and SD are indicated (n = 3).

When SMP-105 was injected into the opposite side, no effect was observed on the primary tumor or axillary lymph node metastases (Figures 3A and B).

3.2. *Time course study of lymph node metastases*

Metastases into a lymph node were pathologically investigated and the Metastasis Score was established as follows:

0: Tumor cells are not observed.
1: Tumor cells can be found as small clusters.
2: Clusters composed of a significant number of tumor cells are clearly observed.
3: Clusters composed of a large number of tumor cells, some of which are undergoing mitosis, are observed.
4: Tumor clusters occupy more than half the area of the lymph node.

Line 10 hepatoma cells were inoculated into the flank region and 60 μg of SMP-105 or vehicle was injected into different sites on days 0, 7 and 14. Clusters of line 10 hepatoma cells emerged by day 8 in the axillary lymph node irrespective whether SMP-105 or vehicle was injected; thereafter, the area and number of clusters developed over time in vehicle-injected guinea pigs, whereas in animals treated with SMP-105, the number and area of tumor clusters diminished and tumor cells finally disappeared on day 18 (Table 1). A period of about 2 weeks was required before the effect of SMP-105 was observed in lymph node metastases.

A lymph node from one of three animals, sampled 21 days after SMP-105 injection was scored as 3, indicating the development of metastases (Table 1). Lymph node metastases failed to be eradicated in this animal, demonstrating that metastases are not always eliminated by treatment with SMP-105.

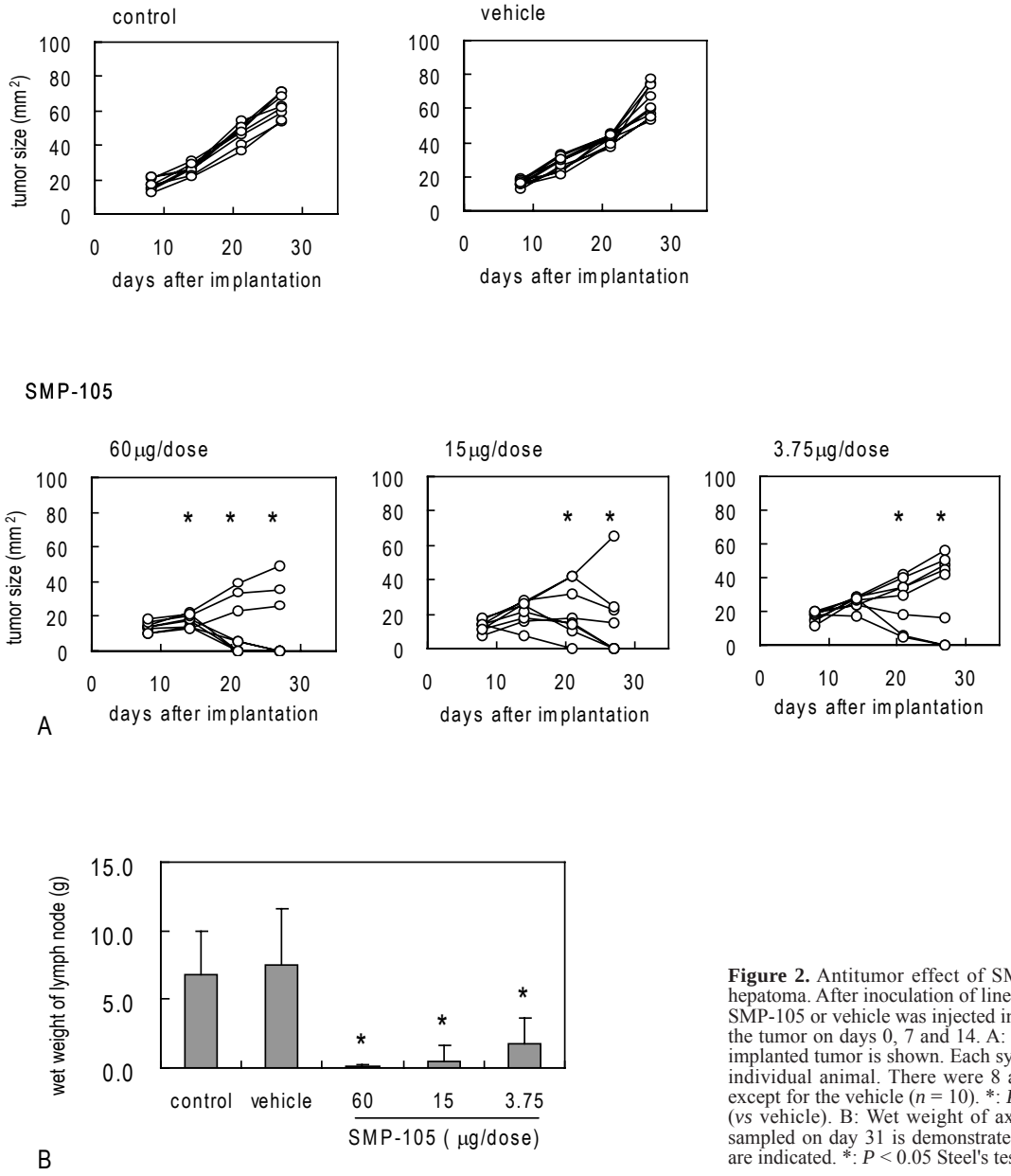

Figure 2. Antitumor effect of SMP-105 on line 10 hepatoma. After inoculation of line 10 hepatoma cells, SMP-105 or vehicle was injected into the same side as the tumor on days 0, 7 and 14. A: Growth of primary implanted tumor is shown. Each symbol represents an individual animal. There were 8 animals in a group except for the vehicle (n = 10). *: $P < 0.05$ Steel's test (*vs* vehicle). B: Wet weight of axillary lymph node sampled on day 31 is demonstrated. Average and SD are indicated. *: $P < 0.05$ Steel's test (*vs* vehicle).

3.3. *Elimination of tumor cells of established micrometastases*

The primary dermal tumor nodule was excised on day 7, and SMP-105 or vehicle was injected. The elimination rate was demonstrated as the number of animals with Metastasis Score 0 to the total number of animals in the group. All animals treated with tumor excision alone or tumor excision and vehicle developed progressively growing tumors in the axillary lymph node, but tumor excision followed by SMP-105 injection eliminated tumor cells from the lymph node in four of eight guinea pigs (Table 2).

3.4. *Distribution of SMP-105 in lymph nodes and spleen*

Immunohistochemical investigation was performed using anti-BCG antibody obtained commercially. The anti-BCG antibody, which was raised against whole BCG bacteria, binds to SMP-105 by *in vitro* binding assay (data not shown). No unspecific staining was observed (Figure 4).

SMP-105 was inoculated intradermally into the right flank region and both right and left axillary lymph nodes and spleen were collected at each sampling time. Three hours post-inoculation, SMP-105 was detected in the marginal sinus of the axillary lymph node from the injection side, and even in the medullary cords of the lymph node 24 h after injection (Figures 4B and C), but not in the opposite lymph node or spleen (Figures 4B-F). Closer observation identified particles

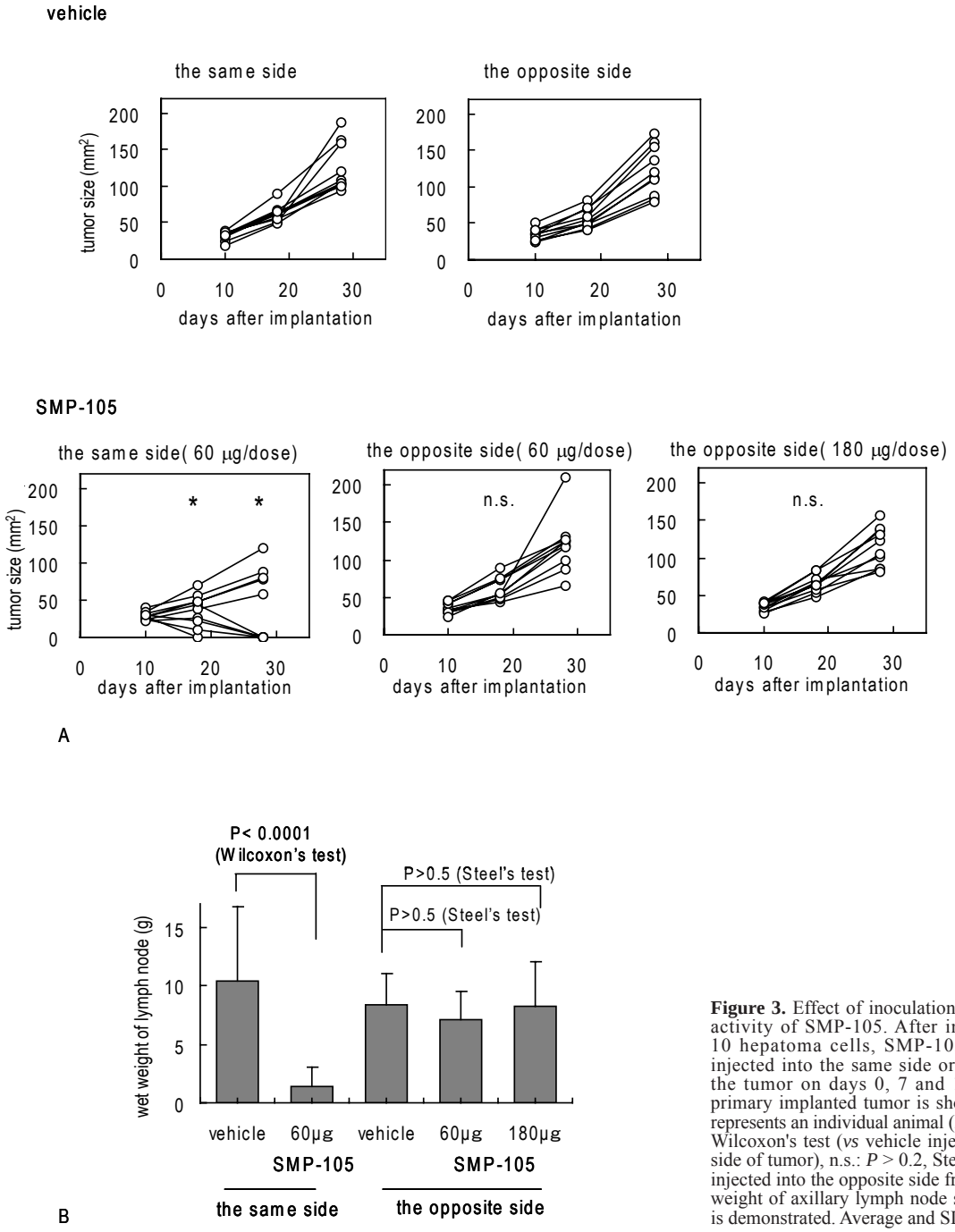

Figure 3. Effect of inoculation side on antitumor activity of SMP-105. After inoculation of line 10 hepatoma cells, SMP-105 or vehicle was injected into the same side or the side opposite the tumor on days 0, 7 and 14. A: Growth of primary implanted tumor is shown. Each symbol represents an individual animal ($n = 10$). *: $P < 0.05$ Wilcoxon's test (*vs* vehicle injected into the same side of tumor), n.s.: $P > 0.2$, Steel's test (*vs* vehicle injected into the opposite side from tumor). B: Wet weight of axillary lymph node sampled on day 28 is demonstrated. Average and SD are indicated.

Table 1. Time course of lymph node metastases

Days after implantation	Vehicle	SMP-105
8	1, 1, 1	1, 1, 1
12	1, 2, 3	0, 0, 1
15	2, 2, 3	0, 1, 3
18	4, 4, 4	0, 0, 0
21	4, 4, 4	0, 0, 3
25	4, 4, 4	0, 0, 0

Metastasis Score of individual animals ($n = 3$) defined in the text is indicated in the table. Line 10 hepatoma cells were inoculated and 60 μg of SMP-105 or vehicle was injected intradermally into a site distal to tumor inoculation on days 0, 7 and 14. Axillary lymph node was collected from tumor inoculation side on the indicated days and fixed with 10% of formaldehyde solution. A slice of the lymph node was stained with hematoxylin and eosin.

Table 2. Effect of SMP-105 on established micrometastases

Treatment	Elimination rate
Control	0/8
SMP-105	5/8
Excision alone	0/8
Excision and vehicle	0/8
Excision and SMP-105	4/8

Line 10 hepatoma cells were inoculated and the primary dermal tumor nodule was excised on day 7. SMP-105 at a dose of 60 μg or vehicle was injected on days 7 and 14. Animals were sacrificed on day 21 and an axillary lymph node was collected. Elimination rate was the number of animals with Metastasis Score 0 to the total number of animals in the group.

