Novel approaches for developing new antiarrythmic agents

Summary of PhD Thesis

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INTRODUCTION

Arrhythmias are a major public health concern. The most common forms of arrhythmias leading to a high risk of cardiac morbidity and mortality are atrial fibrillation (AF) and ventricular tachycardia/ventricular fibrillation (VT/VF). "Arrhythmia" refers to any change in the normal sequence and/or shape of electrical impulses during the cardiac cycle. We focus on ion-channel activation and blockade and the role of intracellular Ca^{2+} as key elements in generating arrhythmias**.**

Challenges in the treatment of arrhythmias

The available antiarrhythmic drugs (AA) can be classified by the Vaughan Williams 4 – level schema (Table 1) or by the mechanistic and clinically relevant Sicilian Gambit. The Vaughan Williams schema is somewhat outdated because antiarrhythmic drugs have complex actions that do not easily fit into 1 of the 4 specified classes of drug effects**.** The Sicilian Gambit, introduced in 1991, was an attempt to provide a classification of antiarrhythmic drugs based on their mechanism of action and on arrhythmogenic mechanism**.**

Class I Drugs that delay fast sodium channel mediated conduction	**Class II** Sympathetic antagonists	**Class III** Drugs that prolong repolarisation	**Class IV** Calcium antagonists
IA Depress phase 0 Delay conduction Prolong repolarisation Disopyramide, Procainamide, Quinidine **IB** Little effect on phase 0 in normal tissue Depress phase 0 in abnormal tissue Shorten repolarisation or little effect Diphenylhydantoin, Lidocaine, Mexiletine, Tocainide **IC** Markedly depress phase 0 Markedly slow conduction Slight effect on repolarisation Flecainide, Moricizine, Propafenone	Acebutolol Betaxolol Bisoprolol Bucindolol Carvedilol Esmolol Metoprolol Nadolol Propranolol Timolol Others	Amiodarone Azimilide Bretylium Dofetilide Ibutilide Sotalol Tedisamil	Diltiazem Nifedipine Nisoldipine Verapamil

Table 1. The Vaughan Williams classification of antiarrhythmic drugs.

In the past, drug treatment of cardiac arrhythmias has proven difficult, both because of inadequate effectiveness and a risk of serious complications. In spite of the important advances in cardiology, the pharmacological treatment of cardiac arrhythmias remained empiric to a large extent because of our incomplete understanding of either physiological and pathophysiological processes underlying the cardiac rhythm disturbances and the mechanisms by which antiarrhythmic drugs prevent, suppress, and in some cases also induce, arrhythmias. Therefore, in order to develop new more effective agents with less proarrhythmic potency, it is important to understand the mechanism of action of antiarrhythmic drugs at the organ, tissue, cellular and also subcellular levels.

Pharmacological treatment of arrhythmias still raises a number of problems, there have been dramatic examples in the past, and studies of the effectiveness of antiarrhythmic drugs at ventricular levels have shown an increase in mortality directly related to drug side-effects. The Cardiac Arrhythmia Suppression Trial (CAST) evaluated the effect of antiarrhythmic therapy (encainide, flecainide, or moricizine) in patients with asymptomatic or mildly symptomatic ventricular arrhythmia (six or more ventricular premature beats per hour) after myocardial infarction in order to see whether antiarrhythmic therapy reduces the risk factor for sudden death in survivors of myocardial infarction with occurence of ventricular premature depolarizations. During an average of 10 months of follow-up, the patients treated with active drug had a higher rate of death from arrhythmia than the patients assigned to placebo. Because of these results, the part of the trial involving encainide and flecainide has been discontinued. It was concluded that neither encainide nor flecainide should be used in the treatment of patients with asymptomatic or minimally symptomatic ventricular arrhythmia after myocardial infarction, even though these drugs may be effective initially in suppressing ventricular arrhythmia.

The SWORD (Survival With Oral D-sotalol) study investigated, whether d-sotalol, a pure potassium-channel blocker with no clinically significant beta-blocking activity, could reduce all-cause mortality in high-risk patients, because other studies have suggested that potassium-channel blockers might reduce the this risk. Among the recruited patients, administration of d-sotalol was associated with increased mortality, which was presumed primarily to be due to arrhythmias. The prophylactic use of the potassium-channel blocker d-sotalol did not reduce mortality, and was associated with increased mortality in high-risk patients after myocardial infarction.

