FTY720P inhibits hepatic Na⁺/K⁺ ATPase via S1PR2 and PGE2

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FTY720P inhibits hepatic Na⁺/K⁺ ATPase via S1PR2 and PGE2

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ABSTRACT

Sphingosine-1-phosphate (S1P) was found previously to inhibit the Na^+/K^+ ATPase in HepG2 cells. Whether fingolimod (FTY720), a S1P receptor “(S1PR)” agonist, similarly inhibits the ATPase is a question that needs to be addressed. The aim of this work was to study the effect of FTY720P, the active form of the drug, on the activity of the Na^+/K^+ ATPase in HepG2 cells and elucidate its mechanism of action. The activity of the ATPase was assayed by measuring the amount of inorganic phosphate liberated in presence and absence of ouabain. FTY720-P (7.5 nM, 15min) reduced significantly the activity of the enzyme. This effect disappeared completely in presence of JTE-013, a specific blocker of sphingosine-1-phosphate receptor 2 (S1PR2) as well as in presence of calphostin and indomethacin, respective inhibitors of Protein kinase C (PKC) and COX-2. The effect of FTY720P was mimicked by prostaglandin E2 (PGE2) and PMA but abrogated by NF- κ B inhibition. When NF- κ B was inhibited, the effect of exogenous PGE2 still appeared but that of PMA was not manifested suggesting that NF- κ B is upstream of PGE2 and downstream of PKC. It was concluded that FTY720P activates via S1PR2, PKC and NF- κ B. The latter induces PGE2 generation and the Na^+/K^+ ATPase inhibition.

INTRODUCTION

We demonstrated previously a decrease in the activity of the Na^+/K^+ ATPase in HepG2 cells [Dakroub et al. 2012] treated with S1P for 15 min. The Na^+/K^+ ATPase, also known as Na^+/K^+ pump, plays a key role in the control of the ionic intracellular milieu, a process that is needed for the regulation of metabolism, proliferation, differentiation and most importantly cell volume. It establishes the Na^+ gradient needed for the transport of bile salts [Erlinger 1982] and drives the activity of many other transporters like the Na^+/H^+ exchanger and the $\text{Na}^+ - \text{K}^+ 2\text{Cl}^-$ cotransporter involved, respectively, in the regulation of intracellular pH and cell volume. Changes in hepatocyte cell volume are associated with changes in bile flow and bile acid transport, cytoplasmic and endosomal pH, metabolism of carbohydrates, proteins and lipids, transcription and translation, and alteration of cytoskeleton components [Graf and Häussinger 1996].

FTY720, an agonist of sphingosine-1-phosphate receptor (S1PR), has been approved recently by the US food and drug administration for the treatment of multiple sclerosis [Brinkman 2010]. FTY720 is phosphorylated *in vivo* by the enzyme sphingosine kinase 2 present abundantly in the liver, and changed into its biologically active form, which acts by binding to S1P receptors also known as “Endothelial Differentiation Gene” receptors (EDG receptors) [Billich 2003].

Five receptor subtypes have been identified (S1PR1-S1PR5). They display tissue specific expressions [Chalfant and Spiegel 2005; Cowart 2008; Spiegel and Milstien 2003; Spiegel and Milstien 2002] and are all coupled to G proteins. S1PR2 (EDG5) and

SIPR3 (EDG3) couple to Gi/o, Gq, and G12/13; SIPR4 (EDG6) and SIPR 5 (EDG8) couple to Gi/o and G12/13; SIPR1 couples to Gi/o only.

Being a S1PR agonist, we predict that the active form of FTY720, FTY720P, will exert a similar inhibitory effect to that of S1P on hepatic Na^+/K^+ ATPase leading to alterations in liver functions. Although various studies identified some side effects of the drug, like nausea, diarrhea, and bradycardia, none investigated the effect of FTY720 on the Na^+/K^+ ATPase in the liver or in any other organ.

The aim of this work was to determine the effect of FTY720P, the active form of the drug, on the Na^+/K^+ ATPase in HepG2 cells, a human hepatocellular carcinoma cell line, and to delineate its signaling pathway. Elucidating the mode of action of the drug will help in circumventing and alleviating any undesirable side effects if present, by inhibiting specifically the mediators involved.