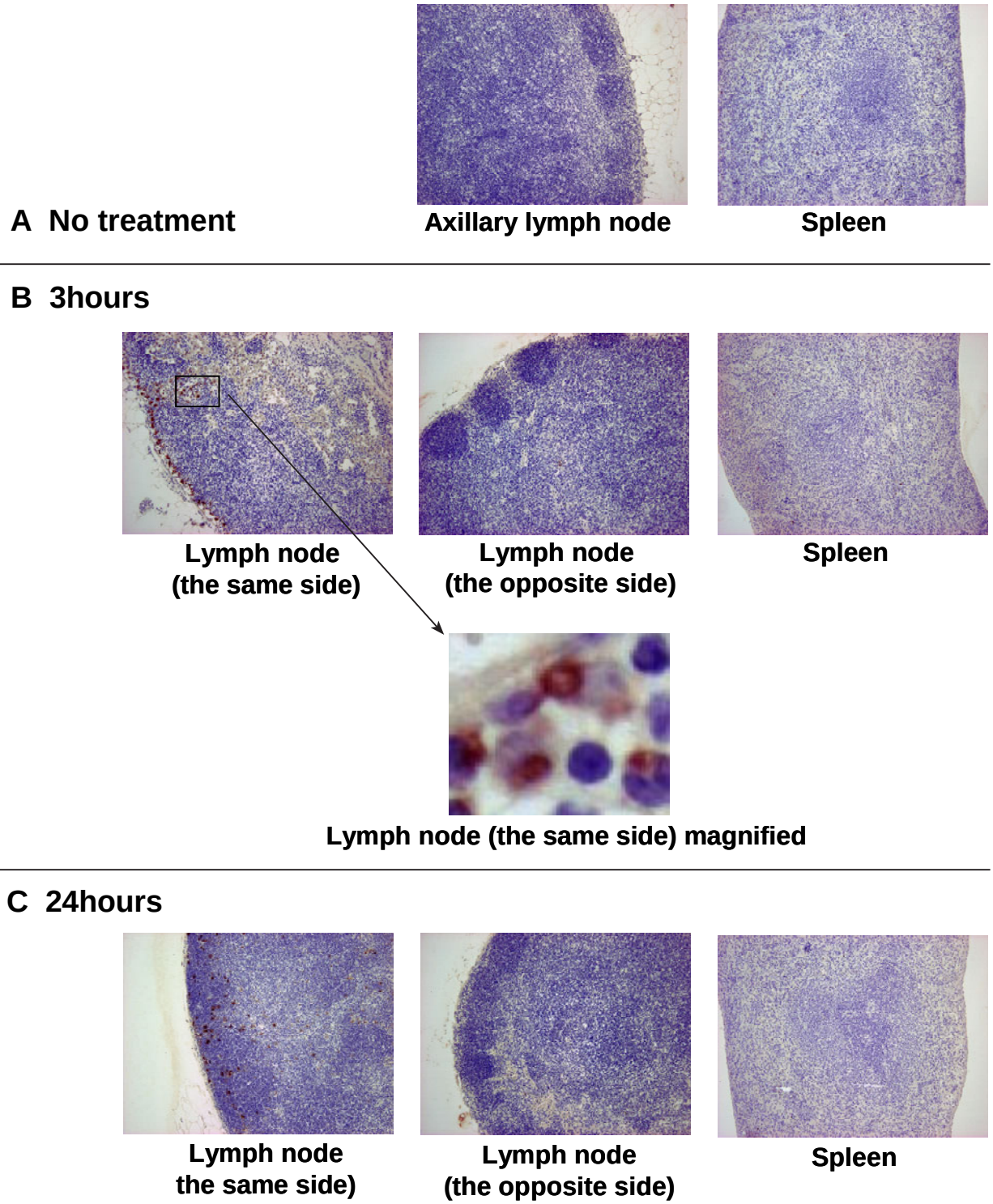

Figure 4. Distribution of SMP-105 in lymph nodes and spleen. SMP-105 was injected intradermally into the thoracic flank region of strain 2 guinea pigs (n = 3) and axillary lymph nodes from both sides and spleen were sampled at the time indicated. Immunohistochemical study was performed using anti-BCG antibody. A, no treatment; B, 3 h; C, 24 h; D, 48 h; E, 3 days; F, 7 days. Representative sections are shown.

with a positive signal at the marginal sinus engulfed by phagocytes with round or oval nucleus (Figure 4B).

3.5. *Generation of systemic tumor immunity*

MMC-treated line 10 hepatoma cells and SMP-105 or vehicle were inoculated separately. Two weeks after treatment, live line 10 hepatoma cells were injected into the opposite side. Strong erythema and edema, considered to be delayed-type hypersensitivity reactions, were induced at the challenge site, and tumor cells were rejected in four of eight animals pre-treated with tumor cells and SMP-105, whereas tumor cell growth was observed in all animals of other groups (Figure 5).

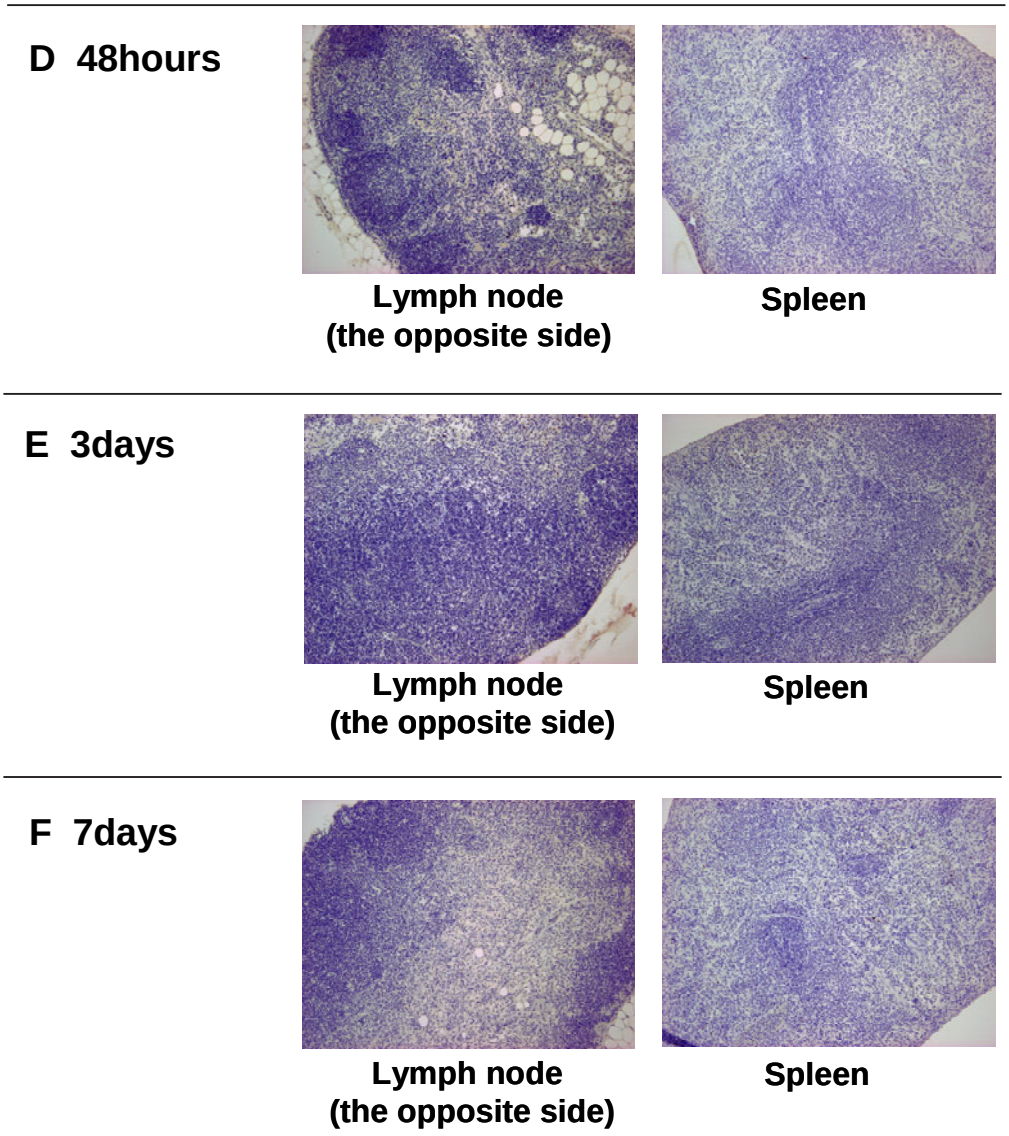

Figure 4. Distribution of SMP-105 in lymph nodes and spleen (continued).

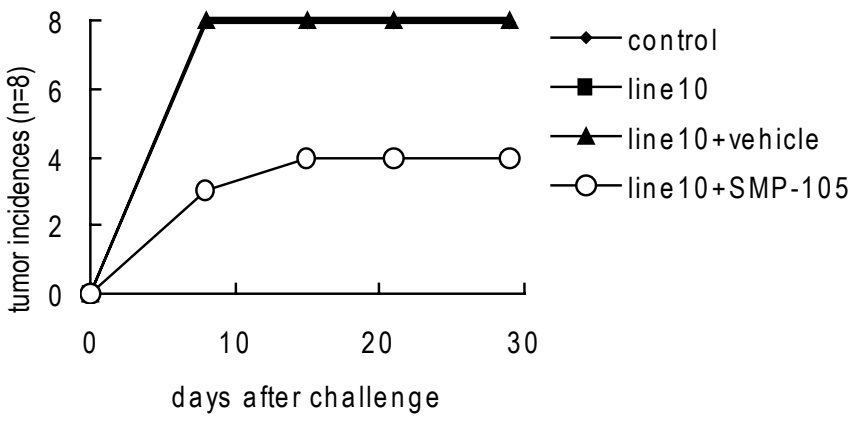

Figure 5. Generation of systemic tumor immunity. Two weeks after separate inoculation of MMC-treated line 10 hepatoma cells and 60 μg of SMP-105 or vehicle, live line 10 hepatoma cells were challenged on the opposite side. Animals were observed for tumor growth. Tumor incidences are plotted against days after challenge (n = 8).

4. Discussion

We prepared a highly purified BCG-CWS (SMP-105) and evaluated its potential for cancer immunotherapy. We thought that if innate immunity is properly activated, tumor antigens distributed endogenously in cancer patients will be exploited to activate tumor immunity. We investigated strain 2 guinea pigs inoculated with line 10 hepatoma, a classical animal model, improving the administration route to exclude the effect of local inflammation on tumor growth.

We injected SMP-105 into sites different, but not distant from the tumor. Metastases in the axillary lymph node and primary skin tumor were eliminated after some time at very low doses in microgram order (Figures 2A and B). But when SMP-105 was injected into the opposite side from the tumor, neither metastases nor primary tumor was eradicated (Figures 3A and B). SMP-105 was observed in the axillary lymph node from the injection side (Figures 4B and C), but was not detected in the axillary lymph node from the opposite side or the spleen (Figures 4B-F). These data indicated that inoculation into sites draining to a sentinel lymph node is crucial for the antitumor activity of SMP-105.

Phagocytes engulfing SMP-105 were observed in the marginal sinus of the axillary lymph node at the injection side (Figures 4B and C). Since SMP-105 is insoluble and formulated into oil droplets of about 2 μm in diameter, three candidates can be listed as phagocytes: polymorphonuclear leukocytes, macrophages and immature dendritic cells (iDCs). From the simple, round or oval shape of the nucleus observed in the lymph node, it is likely that the phagocytes included macrophages and iDC. SMP-105 is therefore considered to activate cells that can potentially process antigens and present them to T cells in the lymph node. Further analysis of phagocytes is necessary. Activation of lymph node cells is investigated using mice in our next paper.

It is not clear whether SMP-105 flowed into the lymph node or was carried by phagocytes. As SMP-105 was injected as insoluble oil droplets and that a large amount of SMP-105 remained for a long time at the inoculation site (our next paper), it is more likely that macrophages or iDCs ingested SMP-105 at the inoculation site and migrated into the draining lymph nodes (*40*,*41*). Investigations as to how SMP-105 reached the lymph node are important to study the formulation for efficient targeting.

Both primary implanted tumors and metastases began to be eradicated about 2 weeks after injection of SMP-105 (Figure 2A, Table 1). When guinea pigs were challenged with live tumor cells 2 weeks after inoculation of tumor cells lacking proliferation activity and SMP-105 separately as antitumor experiments, strong edema and erythema, which was considered to be delayed-type hypersensitivity reactions, developed and the tumor was rejected in the half of the animals (Figure 5). From this evidence it is assumed that systemic tumor immunity was generated during the lag time and eradicated primary and metastasizing tumors.

Taken together, it is considered that SMP-105 activated immune reactions to antigens in the draining lymph node. When a sentinel lymph node was activated, cells capable of processing antigens and presenting them to T cells stimulated by the microbial components would encounter tumor cells and systemic tumor immunity would be generated.

About 30 years ago, when BRMs were intensively studied, lots of animal experiments were tried but systemic administration failed to slow down the growth of the implanted tumor. Then, intratumoral injection and tumor implantation admixed with a BRM were worked out. By these methods, however, antitumor activities are not properly evaluated (*37*,*38*). Our method reported in this paper is a more reasonable one, based on recent development of immunology, for evaluating immunostimulators making use of classical a guinea pig model.

After microscopic metastases settled in the axillary lymph node, the primary tumor was excised and SMP-105 was injected into the same side as tumor excision. Treatment with SMP-105 eliminated lymph node metastases in some animals (Table 2). Shu *et al*. reported that lymph nodes that harbor metastases demonstrate significant suppression in their ability to respond to antigenic stimulation (*42*), but our data show that lymph nodes with metastases recovered their ability to respond to malignant tumor by SMP-105.

Hayashi showed the excellent efficacy of BCG-CWS on head and neck cancer with lymph node metastases (*43*) and Kodama *et al*. reported the long survival of patients with recurrent supraclavicular lymph node metastases of lung cancer by treatment with BCG-CWS (*44*). This evidence may support our idea of exploiting lymph node metastases to generate tumor immunity by microbial immunostimulators.

In conclusion, we suggested that the potential of mono-therapy with a strong immunostimulator and that SMP-105 is one of the most promising agents for cancer immunotherapy by improving the administration route using classical guinea pig models. Although several papers have reported the inoculation of BCG or BCG-CWS as vaccine adjuvants separate from inactivated line 10 hepatoma cells (*45*-*50*), separate inoculation has not been tried for mono-therapy. This is therefore the first report presenting the potential of mono-therapy with BCG-CWS injection into sites different from the tumor. Separate injection from tumor draining to a sentinel lymph node using guinea pigs bearing line 10 hepatoma will be a useful method for investigating immunostimulators.

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Original Article

Immune response against cell-wall skeleton of *Mycobacterium bovis* BCG at the inoculation site and peripheral lymphoid organs

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ABSTRACT: We reported in the previous paper that highly purified cell-wall skeleton of *M. bovis* BCG (SMP-105) eliminated lymph node metastases and primary implanted tumor, presumably by generating tumor immunity, employing guinea pigs. In this paper, we investigated the immune reactions to elucidate the mechanisms of antitumor activity. Twenty-four hours after intradermal injection, inflammatory cells were seen migrating to the inoculation site. Massive infiltrations of lymphocytes were observed on day 7, when a large amount of SMP-105 was still observed in the dermis. Several chemokines attracting neutrophils and monocytes, detected by TaqMan RT-PCR, were induced rapidly and declined 72 h post-injection, but most increased again on day 7, consistent with the pathological findings of lymphocyte infiltration. Activation of lymph node cells was investigated using mice. Upon stimulation by SMP-105 *in vitro*, the draining lymph node cells collected from mice treated with SMP-105 produced interferon-γ (IFN-γ), whereas, lymph node cells did not release IFN-γ when prepared from mice treated with OK-432. This evidence prompted us to assume that SMP-105 functioned as T cell antigens. Intracellular cytokine analysis demonstrated that IFN-γ was mainly attributable to $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells. In conclusion, oil-in-water emulsion of SMP-105 resided for a long time at the inoculation site and activated T cells, probably recognizing SMP-105 itself. The strong tumor eliminating activity of SMP-105 may be explained by the boost of generating tumor immunity *via* positive feed-back from T cells reacting to it, and $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells may distinguish SMP-105 from other synthetic adjuvants.

Keywords: Adjuvant, Cell-wall skeleton of BCG, Immunotherapy, $CD4^-CD8^-\alpha\beta T$ cells

1. Introduction

In the 1980s, bacteria and polysaccharides were intensively studied for cancer immunotherapy and some *e.g.* BCG (*1-4*), OK-432 (*5*), PSK (*6*) were approved as medicines. Recently, agonists of toll-like receptors (TLRs), such as imiquimod and CpG oligodeoxynucleotide (CpG ODN), have been under investigation for cancer treatments (*7-9*).