Importance of cardiac repolarisation reserve

The delayed rectifier potassium current (I_K) is considered to be one of the most important transmembrane ionic current controlling repolarisation in mammalian ventricular muscle. The majority of antiarrhythmic drugs, which exert their effect by lengthening cardiac repolarisation (Class I/A and III) are usually blockers of this current. This current was first described by Noble and Tsien in sheep cardiac Purkinje fibre and has since been identified in various species and cardiac tissue types. In most species I_K consists of two components, I_{Kr} (rapid) and I_{Ks} (slow). These two components differ from each other with respect to their drug sensitivity, rectification and kinetical properties. The characteristics of these currents have been extensively studied using the patch-clamp technique in ventricular myocytes obtained from several mammalian species. These studies have revealed important species differences in the existence and properties of I_K.

I_{Ks} and I_{Kr} have been both generally accepted as having important roles during normal cardiac action potential repolarisation. Specific blockers of I_{Kr} (d-sotalol, dofetilide, E-4031) have been widely shown to lengthen cardiac APD and this is consistent with their strong antiarrhythmic potency. Despite their favourable antiarrhythmic effect, however, a large international study (SWORD study) revealed an increased mortality in patients treated with the pure Class III, selective I_{Kr} blocker antiarrhythmic drug, d-sotalol. Pure Class III drugs (*i.e.* those that block I_{Kr} selectively) increase the inhomogeneity of repolarisation and consequently that of the refractoriness. The reverse use-dependent effect of these drugs is also disadvantageous because at slow heart rate it may cause early afterdepolarisations (EAD), which may lead to *torsade de pointes* type ventricular arrhythmias. Great expectations were raised about the antiarrhythmic potential of the pure I_{Ks} blockers, which were expected to lengthen the action potential duration (APD) in a frequency–independent manner, therefore, without the unfavourable reverse use-dependent properties, which seems characteristics of the I_{Kr} blockers.

For preclinical evaluation of new drugs believed to affect cardiac action potential repolarisation it would be useful to find the most "*human-like*" species. In our study we demonstrate the existence of the I_{Kr} and I_{Ks} in undiseased human heart, and determine their properties by comparing them to those measured in other mammalians. The study gave also the opportunity to perform an extensive study to reveal the exact mechanism and role of I_{Kr} and I_{Ks} in controlling cardiac action potential configuration, and therefore, to determine the main source for initiating final cardiac repolarisation.

Ca^{2+} homeostasis

Ca^{2+} has a central role in excitation-contraction coupling (ECC) , since Ca^{2+} ions entering the cell from the extracellular space (Ca^{2+} - influx) trigger a substantially larger Ca^{2+} release from the sarcoplasmic reticulum (SR). The transient $[Ca^{2+}]_i$ increase (Ca^{2+} transient, CaT) is terminated by both Ca^{2+} reuptake to the SR (via the activity of the SR Ca^{2+} pump, SERCA2a), and Ca^{2+} extrusion (efflux from the cell). Two classes of Ca^{2+} channels (T and L types) exist in cardiac myocytes, but especially the L-type is well expressed. During depolarization L- type voltage dependent Ca^{2+} channels exhibit large conductance.

The sodium/calcium exchanger (NCX) is considered to be a major regulator maintaining the Ca^{2+} homeostasis in the myocardium. In the forward mode, NCX is known to extrude Ca^{2+} from the cell to the extracellular space during the diastole, at relatively low free cytoplasmic Ca^{2+} concentration and negative transmembrane potential. Since the extrusion of one Ca^{2+} is coupled with the entry of 3 Na^{+} into the cell, the forward mode of the NCX is accompanied by a net inward current; when the intracellular Ca^{2+} level is elevated, this can cause substantial depolarization, leading to early (EAD) and delayed (DAD) afterdepolarizations. EAD and DAD are generally thought to play important roles in arrhythmogenesis, especially under conditions when the K^{+} conductance is decreased, as in heart failure. It might be be speculated, therefore, that specific blockers of NCX are potentially antiarrhythmic in dysrhythmias related to a Ca^{2+} overload. The available NCX inhibitors also decreased the L-type Ca^{2+} current (I_{CaL}) which in turn is known to decrease the intracellular Ca^{2+} load, thereby indirectly changing the magnitude of NCX. It has previously been demonstrated that KB-R7943 and SEA-0400, effective inhibitors of NCX, reduced the incidence of ischaemia/reperfusion-related arrhythmias

induced by a Ca^{2+} overload and decreased the pharmacologically induced EAD and DAD in canine ventricular preparations.