MATERIALS AND METHODS

Materials

FTY720P, the NF κ B inhibitor NF- κ B SN50, the primary antibodies anti-EDG-5, anti-GAPDH were purchased from Santa Cruz Biotechnology, CA, USA. Anti-I κ B α mouse monoclonal antibody was obtained from R&D Systems, Minneapolis, MN, USA. JTE-013 and CAY10444 were purchased from Cayman Chemical, MI, USA. Phorbol-12-myristate-13-acetate (PMA) and calphostin C were acquired from Calbiochem, San Diego, USA. All other chemicals and culture media used were purchased from Sigma, Chemical Co, St Louis Missouri, USA.

101 The human liver carcinoma cell line, HepG2, was purchased from American Type
102 Culture Collection (ATCC).

103 ***Culture of HepG2 cells***

104 HepG2 cells at passages 25-45 were grown in DMEM supplemented with 1%
105 penicillin (100µg/ml) and streptomycin (100 µg/ml) and 20% FBS at a density of
106 120,000 cells/ml . The cells were kept in a humidified incubator (95% O₂, 5% CO₂) at
107 37°C and treated at 85-90% confluence after an overnight starvation.

109 ***Dose response study on the effect of FTY720P on the activity of the Na⁺/K⁺ ATPase***

110 Following an overnight starvation, HepG2 cells were treated for 15 min with
111 different doses of FTY720P ranging from 0.0075 nM to 150 nM. An equal volume of the
112 carrier, DMSO, was added to the control. The cells were then washed, scraped, and
113 homogenized in a polytron at 20,000-22,000 rpm. Proteins in the resulting cell
114 homogenate were quantified using the Bradford assay. Samples containing equal amount
115 of proteins were then assayed for the Na⁺/K⁺ ATPase activity as described below.

116 ***Determination of the S1P receptor activated by FTY720P***

117 In order to determine the S1P receptor subtype activated by FTY720P, S1PR2 and
118 S1PR3 were blocked respectively with their specific antagonists JTE-013 [Ohmori 2003]
119 (1µM in DMSO) and CAY10444 [Koide 2002] (17.4 µM in DMF). JTE-013 was
120 reported in the literature to abrogate fully the effects of S1P when used at a concentration
121 of 10⁻⁶ M [Arikawa 2003; Liu 2014] while for CAY 10444, also known as BML-241, the
122 concentrations varied between 10 and 50 micromolar [Lichte 2008; Long ; 2010]. We

therefore chose an intermediate concentration (17.4 μ M) which led to full block of the effect of S1P.

The cells were incubated at 37°C with the blockers for 15 min, and then treated with FTY720P for an additional 15 min, after which they were scraped, lysed in lysis buffer (NaCl: 150 mM; Tris-HCl, pH 7.5: 50 mM; nonidet P-40: 0.01%; sodium deoxycholate: 12mM), and assayed for their Na^+/K^+ ATPase activity.

The Na^+/K^+ ATPase Activity Assay

Na^+/K^+ ATPase activity was assayed as described by Esmann [Esmann 1988]. Cell homogenates were diluted to a concentration of 0.5 μ g/ μ l in histidine buffer (150mM, pH 7.4). Saponin (1%) was then added at a ratio of 1:4(v/v) and the samples were incubated at room temperature for 30 min. Aliquots were then drawn and incubated at 37°C for an additional 30 min in histidine buffer containing NaCl (121.5mM), KCl (19.6 mM), MgCl_2 (3.92 mM), adenosine tri-phosphate (2.94 mM), in presence or absence of ouabain (1.47 mM), a specific inhibitor of the ATPase. At the end of the incubation period, 50% trichloroacetic acid was added at a ratio of 1:10 (v)/v to stop the reaction, and the samples were spun at 3000g for 5 min. The amount of inorganic phosphate liberated in the supernatant was measured colorimetrically at 750 nM according to the method of Taussky and Shorr [1953].

Determination of the mediators involved

S1PR2 couples to Gi , G12/13 and Gq . Gq activates PLC and eventually PKC. To check for any role of PKC in the signaling pathway, the effect of FTY720P on the pump was

146 studied in presence of a specific PKC inhibitor, calphostin C (50nM in DMSO) which
147 was added 15 min prior to FTY720P. The effect of a PKC activator PMA (100nM in
148 DMSO, 15 min) on the ATPase was also studied for further confirmation.