Progress of the science of innate immunity prompted us to reevaluate microbial fractions first examined about 30 years ago for cancer therapy, and we investigated the activity of mono-therapy with highly purified cell-wall skeleton of *M. bovis* BCG (SMP-105), employing strain 2 guinea pigs bearing line 10 hepatocarcinoma, as reported in the preceding paper.

In this report we analyzed immune reactions to elucidate the mechanisms of the strong antitumor activity of SMP-105. First, reactions elicited at the inoculation site are very informative for understanding the potential of agents. The inoculation site was therefore pathologically investigated and the induction of cytokines and chemokines was analyzed by TaqMan-PCR using strain 2 guinea pigs. Next, we inquired into peripheral lymphoid organs, which are specialized to trap antigens and enable the initiation of adaptive immune responses. We observed phagocytes, presumably macrophages or dendritic cells in the draining lymph node with engulfed SMP-105 in the preceding paper. In this report, activation of lymphocytes was investigated using mice by analyzing cytokine production after inoculation of SMP-105 with or without re-stimulation by SMP-105 *in vitro*.

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Two important clinical events reported by Hayashi *et al*. (*10,11*), *i.e*. delayed-type inflammation at the inoculation site of cell-wall skeleton of BCG (BCG-CWS) and transient IFN-γ in blood, will be discussed based on our findings.

2. Materials and Methods

2.1. *Animals*

Male strain 2 guinea pigs, 5 weeks of age, were obtained from Japan SLC, Inc., Shizuoka and used when they were 6 weeks old. Female C57BL/6N mice at 6 or 7 weeks of age were obtained from Japan SLC, Inc., or Charles River Laboratories, Inc. Yokohama, Japan. Animals were maintained under specific pathogen-free conditions. Their maintenance and experiments were conducted with the approval of the DSP Animal Care and Use Committee.

2.2. *Preparation of SMP-105*

SMP-105 is a product of Dainippon Sumitomo Pharma Co., Ltd. and chemical analysis data were reported by Uenishi *et al*. (*12*). Briefly, SMP-105 contained less than 3% (w/w) of sugars and amino acids assumed not to constitute CWS. Both DNA and trehalose dimycolate were removed to less than 0.05% (w/w) and lipopolysaccharide was about 0.0015 EU/mg by gel-clot technique. An oil-in-water emulsion of SMP-105 was prepared and lyophilized on the thousand-vial scale. Each vial contained 1.2 mg of SMP-105, 32 mg of squalane, 20 mg of polysorbate 80 and 100 mg of mannitol. Vehicle preparation used the same formulation except for SMP-105. SMP-105 in emulsified form was used for inoculation, and the suspended form in saline was prepared for *in vitro* use.

2.3. *Pathological investigation*

SMP-105 was inoculated intradermally into the flank region of strain 2 guinea pigs at 60 μg (n = 3). Animals were sacrificed with a high concentration of carbon dioxide at various time points over 7 days, and skin, including the center of the inoculation site was sampled and fixed with 10% of formaldehyde solution. The skin was cut through the inoculation site into two equal pieces and analyzed immunohistochemically using rabbit anti-*M. bovis* BCG antibody (DAKO Japan Co. Ltd., Kyoto). The reaction of anti-BCG antibody to SMP-105 was investigated by binding assay (data not shown).

2.4. *Analysis of induction of chemokines and cytokines at inoculation site by Taq-Man real-time RT-PCR*

SMP-105 was inoculated intradermally into the flank region of strain 2 guinea pigs at 60 μg (n = 5), and the skin at the inoculation site was excised in a circle of ca.1 cm diameter and collected into liquid nitrogen over 7 days. Total RNA was extracted and purified using TRIZOL (GIBCO) and Rneasy Mini kit (Qiagen), respectively, according to each manufacturer's instructions. The quality of extracted RNA was checked by agarose-gel electrophoresis. Complementary DNA was synthesized using TaqMan Reverse Transcription Reagent (Applied Biosystems,). Amplification was performed using an ABI PRISM 7700 Sequence Detection System (PerkinElmer, Applied Biosystems) using the 5'-nuclease method (TaqMan) with a three-step PCR protocol (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min). All primers and probes were designed with the aid of Applied Biosystems Japan, Co., Ltd. (Tokyo, Japan) from cDNA sequences in NCI data base. TaqMan probes were labeled with FAM as reporter dye and MGB as minor groove binder, except for the β-actin probe, which was labeled with VIC as reporter dye. The sequences of all primers and probes used in this study are shown in Table 1.

Relative quantitation of mRNA levels was performed using the standard curve method. The standard curves were created using five serial dilutions (1:2, $1:2^2$, $1:2^3$, $1:2^4$, and $1:2^5$) of cDNA from the skin of guinea pigs inoculated with either SMP-105 or vehicle. The samples were run in duplicate with primers and probes against β-actin and the target mRNA in the same well. Samples without reverse transcriptase treatment were also run to confirm no contamination of genomic DNA. The relative amount of mRNA in each sample was calculated as the ratio between the target mRNA and the corresponding endogenous control β-actin, and induction kinetic curves were drawn as logarithms of the ratio plus 1 against sampling times.

2.5. *Response in weight of draining lymph node and spleen*

SMP-105 was injected intradermally into the flank region of strain 2 guinea pigs at 60 μg and animals were sacrificed with a high concentration of carbon dioxide on day 14. Axillary lymph node and spleen were sampled and weighed. In experiments using mice, SMP-105 was injected into the footpad of C57BL/6N mice at 30 μg. Popliteal lymph nodes and spleen were sampled over 4 weeks.

2.6. *Cytokine production of draining lymph node cells by re-stimulation of SMP-105 in vitro*

SMP-105 or OK-432 (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) was injected intradermally into both sides of C57BL/6N mice (n = 3). Ten days later, axillary and inguinal lymph nodes were collected and pooled, and single cell suspensions were prepared. Lymph node cells from mice treated with SMP-105 were stimulated

Table 1. Sequences of probes and primers

MCP-1	
MGB probe	5'- CCCAGCAGAAACAG -3'
forward primer	5'- ATTGCCAAACTGGACCAGAGA -3'
reverse primer	5'- TTGAAGTTTGAGGTGCAGTTGAG -3'
MCP-3	
MGB probe	5'- CAGTCCTTCTGTGCCTAC -3'
forward primer	5'-CCTTCCACCATGCAAGTCATT -3'
reverse primer	5'-GCTGAAGTTGGCTGCTGTGA -3'
GM-CSF	
MGB probe	5'-CCCTGTCACCCAGTCC -3'
forward primer	5'-GCACTGTGGTTTGCAGCATCT -3'
reverse primer	5'- GTGGCATCCACGTGTTTCC -3'
IL-1β	
MGB probe	5'- CAATGGAGACAATCTGA -3'
forward primer	5'-CCATCACGGAACTGCACACT -3'
reverse primer	5'- CTCCTTGTACGAAGCTCATGGA -3'
IL-8	
MGB probe	5'-TGACAATCGACAGCTCT -3'
forward primer	5'- GACCACGTTGTGCCAACTCA -3'
reverse primer	5'-CACGTCCTGCACCCACTTCT -3'
VEGF	
MGB probe	5'- TCGGAGAGATGAGTTTC -3'
forward primer	5'- TGCAGATCATGCGGATCAA -3'
reverse primer	5'- TTGGTCGCATTCACATTTGC -3'
eNOS	
MGB probe	5'- CGCCCATGACTTTG -3'
forward primer	5'- GCGGCTGCATGACATTGA -3'
reverse primer	5'- GATGGTCGAGTTGGGAGCAT -3'
GRO	
MGB probe	5'- TCCCTTGGACATTTT-3'
forward primer	5'- ACCTTCATGGTATGCAGTCAACA-3'
reverse primer	5'- CAGTTATGGCTAAACAAGGCATTG-3'
RANTES	
MGB probe	5'- CCCACTGCTTAGCAAT -3'
forward primer	5'- TCTAGGTTCCCAGGCCTCTCA -3'
reverse primer	5'- TGCCTTGAAAGATGTGCTGACT -3'
TNF-α	
MGB probe	5'- TCACACTCAGATCAGC -3'
forward primer	5'- CAGCGGGAAGAGCAGTTCTC -3'
reverse primer	5'- GCCACCGGCTTGTCATTAT -3'
IL-12 p40	
MGB probe	5'- CCTGCAACACTGCTG -3'
forward primer	5'- CCTGGAGAGACGGTGGTTCTT -3'
reverse primer	5'- GAGGTCCATGTGATGCCATCT -3'
β-actin	
MGB probe	5'- TGCGTGACATCAAGGA -3'
forward primer	5'- GACGGAGCGTGGCTACAGTT -3'
reverse primer	5'- GCCATCTCCTGCTCGAAGTC -3'

with SMP-105 for 48 h and IL-2, IL-4, IL-10, IL-12 and IFN-γ in culture supernatants were determined using ELISA kits (Endogen Inc., MA) according to the manufacturer's instructions. Lymph node cells from mice treated with OK-432 were stimulated for 48 h with SMP-105 at 1 μg/mL or OK-432 at 0.01 KE/mL and IFN-γ was determined.

2.7. *Analysis of IFN-γ-producing cells*

C57BL/6N mice were treated with SMP-105 at 60 μg/side on days 0, 3 and 6, and axillary and inguinal lymph nodes were collected and pooled on day 10. Single cell suspensions were prepared and incubated in a 24-well plate at 10^7 cells/well for 20 h with or without SMP-105 at 10 μg/mL. Cell-surface antigens and intracellular IFN-γ were stained using BD Cytofix/Cytoperm™ plus the Fixation/Permeabilization kit (BD Biosciences, New Jersey) according to the manufacturer's protocol. Briefly, for the final 5 h, GolgiStop™ (protein transport inhibitor containing monensin) was added to the medium. The cells were then harvested and Fc receptors were blocked with anti-FcγII/III receptor antibody (clone 2.4G 2), followed by staining of FITC-labeled monoclonal antibodies for CD3ε (clone 145-2C11), CD4 (clone RAM4-5), CD8a (clone 53-6.7), CD8b.2 (clone 53-5.8), NK1.1 (clone PK136), TCRβ (clone H57-597), γδTCR (clone GL3), or isotype control antibodies in separate tubes. After a thorough washing, cells were fixed and permeabilized, stained with PE-labeled anti-IFN-γ antibody (clone XMG1.2), washed and analyzed by FACScan. The monoclonal antibodies described above were all purchased from BD Biosciences.

2.8. *Detection of IFN-γ in blood and analysis of producing peripheral lymphoid organs*

SMP-105 was injected into both fore-footpads at 30 μg per pad on days 0, 3 and 6. After the final injection, blood was collected by heart puncture under ether anesthesia at various time points over 24 h. The brachial and axillary lymph nodes and spleen were collected and lymph nodes from each animal were pooled. Cell suspension was prepared and incubated for 48 h with 10 U/mL of interleukin 2 (IL-2) (Genzyme Corporation) without re-stimulation with SMP-105. IFN-γ in the serum and culture supernatant was determined using ELISA kits (Endogen Inc., MA) according to the manufacturer's instructions.

2.9. *Statistical analysis*

Statistical analysis was performed using the SAS system for Windows (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. *Pathological investigation of inoculation site*

Tumor elimination, presumably by generating tumor immunity using SMP-105, was observed in strain 2 guinea pigs bearing syngeneic line 10 hepatocarcinoma. We first pathologically investigated the skin at the

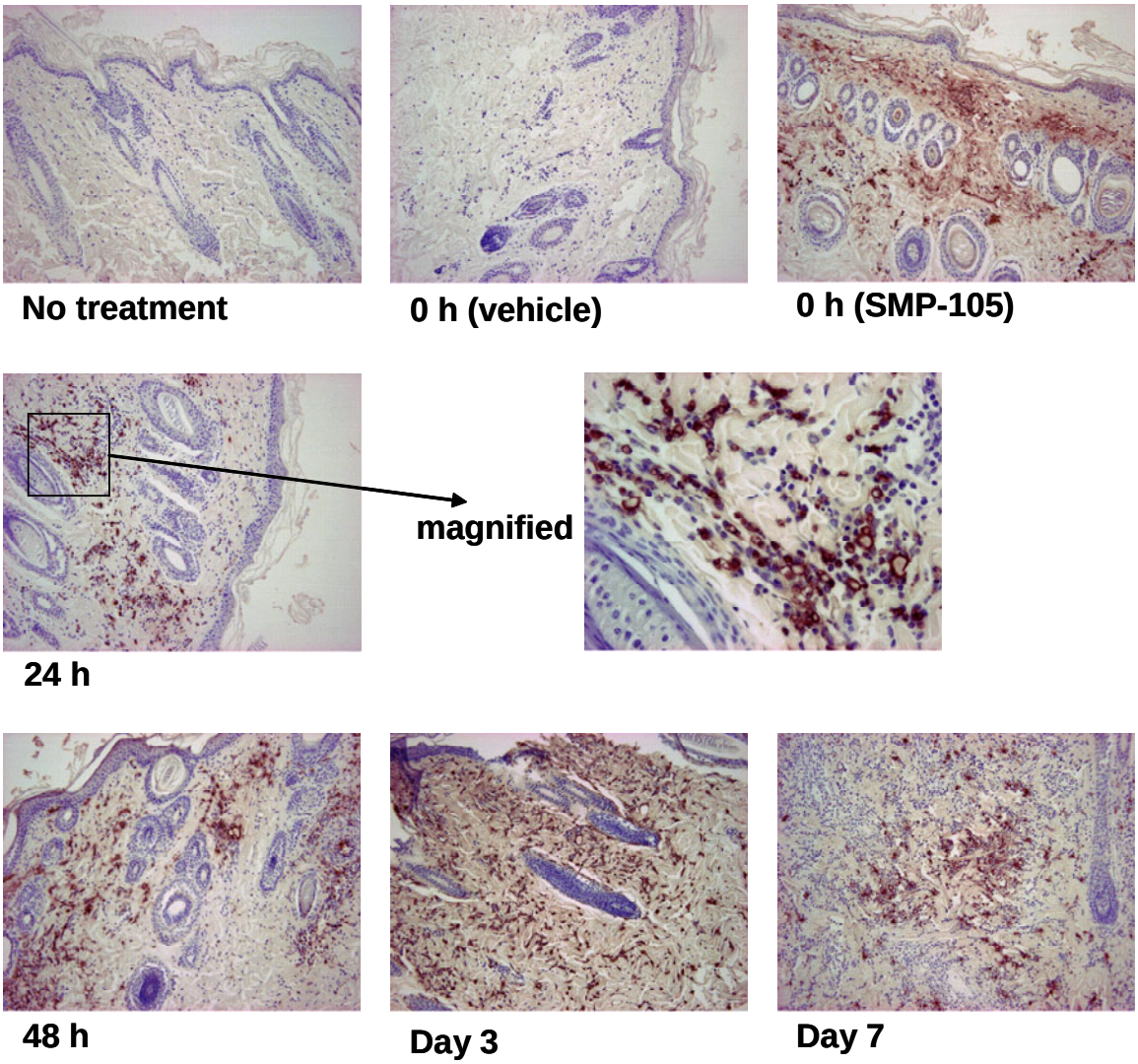

Figure 1. Distribution of SMP-105 at inoculation site (guinea pig). Paraffin-fixed skin tissues from guinea pigs injected with SMP-105 were stained with anti-BCG polyclonal antibody ($n = 3$). Representative sections are shown.

inoculation site using strain 2 guinea pigs. SMP-105 was injected intradermally into the flank region and the skin was sampled over 7 days. Twenty-four hours after injection, inflammatory cells were seen migrating to the inoculation site and engulfing SMP-105 (Figure 1). A large number of positive signals from SMP-105 were still observed on day 7 by immunohistochemical analysis and massive infiltration of lymphocytes was observed into areas where SMP-105 was distributed (Figure 1).