Aims of the study

(1) To analyse the effect of the two optical enantiomers of R-L3 (ZS_1270B and ZS_1271B) on I_{Ks} current in rabbit isolated ventricular myocytes, by applying the whole-cell patch clamp and standard microelectrode techniques.

(2) To investigate and to compare the electrophysiological properties (including amplitudes, current-voltage relationships, activation and deactivation kinetics) of I_{Kr} and I_{Ks} currents in ventricular preparations isolated from dog, rabbit and guinea pig hearts and from undiseased human cardiac muscle.

(3) To investigate the electrophysiological effects of ORM-10103, a newly synthetised specific NCX inhibitor, on the NCX and L-type Ca^{2+} currents and on the triggered arrhythmias (formation of early and delayed afterdepolarizations).

MATERIALS AND METHODS

Species and cardiac preparations

Experiments were carried out in ventricular myocytes enzymatically isolated from dog and rabbit hearts and from undiseased human cardiac ventricular preparations. The protocols used on rabbit, dog and guinea pig myocytes were approved by the Review Board of the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development, Hungary (XII./01031/000/2008 and XIII./1211/2012) and Ethical Committee for the Protection of Animals in Research at the University of Szeged, Szeged, Hungary (approval number: I-74-9-2009) and conformed to Directive 2010/63/EU of the European Parliament.

The experimental protocols used on human myocytes complied with the Declaration of Helsinki (Cardiovascular Research 1997; 35:2-4) and all experimental protocols were approved by the University of Szeged and National Scientific and Research Ethical Review Boards (No. 51-57/1997 OEj and 4991-0/2010-1018EKU (339/PI/010.)). Proper consent was obtained for use of each individual's tissue for experimentation. After explantation, each heart was perfused with cardioplegic solution and kept cold (4 - 6 °C) for 2-4 hours prior to dissection.

Current measurements in whole-cell configuration

Transmembrane potassium currents were recorded in whole-cell configuration of the patch-clamp technique. Micropipettes had a tip resistance of 1.5-2.5 MOhm when filled with pipette solutions.In principle HEPES buffered Tyrode solution was used as normal superfusate, however for when measured special K^+ and or Na^+/Ca^{2+} currents this nutrient soulution was supplemented with different blockers in order to properly select the measured current from the overlapping effect of other transmembrane currents. After establishing a high resistance seal (1-10 GOhm) by gentle suction, the cell membrane beneath the tip of the electrode was ruptured by further suction or by applying 1.5 V

electrical pulses for 1 - 5 ms. Recordings were obtained by using a patch-clamp amplifier and a 333 KHz analogue to digital converter under software control. The experiments were carried out at 37 °C.

Membrane currents were recorded with an Axopatch 200B amplifier (Molecular Devices-Axon Instruments, Union City, CA, USA) using the whole-cell configuration of the patch-clamp technique. After establishing a high (1-10 Gohm) resistance seal by gentle suction, the cell membrane beneath the tip of the electrode was disrupted by suction or by application of 1.5 V electrical pulses for 1-5 ms. The series resistance was typically 4-8 Mohm before compensation (50 - 80%, depending on the voltage protocols). Experiments where the series resistance was high, or substantially increased during measurement, were terminated and the results were excluded from analyses. Membrane currents were digitized using a 333 kHz analog-to-digital converter (Digidata 1320 and 1440, Molecular Devices-Axon Instruments) under softwares control (pClamp 8.0 and pClamp10, Axon Instruments). Analyses were performed using Axon (pClamp 8.0 and pClamp 10) softwares after low-pass filtering at 1 kHz. All patch-clamp data were collected at 37 °C.