149 Since activation of S1PR2 was reported to induce PGE2 production [30], the
150 involvement of PGE2 was also studied by treating the cells 15 min prior to FTY720P
151 with indomethacin (100μM, DMSO), a COX inhibitor or with exogenous PGE2 (1nM, 15
152 min). At the end of each of these treatments, the cells were collected, homogenized and
153 assayed for their Na⁺/K⁺ ATPase activity as described above.

154

155 NF-κB is known to be involved in COX-2 expression and PGE2 release. The
156 transcription factor NF-κB is usually sequestered in an inactive form in the cytoplasm
157 through its interaction with the inhibitory protein IκB which masks the nuclear
158 localization signals. It is only when IκB is degraded that NF-κB is freed and able to
159 translocate to the nucleus and induce the transcription of target genes. To study the
160 involvement of NF-κB in the effect of FTY720P on the Na⁺/K⁺ ATPase, cells were
161 treated, before FTY720P (7.5 nM, 15 min) with NF-κB SN50 (10μg/ml, 15 min), a cell
162 permeable inhibitory peptide that inhibits translocation of the active NFκB complex into
163 the nucleus and blocks the transcription of NF-κB- dependent genes. Changes in the
164 protein expression of IκB were also determined by western blot analysis.

165 Activation of Gq leads to activation of PLC and release of calcium in the cytosol
166 via IP3. To determine any potential role of calcium in the effect of FTY720P on the
167 pump, HepG2 cells were pre-treated 15 min before FTY 720P, with BAPTA-AM, a

calcium chelator, (20nM in DMSO). An equivalent volume of the vehicle (DMSO) was added to the control.

Locating the involved mediators with respect to each other in the signaling pathway

To locate PKC, and NF- κ B with respect to PGE₂, HepG2 cells were treated respectively with PMA in presence of indomethacin, an inhibitor of PGE₂ synthesis, or with PGE₂ in presence of NF- κ B inhibitor.

Western Blot Analysis

The presence of S1PR2 in HepG2 cells was studied by western blot as well as the involvement of NF- κ B in the signaling pathway. Equal amounts of protein (40 μ g) were resolved on 10% SDS polyacrylamide gels, and then transferred to nitrocellulose membranes which were then blocked at room temperature for 40 min in PBS buffer containing 3% skim milk and incubated overnight at 4°C with a mouse anti-I κ B primary antibody or with a rabbit primary anti-EDG5 (anti-S1PR2) at 1:500 dilutions. The membranes were then washed and incubated for 2 hours at room temperature with a goat anti-mouse (I κ B) or goat anti-rabbit (S1PR2) secondary antibody conjugated with horseradish peroxidase. The signal was detected by chemiluminescence using luminol reagent. In the case of I κ B, equal loading was validated by checking, after stripping the membrane, for GAPDH expression. Checking for equal loading in the case of the receptors was not necessary, since the aim of the western was only to demonstrate the presence of the receptor protein.

191 *Statistical Analysis*

192 Results are reported as means $\pm$ SEM and tested for statistical significance by a
193 one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple
194 comparisons test using Instat and Excel Analysis softwares.

196 **RESULTS**

198 *FTY720P causes dose-dependent inhibition of the Na⁺/K⁺ ATPase in HepG2 cells by* 199 *activating S1PR2*

200 HepG2 cells treated with different doses of FTY720P for 15 min, exhibited a
201 significant decrease in the activity of the Na⁺/K⁺ ATPase at concentrations of 7.5 and
202 15nM by around 48% and 42% respectively (Figure 1). The drug did not affect the
203 activity of the ATPase at higher and lower doses. As the highest inhibitory effect was
204 observed at 7.5nM, this dose was adopted in all subsequent experiments.