3.2. *Induction of cytokines and chemokines at inoculation site*

Gene expression of the skin at the inoculation site was investigated by the TaqMan quantitative RT-PCR approach. Tumor necrosis factor-α (TNF-α), IL-1β, growth-related oncogene (GRO), monocyte chemotactic protein 1 (MCP-1) and IL-8 were induced rapidly, and peaked 8 h after inoculation. Regulated on activation normal T cells expressed and secreted (RANTES), which is reported to be up-regulated by TNF-α and IL-1β (*13*), showed a somewhat delayed peak at 24 h (Figure 2). After decreasing once, TNF-α, IL-1β, RANTES, MCP-1 and IL-8 increased again 7 days after injection (Figure 2), suggesting that the immune reactions entered a new phase. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was induced significantly only 7 days after injection (Figure 2). MCP-1, IL-8, GRO, RANTES and endothelial nitric oxide synthase (eNOS) were induced also by vehicle (Figure 2) and in the early phase, the mRNA level of MCP-1 and eNOS stimulated by SMP-105 was explicable by the vehicle, probably due to squalane contained in the vehicle. The induction of chemokines and cytokines by the vehicle may involve adjuvant activity of squalane (*14*). A clear increase or decrease of MCP-3, vascular endothelial growth factor (VEGF and IL-12 p40 was not observed (Figure 2).

3.2. *Marked increase in weight of the draining lymph node but no change in the spleen*

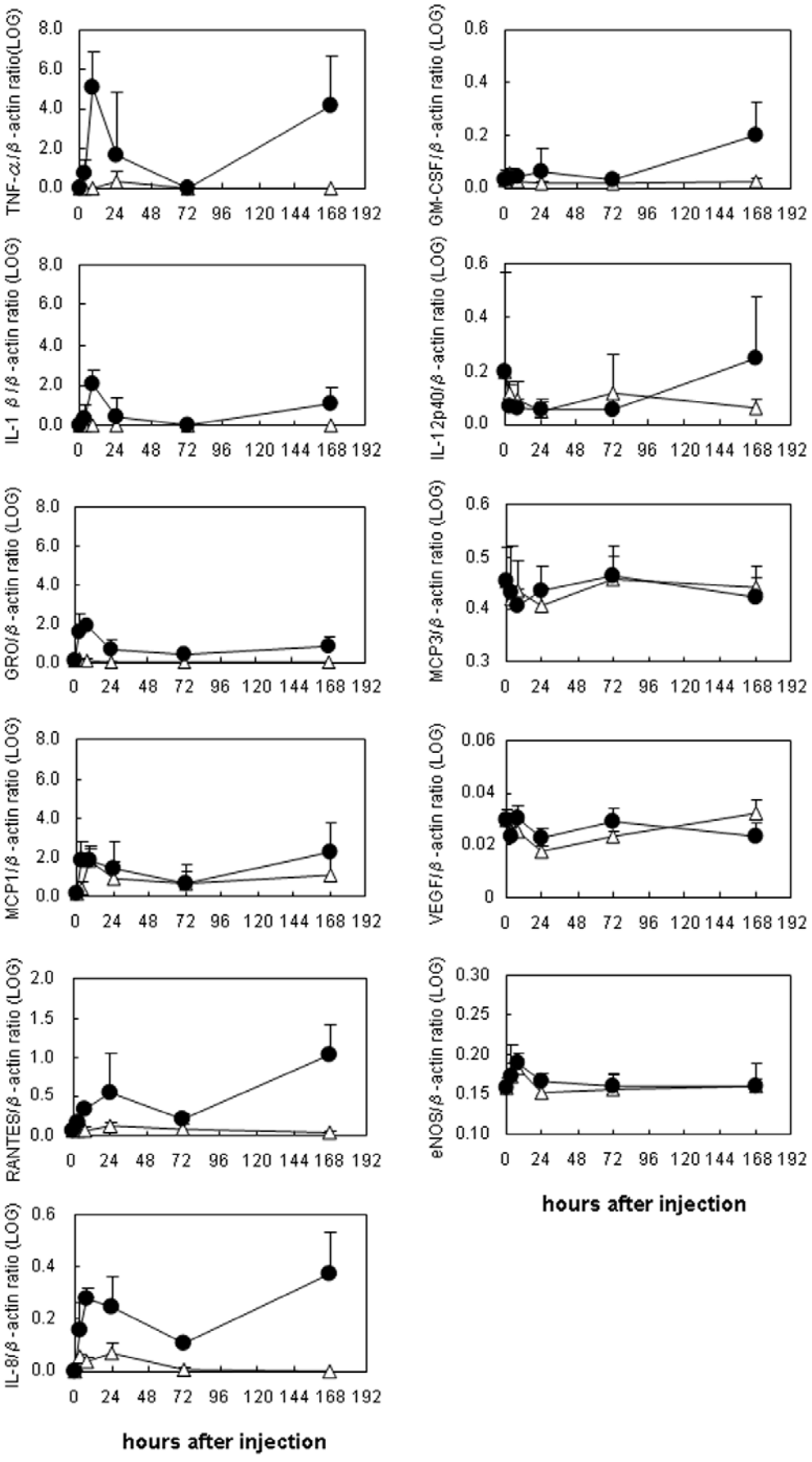

Figure 2. Induction of cytokines and chemokines at inoculation site (guinea pig). Induction of cytokines and chemokines indicated in each figure at the inoculation site was investigated by TaqMan quantitative RT-PCR approach. Average and SD (n = 5) are shown. Circle, SMP-105; triangle, vehicle.

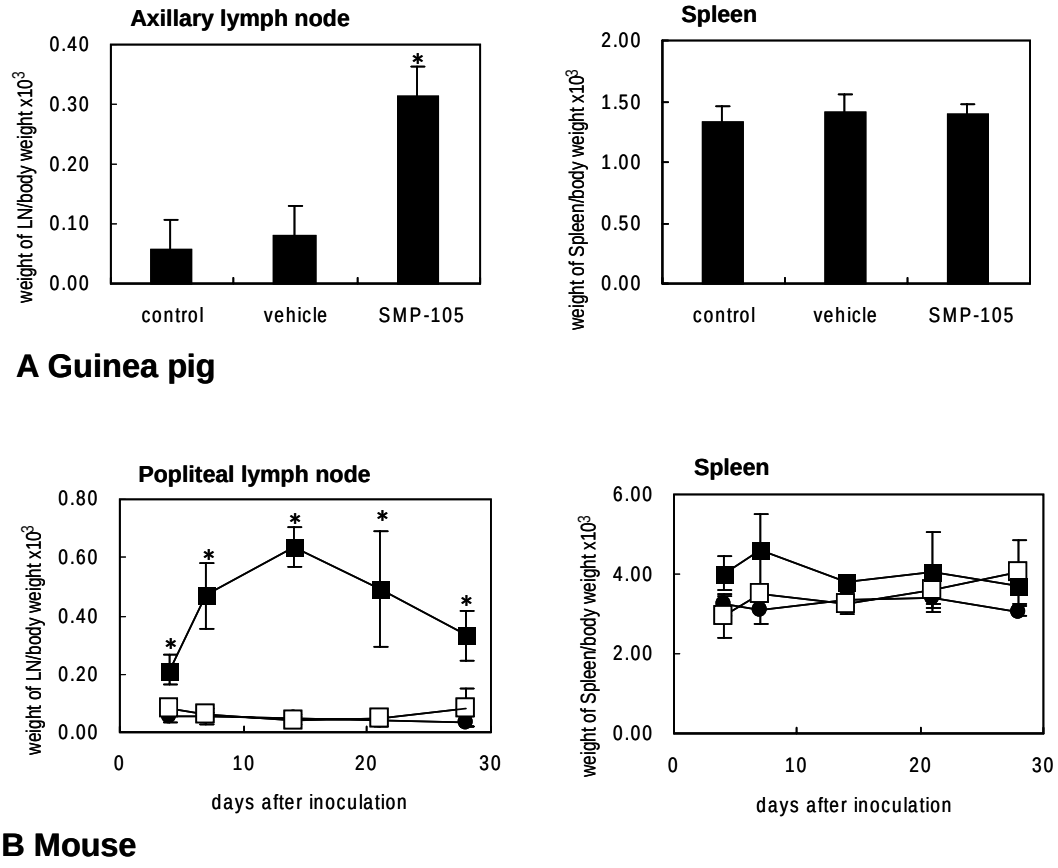

Figure 3. Increase in weight of the draining lymph node (guinea pig and mouse). A: Guinea pig. Wet weight of axillary lymph node and spleen 14 days after SMP-105 injection is demonstrated as the ratio to body weight ($n = 4$); B: Mouse. Wet weight of popliteal lymph node and spleen over 4 weeks is shown as the ratio to body weight ($n = 3$). Closed circle, control; open square, vehicle; closed square, SMP-105. Average and SD are indicated. *: $P < 0.05$ *T*-test (*vs* vehicle).

Next, peripheral lymphoid organs were analyzed. We noticed marked swelling of the draining lymph nodes and wet weight was therefore measured.

Two weeks after injection of SMP-105 into guinea pigs, the wet weight of the draining lymph nodes was remarkably increased, but there was no response in spleen weight (Figure 3A). In experiments using C57BL/6N mice, the same results were obtained, *i.e.* increase in weight of popliteal lymph node was observed for 4 weeks, peaked 2 weeks after inoculation, and there was no response in spleen weight (Figure 3B). Further analysis of lymph node activation was performed using mice, because various well-characterized antibodies were available.

3.3. *Production of IFN-γ, IL-2 and other cytokines in draining lymph node cells by re-stimulation of SMP-105 in vitro*

Activation of the draining lymph nodes by injecting SMP-105 was investigated by cytokine production upon re-stimulation with SMP-105 *in vitro*. The lymph node cells produced IFN-γ dependent on the pre-treatment doses (Figure 4A), demonstrating that SMP-105 injection induced the activation of draining lymph node cells. In addition to IFN-γ, IL-2 was also secreted (Figure 4B). Production of a marginal amount of IL-10 was observed but the induction of either IL-12 or IL-4 was not detected (Figure 4B).

The production of IL-2 indicated that T cells were differentiated in the lymph nodes. When lymph node cells from mice immunized with OK-432 were re-stimulated with OK-432 *in vitro*, a large amount of IFN-γ was released, but little was detected upon stimulation with SMP-105 (Figure 4C). When lymph node cells prepared from mice immunized with SMP-105 were re-stimulated with SMP-105 *in vitro*, an enormous amount of IFN-γ was produced (Figure 4C). This evidence suggested that some portion of IFN-γ is attributable to T cells activated by antigen presentation. This issue will be discussed later.

3.4. *Major populations of IFN-γ-producing cells*

Produced in abundance by re-stimulated lymph node cells, the major populations of IFN-γ-producing cells were investigated. Axillary and inguinal lymph node cells from C57BL/6N mice pre-treated with SMP-105 were re-stimulated *in vitro* with SMP-105, and IFN-γ in cytoplasm was analyzed by FACS using the cellular cytokine detection method. The major populations of IFN-γ-producing cells were $CD4^{-}CD8^{+}\alpha\beta$T cells and

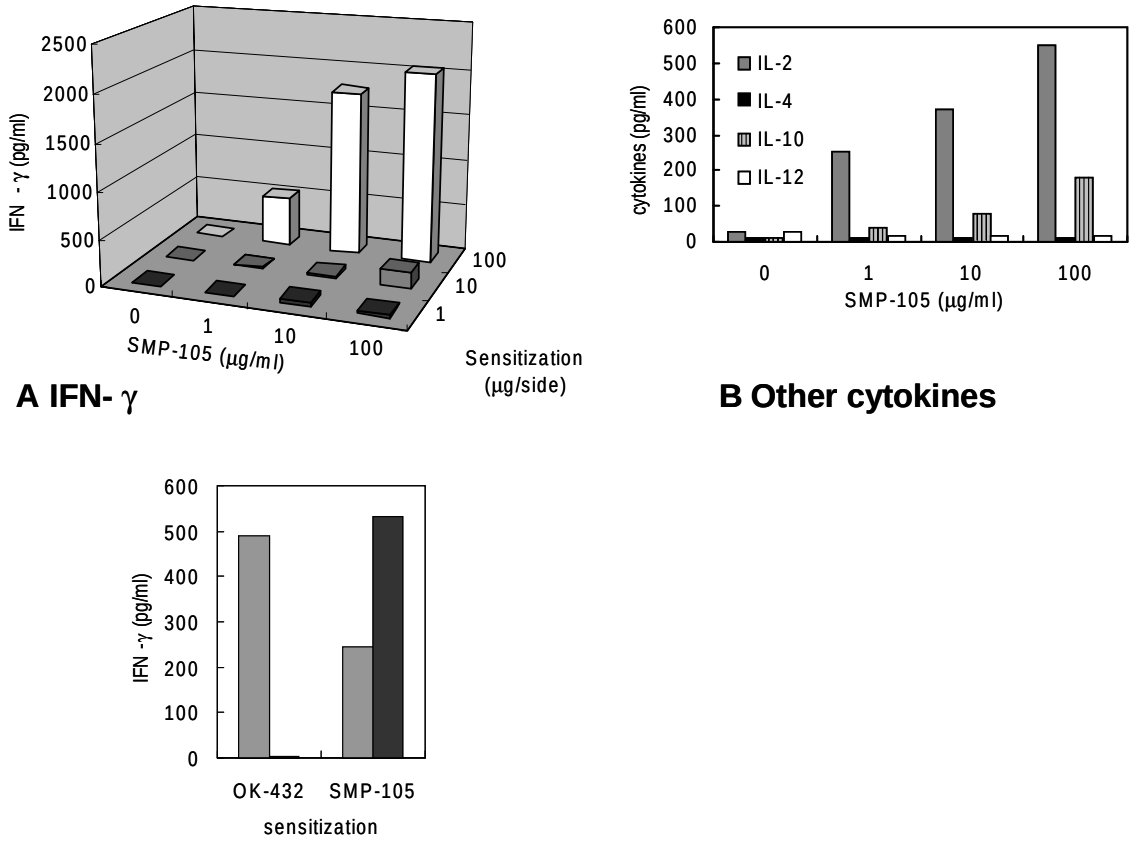

Figure 4. Production of cytokines from draining lymph node cells by re-stimulation of SMP-105 *in vitro* (mouse). Ten days after inoculation of SMP-105 at 1, 10 or 100 μg (1 μg; n = 5, 10, 100 μg; n = 3) or OK-432 at 0.1KE (n = 3), draining lymph nodes were sampled and pooled. Cell suspensions were prepared and stimulated by SMP-105 or OK-432 for 48 h (n = 3). IL-2, IL-4, IL-10, IL-12 and IFN-γ in the culture supernatants pooled from 3 wells were determined. A: Production of IFN-γ. Production of IFN-γ is demonstrated by 3-D figure. X-axis, concentration at re-stimulation *in vitro*; Y-axis, pre-treatment dose; Z-axis, IFN-γ secreted into medium. Shown are representative experiments of more than three; B: Production of other cytokines. Culture supernatant of lymph node cells prepared from mice treated with 100 μg of SMP-105 in the same experiments as described above was assayed for cytokines indicated in the figure; C: Antigen dependency of IFN-γ production. Cell suspensions were stimulated *in vitro* by 1 μg/mL of SMP-105 (black bar) or 0.01 KE/mL of OK-432 (gray bar) for 48 h (n = 3). IFN-γ in the culture supernatants pooled from 3 wells was determined.