Action potential measurements

Action potentials were measured by applying the standard microelectrode technique at 37 ^{o}C. Transmembrane potentials were recorded using sharp glass microelectrodes filled with 3 M KCl and having tip resistance between 20 and 40 MOhm. These electrodes were connected to the input of microelectrode amplifier. Action potentials were digitised for later analysis.

Statistics

All data are expressed as means ± SEM. Statistical analysis was performed with Student's *t*-test for paired data. The results were considered statistically significant when p was < 0.05.

RESULTS AND CONCLUSIONS

The investigation of the effects of the two optical enatiomers of R-L3

A previous study which suggested that L-364,373 has two optical enantiomers that might have adverse modulating effect on the slow delayed rectifier potassium current (I_{Ks}) led us to the major finding of our study. We have analyzed the efficacy of ZS_1270B and ZS 1271B, the two enantiomers of R-L3. The right enantiomer ZS_1270B proved to be a successful activator of I_{Ks} current, at 1 μM it increased by about 26 %, while the left enantiomer ZS_1271B, at 1 μM blocked the current by about 47%. Moreover, it seems that the two enantiomers have these adverse modulating effects at close concentrations. In some previous studies others showed that in guinea pig and rabbit myocytes L-364,373 (R-L3) increased I_{Ks} markedly and in a concentration dependent manner (0.1–1 μM) causing a leftward shift in its activation curve. We have applied the right enantiomer on the action potential repolarization in guinea pig papillary muscle and we showed that 1 μM ZS_1270B shortened $APD_{90,}$ while conversely the left enantiomer ZS_1271B applied on the same concentration of 1 μM significantly lengthened the guinea pig repolarization.

I_{Ks} current is composed by co-assembling expression of the KvLQT1 and MinK proteins that can associate to form functional cardiac I_{Ks} channels. It has been known for many years that the cardiac current I_{Ks} is upregulated following sympathetic stimulation. It would be an important question to discuss at which pathways activates the ZS_1270B compound the I_{Ks} current, *ie.* whether the I_{Ks} activating properties of the enantiomers is or not mediated via sympathetic stimulation. We have checked the chemical structures of isoprenaline and other beta adrenergic agonist compounds, and may say the beta-phenylethylamine structure is an important criteria for beta-adrenergic stimulation on adrenerg receptors. If we looked the structure of the I_{Ks} activator enantiomer ZS_1270B, we found that none of the three critical pharmacophoric groups could be found in the structure of ZS_1270B, therefore, in principle we can conclude that the activating effect of this compound is not mediated via beta-adrenergic stimulation pathways.

Comparison of the properties of the I_{Kr} and I_{Ks} in human, dog, rabbit and guinea pig ventricular myocytes

The available data regarding the I_{Kr} and I_{Ks} components in various species draws our attention to another important question. The different experimental conditions, whether they are related to the isolation procedures or to the recording methods, resulted in significantly different, moreover, often contradictory conclusions. Therefore, our originally presumption was that our human I_K data can be discussed only, if we compare them with results obtained from other species and measured also in identical or at least similar experimental setting.

In our experiments we separated the two components through pharmacological methods, choosing selective blockers in specific concentrations, which were used in earlier studies. In this study the well expressed presence of I_{Kr} and I_{Ks} was identified and characterised in undiseased human ventricle. I_{Kr} exhibited fast activation and slow and biexponential deactivation kinetics. I_{Ks} exhibited slow activation and fast and monoexponentially deactivation kinetics. Earlier reports regarding the existence of I_{Ks} in human ventricular myocytes were controversial, namely in some studies no evidence was found for $I_{Ks.}$ It is also worth noting that the amplitude of the I_{Ks} tail current reported in this thesis is relatively small.