205 In order to identify the receptor subtype(s) mediating the effects of FTY720P,
206 various receptor blockers were used. The inhibitory effect of FTY720P disappeared
207 completely in the presence of JTE-013, a selective S1PR2 antagonist (Fig 2A), but was
208 maintained in presence of CAY10444, a S1PR3 blocker (Fig 2B). JTE-013 alone did not
209 have any effect on the ATPase, while CAY10444 alone mimicked the effect of FTY720P
210 and reduced the activity of the pump. However, this inhibitory effect disappeared when
211 the cells were pre-treated with JTE-013 (Fig 2C).

212 The expression of S1PR2 in HepG2 cells was confirmed by western blot analysis (Fig
213 2D). The receptor consists of 353 amino acids and has a predicted molecular weight of
214 39 KDa. However the molecular weight of the bands observed was 40-46 kDa.

215 ***FTY720P activates PKC but does not alter intracellular calcium levels***

216 Calphostin, a PKC inhibitor, abolished fully the inhibitory effect of FTY720P on
217 the Na^+/K^+ ATPase (Fig 3A), while phorbol 12-myristate 13-acetate (PMA), a PKC
218 activator, imitated the effect of the drug (Fig 3B). The data confirm thus a role of PKC in
219 the signaling pathway.

220 Since S1PR2 is coupled to Gq which activates PLC leading to IP3 production, a
221 role of calcium in FTY720P signaling was predicted. To study the involvement of
222 calcium in the effect of the drug on the Na^+/K^+ ATPase, the effect of FTY720P was
223 studied in cells treated with the calcium chelator, BAPTA-AM. The chelator did not alter
224 the inhibitory effect of FTY720P (Fig 3C), inferring that calcium is not involved in the
225 effect of the drug on the ATPase.

226 ***FTY720P induces PGE2 production and activates NF- κ B***

227 The effect of FTY720P on the Na^+/K^+ ATPase was abrogated by indomethacin, a
228 COX inhibitor, (Fig 4A), and mimicked by exogenous PGE2 (Fig 4B). These results
229 indicate an involvement of PGE2 in the effect of FTY720P on the Na^+/K^+ ATPase.

230 The effect of FTY720P was not manifested (Fig 5A) when the cells were pre-
231 treated with the NF- κ B inhibitor NF- κ B SN50, a cell permeable inhibitory peptide that
232 blocks the translocation of the NF κ B active complex into the nucleus. Moreover, western

blot analysis revealed a decrease in the protein expression level of I κ B following FTY720P treatment (Fig 5B), supporting further the involvement of NF- κ B in this signaling pathway.

NF- κ B and PGE2 are downstream of PKC

The inhibitory effect of PMA did not appear in presence of indomethacin (Fig 6A) or when NF- κ B was inhibited with NF- κ B SN50 (Fig 6C) suggesting that both the transcription factor and PGE2 are downstream of PKC. Inhibiting NF- κ B did not alter however, the effect of PGE2 on the pump (Fig 6B) inferring that NF- κ B is upstream of PGE2.

DISCUSSION

This study revealed that the inhibitory effect of a SIP2R agonist, FTY720P, on the Na⁺/K⁺ pump was dose-dependent, appearing at doses ranging between 7.5 nM and 15 nM (Fig 1). The absence of an effect at higher and lower doses may be attributed to different affinities of the drug to the different types of SIP receptors. Low doses were probably too low to activate the receptors involved in the effect on the pump, while high doses may have activated more than one receptor subtype which acted through opposite signaling cascades, cancelling thus each other's effects and leading to no change in the activity of the Na⁺/K⁺ ATPase. This hypothesis would need however to be verified by future work.

The presence of S1PR2 in HepG2 cells was confirmed by western blot analysis (Fig 2) using antibodies that, according to the manufacturer, are specific and do not cross react with other proteins.

Since the inhibitory effect of FTY720P was completely abolished by the S1PR2 blocker JTE-013, (Fig 2A), it was concluded that the S1PR2s are the sole ones activated by FTY720P in this signaling pathway. Had other receptors been involved, then JTE-013 would have abolished only partially the effect of FTY720P.

A decrease in the activity of the pump alters K^+ homeostasis, induces anti-proliferative effects [Kaplan 1978], and contributes to cell death and apoptosis [Chen et al. 2014] while an increase has been observed to accompany liver cell proliferation [Martinez-Mas 1995]. Our results provide an explanation to the reported S1PR2-mediated anti-proliferative effect of S1P in rat hepatocytes [Ikeda 2003; Ikeda 2009] and to the counteracting effect of S1PR2 activation on the proliferation induced by the platelet derived growth factor (PDGF) in embryonic fibroblasts [Goparaju 2005].