$CD4^{-}CD8^{-}\alpha\beta$T cells, and few positive signals of IFN-γ were detected from $NK1.1^{+}$ or γδT cells (Figure 5).

3.5. *Transient IFN-γ detected in blood after repeated injection of SMP-105*

Hayashi *et al*. reported that IFN-γ was transiently detected in blood after the injection of BCG-CWS from cancer patients and that IFN-γ was an excellent prognostic marker (*10,11*). Detection of transient IFN-γ in blood was therefore attempted using mice and we succeeded by injecting SMP-105 into fore-footpads. IFN-γ was detected from 3 h, peaked at 6 h and then fell markedly 24 h after the final injection (Figure 6).

3.6. *Production of IFN-γ by draining lymph node cells but not the spleen upon incubation without SMP-105*

In order to investigate the origin of IFN-γ in blood, the draining lymph nodes and spleen were collected after repeated injection of SMP-105, incubated without re-stimulation by SMP-105 for 48 h, and the culture supernatant was assayed for IFN-γ by ELISA. IFN-γ was detected from the supernatant of the draining lymph node cells (Figure 7A), but not from that of spleen cells (Figure 7B), suggesting that IFN-γ in the blood was secreted mainly by draining lymph node cells.

4. Discussion

It is reported that BCG-CWS in the emulsified form induces delayed and long-lasting inflammation at the inoculation site (*10,11*). When SMP-105 was injected into the skin, slight redness developed, peaked from 24 to 48 h post-injection, and then disappeared by day 5. Erythema accompanied by edema then developed and lasted for more than a month (data not shown). Within 24 h, inflammatory cells migrated to the inoculation site, probably attracted by chemokines affecting neutrophils, *e.g.*, IL-8, GRO and macrophages, *e.g.*, MCP-1, RANTES and further activated by IL-1β

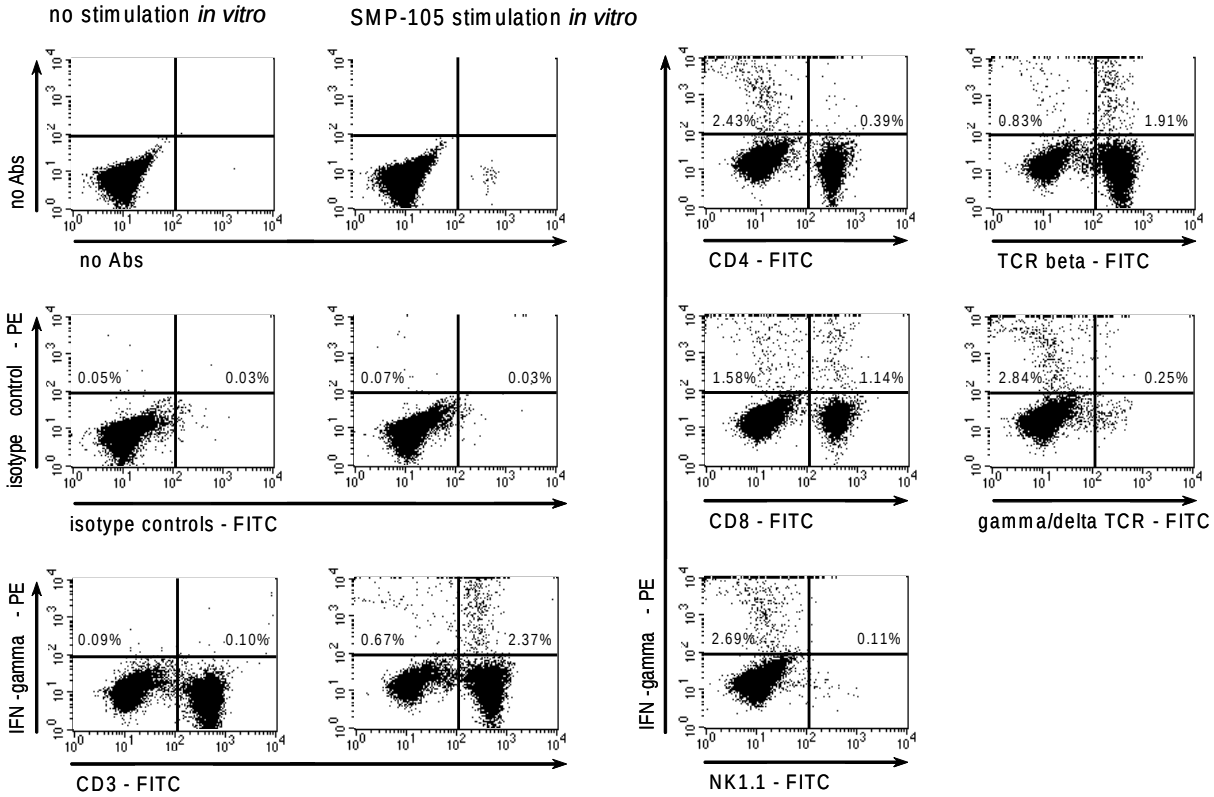

Figure 5. Major population of IFN-γ-producing cells (mouse). Draining lymph node cells from C57BL/6N mice pre-treated with SMP-105 were re-stimulated *in vitro* with SMP-105, and IFN-γ in cytoplasm was analyzed by FACS using the cellular cytokine detection method. Shown are representative experiments of more than five.

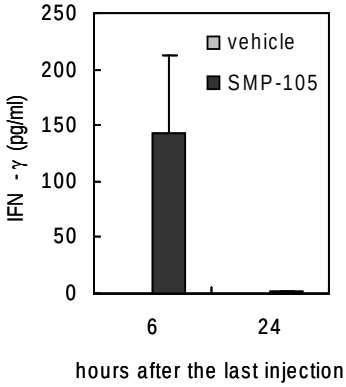

Figure 6. Transient IFN-γ detected in blood (mouse). Blood was collected from mice repeatedly treated with SMP-105 (n = 3), and serum was assayed for IFN-γ. Blood from vehicle-treated mice was collected 6 h after the final treatment. Average and SD are indicated.

and TNF-α (*15-17*) (Figure 2). On day 7, massive infiltration of lymphocytes was seen (Figure 1), probably associated with the secretion of RANTES (*13,15-17*), and RANTES is considered to involve T-cell-mediated chronic inflammation. In fact, at that time, a large amount of SMP-105 remained (Figure 1) and more than a month later, positive signals derived from SMP-105 were still observed (personal communication from Dr. Koji Hanai), consistent with skin reactions. Long-lasting stimulation of immune systems, from innate immunity to acquired immunity, could evoke tumor elimination activity, and

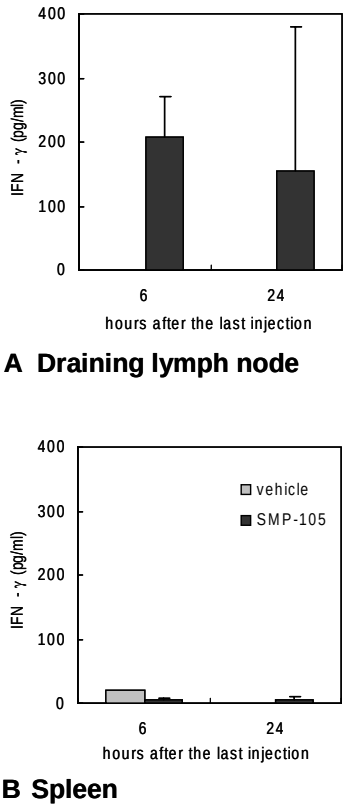

Figure 7. Secretion of IFN-γ by peripheral lymphoid organs (mouse). Brachial and axillary lymph node and spleen were collected from the same mice as described in Figure 6 after repeated inoculation of SMP-105 into fore-footpads (n = 3). Lymph node cells (A) and spleen cells (B) were incubated with 10 U/mL of IL-2 for 48 h and culture supernatant was assayed for IFN-γ. Average and SD are indicated.

the long residence of BCG-CWS emulsified with oil may explain the recurrence of inflammation at past inoculation sites upon fresh injection into the other arm (personal communication from Dr. Akira Hayashi).

The production of IL-2 by draining lymph node cells from mice pre-treated with SMP-105 indicated the differentiation of T cells (Figure 4B). Lymph node cells also produced a large amount of IFN-γ (Figure 4A). There are two pathways to activate T cells; direct activation *via* TCR ligation and indirect activation *via* macrophages stimulated by SMP-105. When lymph node cells from mice immunized with OK-432 were re-stimulated with OK-432 *in vitro*, a large amount of IFN-γ was released, whereas little was detected upon stimulation with SMP-105 (Figure 4C). If T cells from mice immunized with SMP-105 were only activated without antigen presentation, T cells from OK-432-immunized mice would also produce IFN-γ *via* macrophages stimulated with SMP-105. From these observations we consider that some portion of IFN-γ was attributable to T cells activated *via* TCR ligation.

Although proteinous antigens, except some living organisms (*18*), generally induce $CD4^+$ T cell activation, SMP-105 did not activate $CD4^+$ T cells (Figure 5). Furthermore, SMP-105 is a highly purified BCG-CWS containing only a small amount of amino acid residues not constituting peptidoglycan (1%, w/w) (*12*). There is a possibility that T cells releasing IFN-γ recognize non-proteinous antigens. In fact, there are papers reporting that $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells recognize mycobacterial lipid antigens (*18*).

This is one of the prominent features of SMP-105, when considering that there are no reports that synthetic TLR agonists, such as CpG ODN or imiquimod, function as antigens to induce IFN-γ or IL-2. T cells, even though not specific to tumors, will be able to boost the generation of tumor immunity through cell-to-cell interactions, *e.g.*, CD40L-CD40 and secreted cytokines, *e.g.*, IFN-γ (*19-21*). IL-2 activates other T cell populations, including precursor CTL to tumors. Multi-pathways for activating immune reactions may be necessary for patients suffering from malignant neoplasm in immuno-compromised conditions.

Hayashi *et al*. reported that transient IFN-γ in peripheral blood after inoculation of BCG-CWS was associated with survival (*10,11*). Our investigation demonstrated that IFN-γ was mainly produced by $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells in draining lymph nodes and suggested that transient IFN-γ in the blood was a marker indicating that lymph node functions to activate T cells were retained or retrieved. As for delayed-type skin inflammation, lymphocytes infiltrating tissues to which SMP-105 was distributed may include $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells. Delayed chronic inflammation therefore indicated immunophysiological states of the host, *e.g.*, draining lymph node retains or retrieves functions for differentiating T cells, and the immune system is not lost, at least at the site of chronic inflammation. In fact, skin inflammation was weak or not observed in guinea pigs, enabling the growth of implanted line 10 hepatoma. Analysis of the infiltrating lymphocytes is required.

In conclusion, oil-in-water emulsion of SMP-105 resided for a long time at the inoculation site and activated T cells, probably recognizing SMP-105 itself. The strong tumor-eliminating activity of SMP-105 may be explained by the boost of generating tumor immunity *via* positive feed-back from T cells reacting to it, in addition to direct activation of macrophages and dendritic cells. $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells may distinguish SMP-105 from other synthetic adjuvants. Further investigation is needed to clarify the mechanisms of T cell activation, including TCR-dependency, the antigen structures and presenting molecules, and then the contribution of T cells to the therapeutic effect.

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Original Article

Preliminary research on abating rat testicle toxicity due to triptolide after oral polymer nanoparticle delivery

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ABSTRACT: This study was undertaken to investigate the effect of triptolide (TP) on male rats after oral polymer nanoparticle delivery (TP-loaded poly(D,L-lactic acid) nanoparticles, TP-PLA-NPs). Free TP and TP-PLA-NPs were administered orally at doses of 0.2 and 0.6 mg/kg for 15 days and rats were sacrified at end of the dosage period. All rat testes were weighed, fructose content and activity of acid phosphatase (ACP) were assayed, and testis tissues were observed histopathologically. Testis weight, testis index, ACP activity, and fructose content of the treated animals were lower than those of the control group. Moreover, ACP activity and fructose content were markedly decreased for free TP of 0.6 mg/kg in comparison to the same dose of TP-PLA-NPs. Obvious pathological changes were not observed for a dose of 0.2 mg/kg free TP and TP-PLA-NPs at the end of 15 days. At a dose of 0.6 mg/kg, however, free TP caused more serious damage to the testes than TP-PLA-NPs. These results revealed that TP-PLA-NPs might decrease the testis toxicity of TP because TP was slowly released from polymer nanoparticles. Further research on this mechanism of abated toxicity is in progress.