The kinetic properties of I_{Kr} and I_{Ks} measured by us in rabbit and dog ventricular myocytes were similar to those determined in human. In both species, I_{Kr} exhibited fast activation and slow and biexponential deactivation kinetics, while I_{Ks} current exhibited slow activation and fast monoexponential deactivation. The kinetic parameters of the I_{Kr} and I_{Ks} measured in rabbit and dog were similar to those measured in human**.** The amplitude of the I_{Kr} current in dog was similar to that measured in human myocytes, while in rabbit myocytes the amplitude was greater. The amplitude of I_{Ks} in human seems to be smaller than that measured in dog and rabbit. If we compare our results with those published in literature, we can conclude, that the amplitude of the I_{Kr} and I_{Ks} in rabbit and dog is similar to those measured by other groups. We observed an important difference in our guinea pig experiments. In guinea pig myocytes I_{Kr} exhibited fast activation and slow triexponential deactivation. The characteristics of the I_{Ks} current obtained in guinea pig were distinct from those of other three species. The I_{Ks} current activated slowly, biexponentially and the deactivation had two components: a fast one. It is well known that even the partial (30-50%) blockade of the I_{Kr} current resuls in a substantial but reverse use

dependent lenghtening of the APD. In our experiments we showed that in all four species the I_{Kr} blocker E-4031, while in different proportion, but significantly lenghtened the APD. In our experiments the selective I_{Ks} blocker L-735,821 (100 nM) did not lenghten the APD in human, dog and rabbit ventricular muscle. These results can be best explained by the kinetic parameters of the I_{Ks} current, *ie*. I_{Ks} activated slowly and the current amplitude is relatively small. Due to its slow activation kinetics the I_{Ks} current under voltages relevant to an action potential plateau phase activated only in a small quantity, thereby its selective inhibiton did not resulted in a measurable APD lenghtening.

Effects of the ORM-10103 on the NCX, delayed rectifier potassium current and on the early and delayed afterdepolarizations

In our experiments NCX current is defined as Ni^{2+}-sensitive current. ORM-10103 inhibited I_{NCX} in canine ventricular myocytes at relatively low concentrations, with an estimated EC_{50} of 780 - 960 nM. In the same cells, even at the high concentration of 10 μM, ORM-10103 did not influence I_{CaL} measured by the patch clamp technique, or I_{Na} estimated as dV/dt_{max} by the conventional microelectrode measurements. Consequently, decreases of the inward currents and thereby diminution of the Ca^{2+} load via the I_{CaL} and I_{Na} can not explain the effects of ORM-10103 on the amplitudes of EAD and DAD.

The main finding of this study was that ORM-10103 effectively inhibited the NCX current without affecting I_{CaL}, and this effect was associated with decreases in the amplitudes of EAD and DAD evoked in canine ventricular papillary muscle and cardiac Purkinje fibres, respectively.

ORM-10103 did not change considerably I_{to}, I_{K1} and I_{Ks}, and regarding I_{Kr} a slight but significant decrease of the current was found in the presence of ORM-10103. The reduction in outward potassium currents resulting in prolongation of repolarization would rather increase than decrease the liability to afterdepolarizations. Therefore, participation of the rapid delayed rectifier K^+ current in the mechanism whereby ORM-10103 decreases EAD and DAD is unlikely.

The possible therapeutic implications of our study appear to be rather complex. Suppression of EAD and DAD may be antiarrhythmic in both ventricles and atria during a Ca^{2+} overload, as in heart failure, digitalis intoxication, and at the beginning of atrial flutter and fibrillation, especially when K^+ currents have been downregulated and the NCX current upregulated. It has been considered that, on reperfusion after myocardial ischaemia, Ca^{2+} influx occurs via NCX in the reverse mode contributing to Ca^{2+} overload and the release of Ca^{2+} from the sarcoplasmic reticulum and thereby causing cardiac arrhythmias. Accordingly, blockade of the reverse mode of the NCX current may be beneficial.

Further research is clearly needed with both *in vitro* and *in vivo* methods in order to elucidate the potential therapeutic targets and, in a wider sense, the possible beneficial effect of specific NCX inhibition.

SUMMARY AND POTENTIAL SIGNIFICANCE

(1) We have shown that the two optical enantiomers of the benzodiazepine R-L3 (ZS_1270B and ZS_1271B) have adverse modulating effects on I_{Ks} in the same concentration range. ZS_1270B is a potent activator of I_{Ks}, therefore, this substance is adequate to test whether I_{Ks} activators are indeed ideal tools to suppress ventricular arrhythmias originating from prolongation of action potentials.