Although the effect of FTY720P was not altered by the S1PR3 blocker CAY10444, the blocker alone exerted an inhibitory effect on the ATPase (Fig 2C) that disappeared in presence of the S1PR2 antagonist JTE-013, suggesting that CAY10444 acts on the S1PR2. This observation points to a role of CAY10444 as a S1PR2 agonist at the concentration used in this study (17.4 μ M). Instances of ligands acting as a blocker for one S1P receptor and an agonist for another have been previously reported in the literature. For example, the S1PR2 blocker, JTE-013, was shown to augment the excitability of sensory neurons by activating the S1PR1 [Li 2012]. CAY10444 seems to

277 play an analogous role: it acts as an antagonist for S1PR3 and an agonist for S1PR2. Such
278 a dual role for CAY10444 has not been reported before and warrants further
279 investigation.

280 The S1PR2 is known to be coupled to three different types of G-proteins namely,
281 Gq, Gi, and G12, 13 [Goparaju 2007]. Gq activates the phospholipase C enzyme which
282 hydrolyzes PIP2 into DAG and IP3. DAG activates PKC directly while IP3 activates it
283 indirectly [Rhee 2001], by increasing cytosolic calcium. We investigated the role of Gq,
284 by studying the involvement of PKC in the effect of FTY720P on the pump. The
285 complete disappearance of the effect of FTY720P in presence of calphostin C, (Fig 3A)
286 and the inhibitory effect of PMA on the pump (Fig 3B) provided sufficient evidence for
287 an implication of Gq and PKC in the signaling pathway. Moreover, the complete
288 abolishment of the inhibitory effect of FTY720P by the PKC inhibitor indicates that Gq is
289 the only G protein activated by S1PR2s. Several works reported a PKC-induced decrease
290 in the activity of the Na^+/K^+ ATPase. Dopamine for example was found to exert a PKC-
291 mediated inhibitory effect on the Na^+/K^+ ATPase in MDKC cells that was mimicked by
292 PMA and abrogated by two different PKC inhibitors [Shahedi 1992]. Since BAPTA-AM
293 did not alter the inhibitory effect of FTY720P on the Na^+/K^+ ATPase (Fig 3C), it can be
294 concluded that the signaling pathway is calcium-independent and that the PKC isozyme
295 involved belongs to the group of novel PKCs which can be solely activated by DAG
296 [Bollag 2009].

297 Our results indicate also that PGE2 mediates the inhibitory effect of FTY720P on
298 the pump (Figs 4A& 4B). Such an effect of the prostaglandin has been reported before.
299 For example, the inhibition of the Na^+/K^+ ATPase by endothelin in cells of the inner

300 medullary collecting duct was blocked by ibuprofen, a COX enzyme inhibitor, indicating
301 a role of PGE₂ in this inhibition [Zeidel 1989]. Moreover, indomethacin, another COX
302 enzyme inhibitor, abolished the inhibitory effect of TNF- α on Na⁺/K⁺ ATPase in HepG2
303 cells while exogenous PGE₂ mimicked TNF- α 's action by activating EP₂ receptors
304 [Kreydiyyeh 2007].

305 PGE₂ was also shown to participate in many of the S1P-induced signaling
306 pathways. S1P up-regulated COX-2 expression in rat and human mesangial cells by
307 activating S1PR₂s [Völzke 2014]. It also increased COX-2 expression and PGE₂
308 production in L929 fibroblasts; these effects were abrogated by RNAi against
309 sphingosine kinase 2 and enhanced by RNAi against S1P lyase and S1P phosphatase
310 [Pettus 2003].

311 Since in this work, the S1PR₂ was shown to act solely via Gq, then PKC is
312 expected to be directly downstream of S1PR₂ and upstream of PGE₂. This was verified
313 by the complete disappearance of the inhibitory effect of PMA in the presence of
314 indomethacin (Fig 4A). Many works reported a similar effect of PKC on COX enzymes
315 in various cell types. COX-2 was shown to be activated by PKC in RAW 264.7 cells
316 [Giroux and Descoteaux 2000]), as well as in non-pigmented ciliary epithelial cells
317 [Delamere 1997].