Keywords: Triptolide, Testis, Polymer nanoparticle delivery, Poly(D,L-lactic acid)

1. Introduction

Triptolide (TP, Figure 1), a purified diterpenoid triepoxide compound that is extracted from the Chinese medicinal plant *Tripterygium wilfordii* Hook.f. (TWHf), has several forms of pharmacological activity including an immunosuppressive effect (*1*,*2*), anti-cancer activity (*3*), and anti-inflammatory activity (*4*). However, the clinical use of TP is known to present several practical disadvantages mainly due to its lower water solubility and severe toxicity. The incidence of adverse drug reactions (ADRs) due to TP is higher than due to other drugs in China. The organic systems affected by ADRs of TP relate to the digestive, urogenital, and circulatory systems as well as bone marrow.

In recent years, the effect of TWHf or TP on the testis tissue of male rats or men has been extensively noted (*5-8*). Oral administration of TP at a dosage of 0.1 mg/kg daily for 70 days left male rats completely infertile, with sperm motility reduced to zero, and cauda epididymal sperm content decreased to 68% (*6*). TP was reported to impair the cauda epididymal sperm ultrastructure with minimal abnormalities in the fine structural cytology of the testes (*9*). Daily oral doses of 20-30 mg extract of TWHf for 2 months caused a sharp decline in the epididymal sperm number and motility for Chinese men; interestingly, though, fertility returned to normal between 1 and 2 months after the cessation of administration (*10*). Therefore, these results suggest that TWHf or TP might be developed as a promising male contraceptive.

Earlier immunological studies have indicated that TP does not have an immunosuppressive effect at dose levels inducing infertility; however, higher doses (5 to

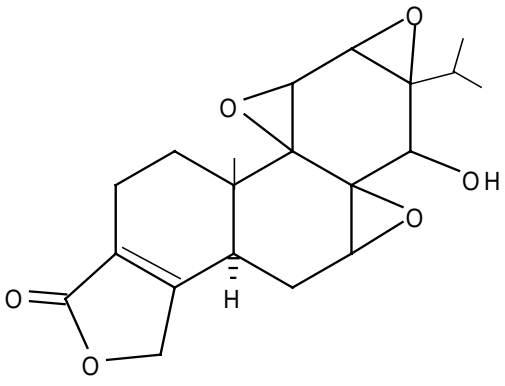

Figure 1. Chemical structure of triptolide.

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12 times higher than its antifertility doses) can produce immunosuppressive effects (*11,12*). However, higher dosages of TP might induce more severe toxicity in animals or man. The question is how to retain or improve the pharmacological activity of TP while decreasing its toxicity on the testes.

Over the last few decades, various drug delivery systems (DDS) such as liposomes, microemulsion, and polymer micro/nanoparticles have shown great promise in controlled release and targeted drug delivery in order to decrease or avoid a drug's side effects (*13-15*). Fortunately, novel types of delivery systems for TP were widely developed in China. Loaded-TP solid lipid nanoparticles efficiently penetrated the skin, had desirable anti-inflammatory activity, and significantly decreased hepatotoxicity in comparison to control groups (*16,17*). Microemulsions containing TP significantly increased the in vitro permeation rate of TP through mouse skin and reduced skin irritation when compared to a TP solution (*18*).

Poly(D,L-lactic acid) (PLA) has been universally used as a microsphere/nanoparticles carrier in DDS due to its desirable biocompatible and biodegradable properties; in particular, it was approved by the US Food and Drug Administration for clinical use. In previous studies, TP-loaded PLA nanoparticles (TP-PLA-NPs) were successfully prepared and characterized (*19*). Animal experiments indicated that TP-PLA-NPs significantly inhibited adjuvant-induced arthritis and had a desirable anti-inflammatory effect with long-time administration (*4*). The main objective of the present study was to investigate if TP-PLA-NPs would decrease the testis tissue toxicity caused by TP in male rats after oral administration.

2. Materials and Methods

2.1. *Materials*

TP (purity > 99%) was supplied by Fujian Chinese Medical Research Institute (China). PLA (MW, 10,000 Da) was obtained from Shangdong Medical Treatment and Instrument Institute (China). Poloxamer 188 was purchased from Jiqi Medicine Co., Ltd., Shenyang Pharmaceutical University (China). Methanol was of HPLC grade and other reagents were of analytical grade.

2.2. *Preparation of TP-PLA-NPs and characterization*

TP-PLA-NPs were prepared according to the method previously described (*4*). Briefly, an amount of PLA/TP (20:1, w/w) was co-dissolved in a mixture of acetone/ethanol (9:6, v/v). The solution obtained was then added dropwise to 40 mL of poloxamer 188 solution (1%, w/v) with continuous gentle stirring at ambient temperature until complete evaporation of the organic solvent. The obtained suspension was then used for animal experiments after adjustment of the drug concentration (calculated according to TP). The produced nanoparticles were collected by ultracentrifugation (12,000 rpm, 3 h, 4°C) using a GL 20 centrifuge (Xiangxi, China) and freeze-dried (LGJ 0.5, Beijing, China) to obtain white, powdered nanoparticles.

The mean particle size, size distribution, and polydispersity index of nanoparticles were assessed by dynamic light scattering with a particle size analyzer (Zeta plus, Brookhaven). The shape and surface morphology of nanoparticles were observed with a transmission electron microscope (JEM-2000 CX-II, JEOL).

The amount of drug incorporated into nanoparticles was determined by HPLC (Agilent 1100) using a reverse phase Lichrospher ODS column (5 μm, 250 × 4.6 mm i.d.). A release experiment for release of TP from nanoparticles was performed using a dialysis bag technique. Analysis of drug content and the release experiment are described in detail elsewhere (*19*).

2.3. *Animals and treatment*

Healthy male Wistar rats (n = 30) weighing 152-171 g were obtained from the Laboratory Animal Center, Tongji Medical College, Huazhong University of Science & Technology (Wuhan, China). The animals were housed in stainless steel cages in the animal room. The room temperature and the relative humidity were controlled at 23 ± 2°C and 50 ± 10%, respectively. All animals were allowed free access to drinking water and feed throughout the study.

Animals were randomly divided into five groups with six per group. The control group was given only saline every day. TP-PLA-NPs groups were given a 0.05% CMC (sodium carboxymethyl cellulose) suspension of TP-PLA-NPs (0.2 and 0.6 mg(TP)/kg), respectively, by gavage. Free TP groups were given a 0.05% CMC suspension of free TP (0.2 and 0.6 mg(TP)/kg), respectively, by gavage. All animal experiments followed a protocol approved by the Institutional Animal Care and Use Committee of this Center.

2.4. *Organ weight and preparation of testis serum*

All rats were weighed on the 15th day and immediately sacrificed under ether anesthesia. Testes were quickly removed, cleared of fat and connective tissue, washed with normal saline (0.9% sodium chloride) and weighed after removing water on their surface. One gram of testes was placed in 10 mL phosphate buffered saline (1 mM, pH 7.0) and homogenized by 2 min of sonication (Scientz-IID, Ningbo, China) under low temperature conditions (4°C). The suspension obtained was

centrifuged for 10 min at 7,000 rpm and the supernatant was stored at -20°C before biochemical analysis.

2.5. *Enzyme assay and fructose content*

Acid phosphatase (ACP) activity was assayed using *p*-nitrophenyl phosphate as a substrate at pH 4.8 (*20*). One unit of enzyme is defined as μmoles of *p*-nitrophenol released per min and per gram protein at 37°C.

Fructose levels from the seminal vesicles were quantified colorimetrically by employing a resorcinol reagent (*21*).

2.6. *Histopathological analysis*

The excised testes were fixed in 10% buffered formalin (0.01 mM, pH 7.4) and dehydrated in aqueous alcohol (25%, 50%, 70%, and 90% EtOH). The testes were embedded in paraffin blocks and sectioned perpendicular to the longest axis of the testis at a thickness of 5-8 μm. The sections were mounted onto regular glass slides and stained with hematoxylin and eosin prior to observation by light microscopy (Olympus, BH-2, Japan).

2.7. *Statistical analysis*

Data were expressed as mean ± standard deviation (S.D.) and analyzed using the Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

3. Results and Discussion

TP is a potent anti-inflammatory and immunosuppressive agent. However, thousands of adverse events related to TWHF or TP are reported in China. The patients usually experience nausea, vomit, dyspnoea, hepatomegaly, duodenal ulcer, and gastrointestinal bleeding in the digestive system if taking the drug for a long time. Some organic systems including the gastrointestinal, urogenital, cardiovascular, and circulatory systems as well as bone marrow and skin can also be affected by these systems' toxic reactions to TP (*2*).

TP is reported to not be immunosuppressive at dose levels inducing infertility (*11*), though doses about 5 to 12 times higher than infertility doses can have immunosuppressive effects (*12*), TP also has potent toxicity associated with the renal, cardiac, hematopoietic, and reproductive systems. In recent years, several advanced dosage forms have been developed in order to effectively improve the immunosuppressive effect of TP and decrease its toxicity (*4,17,18*). The current study mainly investigated the effect of TP-PLA-NPs on the testes of male rats after continuous oral administration at different dosages for 15 days.

3.1. *Characterization of nanoparticles*

TP-loaded PLA nanoparticles showed a narrow size distribution, low polydispersity index of 0.088, and mean particle size of about 150 nm. Moreover, nanoparticles appeared to be a fine spherical shape with smooth surfaces and without any aggregation or adhesion. Drug encapsulation efficiency into nanoparticles was 85.7 ± 4.3% (n = 3). The *in vitro* release profiles of TP from nanoparticles showed a biphasic release phenomenon, namely an initial burst release and then a slow release. The initial burst release resulted in 11.45% of the cumulative amount of TP being released from nanoparticles at the first sampling time of 0.5 h. In contrast, about 22.35% of the cumulative amount of TP was released from nanoparticles within 48 h during the constant slow release process. The above results are shown for reference (*4*).

3.2. *Body and testis weight, testis index, and biochemical parameters of testes serum*

As shown in Table 1, the results revealed that there were no changes in body weight between each group for 15 days. However, testis weight and the testis index were reduced by administration of different dosage forms and decreased significantly ($P < 0.05$) for free TP of 0.6 mg/kg in comparison to the control group.

Monitoring of body weight, testis weight, and testis index provides an index of the general health of animals and such information may also be important for the

Table 1. Effect of different dosage forms on the body weight and testis weights of rats and testis index (gavage daily for 15 days)

Groups	Body weight before experiments (g)[a]	Body weight after experiments (g)[a]	Testis weight (g)[a]	Testis index (g/g, %)[a,b]
Control (n = 6)	164.6 ± 3.4	207.1 ± 3.3	3.44 ± 0.17	1.67 ± 0.2
Free TP (mg/kg)				
0.2 (n = 6)	164.2 ± 5.1	201.6 ± 5.2	2.51 ± 0.13	1.25 ± 0.2
0.6 (n = 5)	164.7 ± 3.6	198.3 ± 3.9	1.81 ± 0.26[c]	0.91 ± 0.13[c]
TP-PLA-NPs (mg/kg)				
0.2 (n = 6)	164.8 ± 4.6	203.1 ± 2.7	2.38 ± 0.18	1.18 ± 0.14
0.6 (n = 6)	164.2 ± 4.3	202.3 ± 4.1	2.22 ± 0.12	1.1 ± 0.13

[a] Values are mean ± S.D.
[b] Testis index: the percent weight of the testis to body weight after 15 days.
[c] Significant difference from control ($P < 0.05$).

interpretation of reproductive health (*22*). The results obtained revealed that TP might be responsibility for the rats' health. This decrease after administration is understandable, though, because TP might greatly affect the digestive system, causing symptoms such as nausea (*2*). Furthermore, testis weight and testis index of free TP decreased less with free TP of 0.6 mg/kg than with the same dose of TP-PLA-NPs. The explanation might be that TP enveloped into polymer nanoparticles was slowly released (*4*).

As shown in Table 2, ACP activity and fructose content of the treated animals decreased significantly ($P < 0.05$) for free TP of 0.2 mg/kg and TP-PLA-NPs of 0.6 mg/kg and very significantly ($P < 0.01$) for free TP of 0.6 mg/kg in comparison to the control group. Moreover, they decreased markedly ($P < 0.05$) for free TP of 0.6 mg/kg when compared with the same dose of TP-PLA-NPs.

Fructose content has been used as a parameter to evaluate the function of sexual glands because fructose in the seminal plasma serves to induce the glycolytic metabolism of spermatozoa (*23*). The enrichment of ACP in human sperm has been proven to restrain the activity of neutrophils and NK cells and provide better immunosuppression to avoid the spermatozoa excluded in the vagina of females (*24*). The fructose content and ACP activity of the treated animals decreased significantly for free TP in comparison to the control group. The explanation might be that TP hampered the glycolytic metabolism of spermatozoa and resulted in abnormal sperm function, ultimately giving rise to complete male sterility. Moreover, fructose content and ACP activity decreased markedly for free TP of 0.6 mg/kg when compared with the same dose of TP-PLA-NPs. The results might be because TP was slowly released from polymer nanoparticles matrix and polymer nanoparticles helped to prevent the testes from being harmed by TP. These changes in parameters were well supported by histopathological observations.

Recent studies have shown that long-term administration of TP (over a period of 70 days or more) can result in male rat infertility due to glutathione depletion and a decrease in microsomal epoxide hydrolase activity (*7*). Moreover, glutathione conjugation and microsomal epoxide hydrolase can detoxify the reactive metabolites present in the chemical constituents of triptolide, such as glycosides, diterpenoids, and epoxide (*11*,*25*,*26*). Glutathione transferases also play an important role in sperm motility and epididymal sperm counts (*27*,*28*), which should be further investigated in subsequent research.

Table 2. Biochemical parameters of testis serum of treated and control rats (gavage daily for 15 days)

Groups	ACP (U/g protein)[a,b]	Fructose (mg/g of testis)[a]
Control ($n = 6$)	350 ± 12	0.32 ± 0.03
Free TP (mg/kg)		
0.2 ($n = 6$)	150 ± 11[c]	0.19 ± 0.02[c]
0.6 ($n = 5$)	80 ± 13[d,e]	0.09 ± 0.01[d,e]
TP-PLA-NPs (mg/kg)		
0.2 ($n = 6$)	190 ± 12	0.23 ± 0.02
0.6 ($n = 6$)	170 ± 14[c]	0.2 ± 0.02[c]

[a] Values are mean ± S.D.
[b] Enzyme activity in Table 2 is expressed in U/g protein where one unit is defined as μmoles of *p*-nitrophenol released per min and per gram protein under specified assay conditions.
[c] Significant difference from control ($P < 0.05$).
[d] Very significant difference from control ($P < 0.01$).
[e] Significant difference from the same dose of free TP-PLA-NPs ($P < 0.05$).

3.3. *Histopathology*

Seminiferous tubules, spermatogenic cells, and Sertoli cells appeared to be normal in the control rats. Multi-nucleation was seen in some tubules. Scattered Leydig cells and blood vessels were found in the interstitial connective tissue between the tubules. Meanwhile, the seminiferous tubules appeared uniform in size and shape (Figure 2A).

As shown in Figure 2, histopathological changes in rat testes occurred as a result of oral administration of different dosage forms after 15 days. Obvious pathological changes were not observed in seminiferous tubules and interstitial tissue at a dose of 0.2 mg/kg free TP and TP-PLA-NPs at the end of 15 days (Figure 2B and 2C). However, spermatogenic cells in some seminiferous tubules of testes decreased slightly in comparison to normal testis tissue.

At a dose of 0.6 mg/kg TP-PLA-NPs, the testes showed slight shrinkage of tubules particularly in the central region, while the number of spermatogenic cells was reduced in some seminiferous tubules. Some tubules appeared to have moderate degeneration (Figure 2D). At a dose of 0.6 mg/kg free TP, fewer spermatid and secondary spermatocytes were observed in some of the seminiferous tubules. Some tubules appeared to have complete degeneration. Meanwhile, necrosis was observed in some seminiferous tubules and edema was observed in interstitial tissue. Large multinuclear cells were found in the lumen of some testes (Figure 2E). During the experiment, one rat died in the group given 0.6 mg/kg free TP.

TP affected the normal configuration of testis tissue; at a high dose of free TP, TP severely harmed the testes, causing fewer spermatid and secondary spermatocytes, degeneration, necrosis, and large multinuclear cells (Figure 2E). Interestingly, histopathological observation revealed that TP-PLA-NPs greatly deceased the damage to rat testes by TP when compared to the same dose of free TP (Figure 2D and 2E).

In conclusion, the results obtained indicated that TP-PLA-NPs can decrease the testis toxicity caused by TP because TP is slowly released from the polymer nanoparticle matrix. Further work is required to investigate the mechanism of decreased toxicity and tissue distribution of TP-PLA-NPs in male rats.

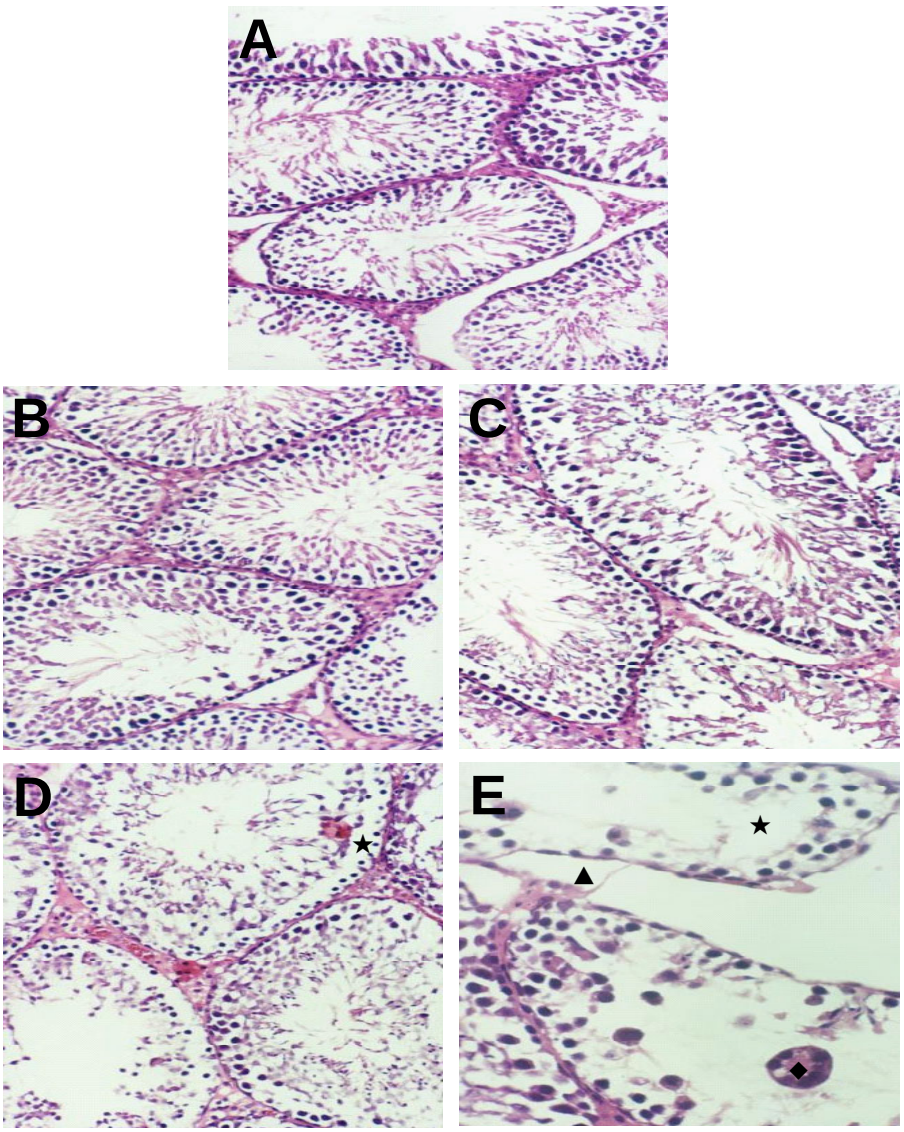

Figure 2. Histopathological changes in the testicle tissue after administration of triptolide and its nanoparticles (gavage daily for 15 days). (A) Normal pattern for control rats. (B and C) Only a slight decrease in spermatogenic cells at a dose of TP-PLA-NPs and TP (0.2 mg/kg). (D) Moderate degeneration, slight necrosis (★), and a decrease in spermatogenic cells at a dose of 0.6 mg/kg TP-PLA-NPs. (E) Severe pathological changes with complete degeneration, necrosis (★) in seminiferous tubules, edema (▲) in interstitial tissue, and appearance of large multinuclear cells (◆) at a dose of 0.6 mg/kg free TP. Magnifications: (A) ×200, (B) ×200, (C) ×200, (D) ×200, (E) ×400.

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Original Article

Questionnaire on the awareness of generic drugs among outpatients and medical staff

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ABSTRACT: Generic drugs are not as widely used in Japan as they are in the West. The objective of this study was to survey the awareness of generic drugs among outpatients and medical staff and propose methods of promoting the use of generic drugs. Our survey showed that 86.7% of respondents were aware of generic drugs. This is a higher awareness rate than that in a survey of other groups conducted last year. One reason to explain this higher awareness is the recent increase in generic drug advertisements both in newspapers and on television. However, a point of note is that generic drug usage has not increased.

Our survey also showed that generic drug awareness was differed widely among age groups, as younger respondents were much more aware of generic drugs than older respondents. Still, about 40% of respondents who were aware of generic drugs did not realize that they were less expensive than name-brand drugs – including 30% of medical staff.

In addition to continuing advertisement of generic drugs in the media, medical doctors and pharmacists should also be encouraged to endorse the use of generic drugs. Furthermore a new system allowing for substitution prescriptions started in April 2008 and consequently pharmacists can now play an important role in promoting the use of generic drugs.

Keywords: Generic drugs, Awareness, Questionnaire, Prescription substitution

1. Introduction

With Japan's rapidly aging society, medical expenditures reached a record level of some 32 trillion yen for the 2005 fiscal year (*1*) and estimates are that this cost will climb to as much as 48 trillion yen by 2025 and continue to grow beyond that date (*2*). Generic drugs are thus seen as one way to reduce medical expenditures (*3*). For example, since 2003 the Diagnosis Procedure Combination (DPC) has been used in 82 key hospitals. Furthermore, the prescription system was revised in April 2006 so that physicians could designate if a prescribed medication could be substituted with generic drugs and dispensed at pharmacies. However, a study of 126 pharmacies around Japan by the Society of Japanese Pharmacists conducted from April – May 2006 showed that while 18% of prescriptions could have been substituted with generic drugs, less than 2% actually had been (*4*). Similarly, in October and November 2006 only 2.2% of prescriptions had been substituted with generic drugs at some 617 pharmacies around Japan. The Ministry of Health, Labor, and Welfare (MHLW) also noted that on average about 17% of prescriptions could have been substituted with generic drugs at 635 pharmacies in October 2006 (*5*). All previous surveys showed that less than 10% of all prescriptions had actually been substituted with generic drugs (*4-7*). Thus, despite efforts to promote the use of generic drugs, they still are not being used as widely in Japan as they are in the West (*8*).

A questionnaire on generic drugs was distributed to survey awareness among outpatients and medical staff. This was used to identify several key reasons why generic drugs were not widely used in Japan and subsequently make recommendations on how to improve this situation.

2. Materials and Methods

Between the period of August and December 2007, a questionnaire was distributed both to outpatients, aged 16 years or older, who had received a prescription for one or more drugs, and to medical staff at pharmacies and hospitals in the Kanto area of Japan to determine their awareness of generic drugs. The completed survey was either collected on the same day it was completed or on the next visit. Pharmacies were selected from the Ain Pharmacies Group and were located in Omiya, Kawasaki, Ueno, Yugawara, and Isezaki. Medical

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staff surveyed were from Tokyo University Hospital, Hanzomon Hospital, Fukagawatachikawa Hospital, Tajima Hospital, Doai Memorial Hospital, and Minamiyamato Hospital – all of which were affiliated with this university hospital. Medical staff surveyed were doctors, nurses, clinical laboratory technicians, radiological technicians, and medical clerks.

3. Results

In total, 614 questionnaires were distributed to outpatients and medical staff and a total of 514 responses were received, indicating a response rate of 83.7%. Of the 514 respondents, 229 were men (44.6%) and 285 were women (55.4%); 12 were teens (over 16 years old) (2.3%), 99 were in their twenties (19.3%), 101 were in their thirties (19.6%), 71 were in their forties (13.8%), 109 were in their fifties (21.2%), 72 were in their sixties (14.0%), and 50 were over seventy (9.7%). In total, 457 respondents were outpatients (74.4%) and 175 respondents were medical staff (25.6%).

A total of 51.3% of respondents thought medicines were expensive. Answers for outpatients and medical staff were similar, but answers among age groups differed widely – the older the respondents were, the more they tended to answer that medicines were expensive. Interestingly the exception was those over seventy (38.8% of those in their twenties and 77.1% in their sixties answered that medicines were expensive, but only 26.0% of those over seventy thought so) (Figure 1).

Although 86.7% of respondents indicated that they knew about generic drugs, only 17.0% had used them. A higher percentage of medical staff (96.9%) knew about generic drugs than outpatients (82.2%). In other words, 3.1% of medical staff did not know about generic drugs. Many more medical staff (26.9%) had used generic drugs than outpatients (11.3%). The awareness rate for generic drugs was almost parallel to the age groups – that is to say, the younger the age group, the higher the awareness rate. (The awareness rate for those over seventy was 64.0%, while the rate for those in their twenties was 96.0%). Younger age groups tended to have used generic drugs, except for teens (20.2% of those in their twenties and 12.0% of those over seventy had used generic drugs. No teens had used generic drugs) (Figure 2).

A total of 55.3% of respondents indicated that they did not know how inexpensive generic drugs were compared to name-brand drugs (Figure 3). Since 82.2% of respondents knew about generic drugs (Figure 1), about 40% of those who knew about generic drugs were not aware of the price difference between generic drugs and name-brand drugs. The number of outpatients who were unaware of the price difference (65.4%) was double that of medical staff (32.2%). Older age groups tended to answer that they did not know the price difference between generic drugs and name-brand drugs, although this also included teens (41.8% of those in their twenties and 69.4% of those over seventy indicated that they did not know how inexpensive generic drugs were compared to name-brand drugs) (Figure 3).

A total of 61.6% of respondents stated that they would prefer to substitute generic drugs if they were less expensive, 28.4% said they did not know if they wanted to substitute, and only 10.0% of respondents said they did not want to substitute generic drugs regardless of the price difference. A higher percentage of medical staff (65.6%) than outpatients (59.9%) said they would prefer to substitute generic drugs. The preference for substituting generic drugs decreased with age groups

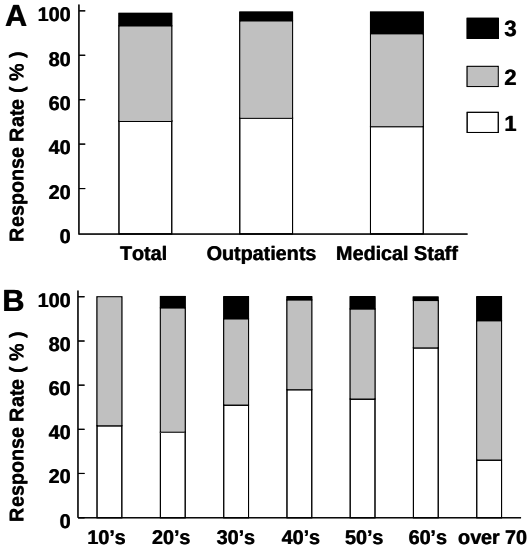

Figure 1. What do you think about the price of medicines? A: Result according to outpatients and medical staff. B: Result according to generations. 1: expensive. 2: dubious (neither expensive nor cheap). 3: cheap.

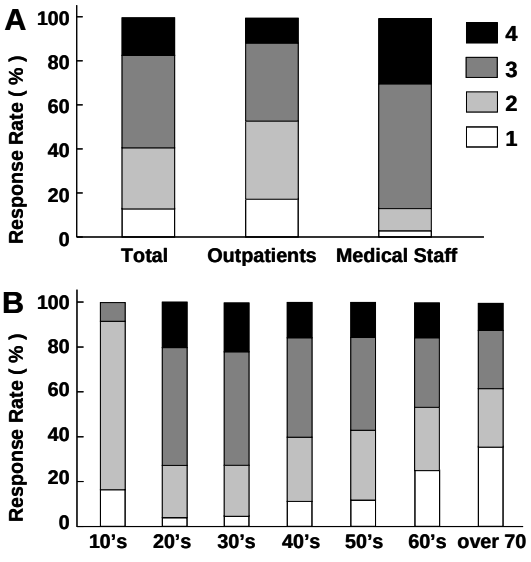

Figure 2. Do you know about generic drugs? A: Result according to outpatients and medical staff. B: Result according to generations. 1: I do not know. 2: I know the name, but do not know fully. 3: I know, but do not take generic drugs. 4: I know and take generic drugs.

except for teens (72.2% of those in their twenties and 36.6% of those over seventy preferred to substitute generic drugs). The number of respondents who were undecided increased in parallel to age groups, with teens again being the exception (22.7% in their twenties and 55.6% seventy and over answered that they did not know if they wanted to substitute) (Figure 4).