318 The fact that inhibition of NF- κ B abrogated the effect of PMA but not that of
319 PGE₂ suggests that the transcription factor acts upstream of PGE₂ and downstream of
320 PKC. NF- κ B has been reported previously to be involved in the up-regulation of COX-2
321 in many cell types. In human synovial fibroblast cells, the TNF- α – induced increase in

COX-2 expression and PGE2 production disappeared in presence of PDTC, an inhibitor of NF- κ B [Ke 2007]. Similarly, in Swiss 3T3 fibroblasts, the up-regulation of COX-2 enzyme by the Proinsulin C peptide was blocked by specific PKC and NF- κ B inhibitors, indicating that the effect of the peptide is mediated through a PKC/NF- κ B dependent pathway [Kitazawa 2006]. Our results suggest a similar involvement of NF- κ B downstream of PKC (Fig 6A) and upstream of PGE2 (Fig 6B) in the FTY720P signaling pathway. Whether NF- κ B increases directly the protein expression of COX-2 or indirectly by promoting the expression of an upstream intermediate cannot be determined from the current results.

It can be concluded that FTY720P inhibits hepatic Na⁺/K⁺ ATPase only at certain doses by binding to S1PR2s, which are coupled to Gq protein, and activates PKC and NF- κ B. The latter increases the expression of COX-2 and leads to PGE2 secretion. The prostaglandin then exerts an inhibitory effect on the Na⁺/K⁺ ATPase.

The signaling pathway of FTY720P is summarized in Fig 7.

Conflict of interest

The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig 1 Dose response study on the effect of FTY720P on the activity of the Na⁺/K⁺ ATPase. Cells were treated with different doses of FTY720P for 15 min. Values are means ± SEM of 6 observations. Bars not sharing the same letter are considered significantly different from each other at p<0.05.

Fig 2 Determination of the type of S1P receptor involved. Effect of FTY720P (7.5 nM, 15 min) on the pump in presence of: (A,) JTE-013, a blocker of S1PR2 and (B,) CAY10444, a blocker of S1PR3. (C,) CAY10444 mimicked the effect of FTY720P. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at p<0.01. FTY=FTY720P; JTE=JTE-013; CAY= CAY 10444. (D,) S1PR2 is expressed in HepG2 cells (protein loaded: 40µg).

Fig 3 PKC is involved but not calcium in the signaling pathway. Effect of FTY720P (7.5 nM, 15 min) in presence of: (A,) calphostin , a PKC inhibitor; (B,) PMA, a PKC activator, (C,) Bapta-AM, a calcium chelator. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at p<0.01. FTY= FTY720P; cal= calphostin;

Fig 4 Involvement of PGE2 in FTY720P signaling. (A,) Effect of FTY720P (7.5 nM, 15 min) on the activity of the Na⁺/K⁺ ATPase in presence of indomethacin, a COX inhibitor; (B,) Effect of exogenous PGE2 (1nM, 15 min) on pump's activity . Values are means ± SEM of 4 observations. Bars not sharing the same letter are considered significantly different from each other at p<0.01.

Fig 5 FTY720 acts via NF-κB . (A,) Effect of FTY720P (7.5 nM, 15 min) on the ATPase activity in presence of NF-κB inhibitor. Values are means ± SEM of 3 observations. Bars not sharing the same letter are considered significantly different from each other at p<0.01. (B,) Effect of FTY720P (7.5 nM, 15 min) on the protein expression of IκB. The blot is representative of an experiment repeated 3 times.

Fig 6 Positioning the different mediators along the pathway. (A,) Effect of PMA when PGE2 synthesis is inhibited. (B,) Effect of PGE2 when NF-κB is inhibited. (C,) Effect of PMA when NF-κB is inhibited. Values are means ± SEM of 5 observations. Bars not sharing the same letter are considered significantly different from each other at p<0.01.

Fig 7 Proposed signaling pathway of FTY720P.

Fig 1

Fig 2

Fig 3

Fig 4

A

B

Fig 5

Fig 6

Fig 7