The primary reasons why outpatients did not use generic drugs were simply because their doctor did not substitute generic drugs and patients did not know about them. Concern over the effects of generic drugs was a third reason. Medical staff expressed more concern about the effect and quality of generic drugs and exhibited more anxiety about generic manufacturers than outpatients (Figure 5). Still, only 32.8% of respondents were concerned about generic drugs.

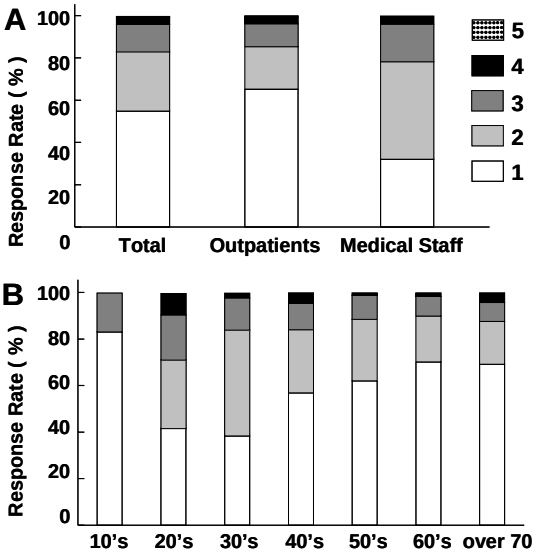

Figure 3. What do you think about the price difference between generic drugs and brand drugs? A: Result according to outpatients and medical staff. B: Result according to generations. 1: I do not know. 2: I think that generic drugs are more than 20% cheaper than brand drugs. 3: I think that generic drugs are about 20% cheaper than brand drugs. 4: I think that generic drugs are about 10% cheaper than brand drugs. 5: I think that both generic drugs and brand drugs are similar price.

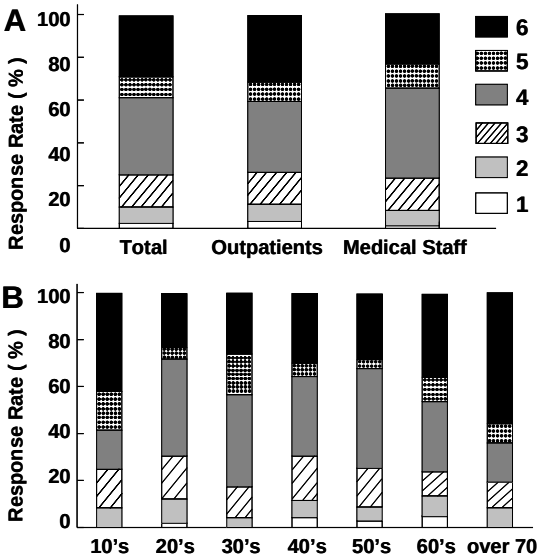

Figure 4. Are you going to take generic drugs? A: Result according to outpatients and medical staff. B: Result according to generations. 1: I am going to take generic drugs even if generic drugs and brand drugs are similar price. 2: I am going to take generic drugs if generic drugs are 10% cheaper than brand drugs. 3: I am going to take generic drugs if generic drugs are 20% cheaper than brand drugs. 4: I am going to take generic drugs if generic drugs are 30% cheaper than brand drugs. 5: I am not going to take generic drugs regardless of their prices. 6: I do not know.

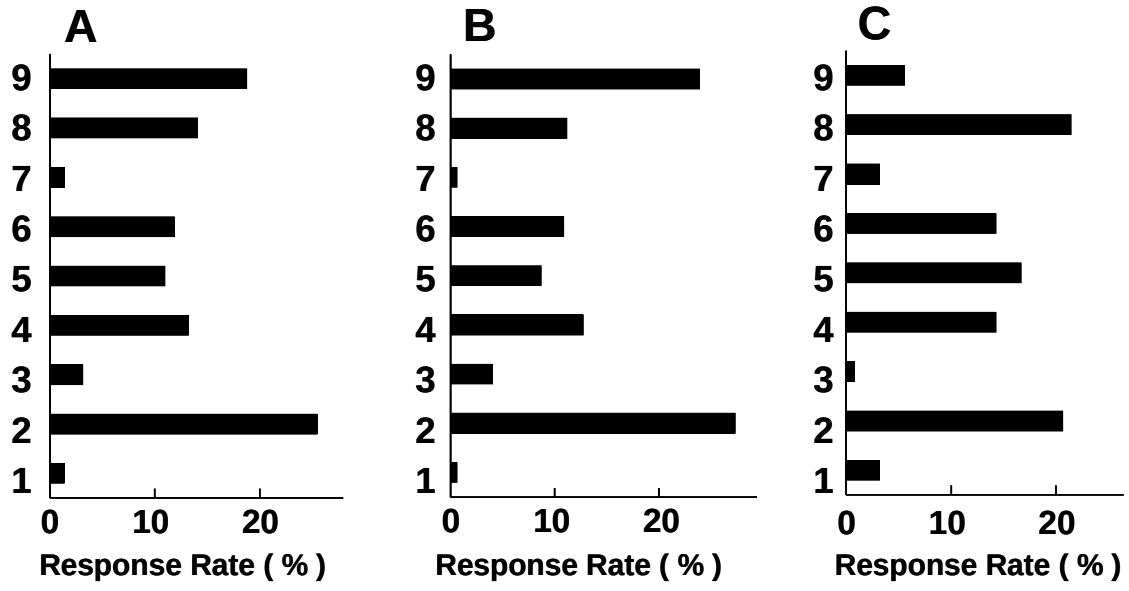

Figure 5. Why do not you take generic drugs? A: Result of total responders, B: Result of outpatients, C: Result of medical staff. 1: Because generic drugs and brand drugs are similar prices. 2: Because doctors do not prescribe. 3: Because pharmacies do not have stock. 4: Because I am concerned about their efficacy. 5: Because I am concerned about their quality. 6: Because I am concerned about generic makers. 7: Because doctors reject my request for a generic drug prescription. 8: none of these. 9: Because I do not know about generic drugs.

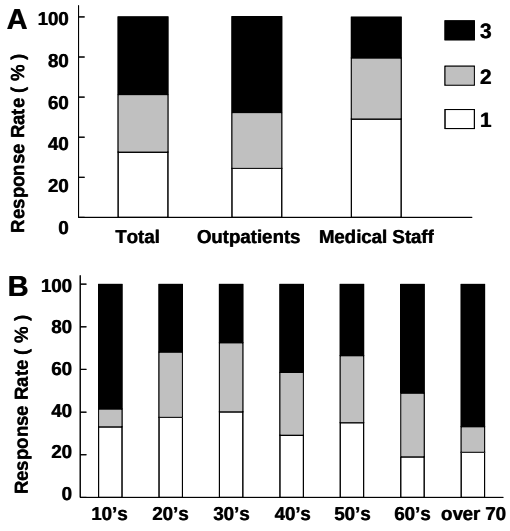

Figure 6. Are you concerned about generic drugs? A: Result according to outpatients and medical staff. B: Result according to generations. 1: Yes, I am. 2: No, I am not. 3: I do not know.

However, of those concerned considerably more were medical staff (49.4% *vs*. 24.7% of outpatients). Younger age groups were more concerned than older age groups (37.8% in their twenties and 21.4% over seventy were concerned about generics). A higher percentage from the older age groups tended to answer that they did not know if they wanted to substitute (31.6% in their twenties and 66.7% over seventy responded that they did not know if they wanted to substitute) (Figure 6).

4. Discussion

While there have been numerous surveys on the awareness of generic drugs, this is to the authors' knowledge the first survey conducted simultaneously with outpatients and medical staff. In the summer of 2006, a survey of some 1,000 consumers conducted by the Fair Trade Commission showed that 78% of consumers were aware of generic drugs, and that more than 90% preferred generic drugs (*9*). However, a 2006 survey of 3,031 outpatients in Miyazaki prefecture showed that 49.4% of outpatients knew about generic drugs (*6*). These differing results in the awareness of generic drugs may be a result of differences in the nuance of the surveys but are more likely a result of the difference in age groups. Generally, the average age of consumers tends to be much younger than outpatients, and the current survey showed that there were considerable differences in awareness of generic drugs between younger and older age groups (Figure 2). Accordingly, the current results should be compared with those from the survey among outpatients in Miyazaki Prefecture, even though the geographical locations of the surveys differ. The awareness rate for generic drugs among outpatients (82.2%) in a 2007 survey (Figure 2) was much higher than in the 2006 survey by Sasaki (49.4%). This would indicate that

Table 1. Share of generic drugs in Western countries and in Japan

Fiscal year	America	Germany	England	France	Japan
2002 (%)	52.0	50.0	52.0	12.0	12.0
2005 (%)	56.0	57.3	59.3	15.7	17.1

• Share is calculated based on the amount of quantity;
• Data are cited from references *8*, *11*, and *12*.

awareness of generic drugs is increasing each year, with the primary reason for this being the impact of recent advertisements both in newspapers and on television. Tanaka *et al*. noted that awareness of generic drugs among older age groups tends to be lower than among younger age groups (*10*), and the current survey supports that conclusion. The awareness rate for those in their twenties was 96.0%, whereas it was 64.0% for those over seventy (Figure 2). Thus, the necessity seems to be to focus on older age groups in order to encourage use of generic drugs. As simply running newspaper and television advertisements is unlikely to achieve this, doctors and pharmacists should recommend generic drugs in their consultations, and generic drug posters should be displayed in hospitals and pharmacies and pamphlets should be distributed to outpatients. The current survey showed that outpatients received information about their prescribed drugs from the Internet or drug manuals, but they actually wanted to direct counseling about their prescriptions from either their doctor or pharmacist (data not shown).

The use of generic drugs is being promoted in order to limit sharp increases in medical expenses. For example, the DPC was implemented in 2003 and a new prescription system was adopted in April 2006 so that physicians could indicate if the prescribed medication could be substituted with generic drugs. Still, the share of the generic drug market in Japan is low when compared to the West, with France being the exception (Table 1). For example, the use of generic drugs in the US has now reached over 50%, while the generic market in Japan was 16.4% in 2003, 17.1% in 2005, and 16.9% in 2006 (*11*). While Japan has adopted many of the systems used in the US with some differences, the share of generic drugs has not increased. This suggests that the promotion of the awareness of generic drugs does not necessarily result in promotion of the use of generic drugs. The results of the current survey indicate that while the total awareness of generic drugs has increased, there is still a considerable difference in awareness between younger and older age groups. Specifically the rate of awareness among people over seventy (who account for the majority of medical expenses) is still low (Figure 2). Furthermore, 74.0% of people over seventy did not think that medicines were expensive (Figure 1) and had very little knowledge or understanding of generic drugs. In fact, the current survey showed that 55.3% of respondents did not

know how inexpensive generic drugs were compared to name-brand drugs (Figure 3). Thus, individual and direct promotion of generic drugs as described above is necessary. Many more medical staff (49.4%) expressed concern about generic drugs than outpatients (27.4%) (Figure 6), so simply describing the cost benefits of generic drugs is insufficient. Patients should also be informed about the safety and efficacy of generic drugs.

The prescription substitution rate can be calculated using the following formula:

Prescription substitution rate = *[Non-hospital prescription rate] × [Rate of physician designation] × [Preference rate among outpatients] × [Preference rate among pharmacists] × (1 - [Off label rate])
(*A non-hospital prescription means a prescription that is dispensed at a community pharmacy and not a hospital pharmacy)

In 2007, the average non-hospital prescription rate was about 50% in Japan (*7*). Using Nikkei Drug information, the rate of physician designation was 31% in April 2006. The new prescription system that was implemented in April 2008 required physicians to indicate if the prescribed medicine could not be substituted with a generic. This means that if there is no designation from the physician, all prescriptions can automatically be substituted with generic drugs. The current survey showed that although more medical staff had concerns about generic drugs than outpatients (Figure 6), more medical staff (65.5%) preferred to use the less expensive generic drugs than outpatients (59.9%) (Figure 4). Actually, all eleven medical doctors working in hospitals who participated in this survey answered that they would themselves use generic drugs if prescribed (data not shown). Thus, the number of prescription drugs that can be substituted with generic drugs could increase dramatically after April 2008. While many generic drugs have off-label problems, generic drugs that are frequently used do not have such issues (*7*). This survey demonstrated that outpatients did not use generic drugs primarily because they did not know about them and because their physician did not prescribe generic drugs (Figure 5). As mentioned above, the new prescription system started in April 2008, so the rate of physician designation should no longer be a limiting factor. Further, this survey showed that 59.9% of outpatients preferred to substitute generic drugs if they were less expensive (Figure 4). Thus, the preference rate among outpatients should not be a limiting factor either. A final key factor limiting the use of generic drugs is likely to be the preference rate among pharmacists. Muto showed that the preference rate for generic drugs among medical doctors was 68%, which is very similar to the current data (65.6%, Figure 4), while the preference rate among pharmacists was only 25% (*7*). The reason for the lower preference rate among pharmacists is primarily an issue of responsibility. That is to say, if trouble occurs after a prescription has been substituted with a generic drug, then the question of who should be responsible – the physician or the pharmacist – is unclear (*13*).

The MHLW has adopted many systems for a stable supply of generic drugs to ensure the quality of generic drugs and it changed the prescription system format in order to encourage the use of generic drugs. The MHLW has stated that the share of generic drugs should increase to more than 30% by 2012. However, the share of generic drugs remains at around 16% and is not increasing (*12*). In the US, the share of generic drugs has increased to over 50% (Table 1), but this is not because physicians prefer generic drugs. Actually, over 80% of physicians prescribe name-brand drugs (*14*). However, prescription substitution laws in states such as Massachusetts require pharmacists to use generic drugs. And, for example, under California legislation, pharmacists are not responsible for side effects of generic drugs (*11*). As a result, generic drugs are much more widely used in the US. In Japan, the preference rate among pharmacists is likely to be a limiting factor and consequently legislation similar to that in the US should be implemented to motivate pharmacists to use more generic drugs.

Research has also suggested that information about generic drugs from generic manufacturers is inadequate (*15*). The MHLW also requires generic manufacturers to provide adequate information to medical sites (*16*). Generally, however, generic manufacturers are smaller in scale than name-brand manufacturers and as such may be unable to provide the same amount of information. In the US, the Food and Drug Administration (FDA) guarantees the efficacy and safety of generic drugs. As a result, generic manufacturers do not actively collect efficacy and safety-related data (*17,18*). In Japan, the MHLW should also establish a system to guarantee the efficacy and safety of generic drugs. As a consequence, the share of generic drugs may indeed exceed 30% by 2012.

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