(2) The rapid (I_{Kr}) and the slow (I_{Ks}) components of the delayed rectifier potassium current are both expressed in the undiseased human cardiac ventricle. I_{Kr} activates fast (within 50 ms) and deactivates slowly (several seconds) and biexponentially, while, conversely I_{Ks} activates slowly and deactivates rapidly. Considering these kinetic properties it is concluded that the human cardiac delayed rectifier potassium currents best resemble those measured in the dog ventricle and rabbit heart, but are dissimilar to the kinetic properties of I_{Kr} and I_{Ks} found in guinea pig. Based also on our studies the I_{Kr} current plays the most important role in cardiac repolarization, and our new findings suggest that the dog and the rabbit are suitable species for preclinical evaluation of new drugs believed to affect cardiac repolarisation.

(3) ORM-10103, a newly synthetised NCX selective blocker significantly reduced both the inward and outward NCX currents at submicromolar range. Even at a high concentration (10 μM), ORM-10103 did not significantly change the L-type Ca^{2+} current or the fast inward Na^+ current. ORM-10103 did not influence Na^+/K^+ pump and the main K^+ currents of canine ventricular myocytes except the rapid delayed rectifier K^+ current, which is slightly diminished by the drug at 3 μM concentration. The amplitude of pharmacologically induced early and delayed afterdepolarizations (EAD and DAD) were significantly decreased by ORM-10103 (3 and 10 μM) in a concentration-dependent manner. In conclusion, our study has furnished evidence of the strong NCX-inhibitory activity of ORM-10103 and its potential to suppress elementary arrhythmogenic phenomena, such as EAD and DAD.

PUBLICATIONS

List of publications related to the subject of the PhD thesis

Full length papers

I. *Corici C, Kohajda Z, Kristóf A, Horvath A, Virág L, Szél T, Nagy N, Szakonyi Zs, Fülöp F, Muntean DM, Varró A, Jost N.* R-L3 enantiomers have adverse modulating effects on I_{Ks} in rabbit ventricular myocytes.
Canadian Journal of Physiology and Pharmacology, 91(8), 648-656, 2013.
IF.: 1,546

II. *Jost N, Nagy N, Corici C, Kohajda Zs, Horváth A, Acsai K, Biliczki P, Levijoki J, Pollesello P, Koskelainen T, Otsomaa L, Tóth A, Papp JGy, Varró A, Virág L.* ORM-10103, a novel specific inhibitor of the Na^+/Ca^{2+} exchanger, decreases early and delayed afterdepolarizations in the canine heart.
British Journal of Pharmacology, 170, 768-778, 2013.
IF.: 4,990

Published abstracts

III/A. *Kohajda Zs, Kristóf A, Corici C, Horváth A, Virág L, Fülöp F, Varró A, Papp JGy, Jost N.* R-L3 enantiomers have adverse modulating effects on I_{Ks} in rabbit ventricular myocytes
Cardiologia Hungarica, 42:(Suppl..A), A27, 2012.

III/B. *Kohajda Zs, Kristóf A, Corici C, Virág L, Fülöp F, Varró A, Jost N.* R-L.3 enantiomers have adverse modulating effects on I_{Ks} in rabbit ventricular myocytes.
Cardiovascular Research, 93:(Suppl.1), S23, 2012.

IV. *Corici C, Kohajda Zs, Horváth A, Bitay M, Bogáts G, Papp JGy, Virág L, Varró A, Muntean DM, Jost N.* The investigation of the kinetics of rapid and slow delayed rectifier currents in undiseased human, dog, rabbit and guinea pig ventricular myocytes by applying modern electrophysiological techniques
*Abstract of the 21st Meeting of the Alpe Adria Association of Cardiology***,** Trieste, Italy, June, 7-8, 2013.

Other papers not related to the thesis

1. *Kohajda Z, Kristóf A, Corici C, Virág L, Muntean DM, Varró A, Jost N.* Novel pharmacological strategies for antiarrhythmic therapy in atrial fibrillation. *In Treatment Strategies in Cardiology* (ed. Laura Hajba; ebook), Volume 3, Issue 2, pp. 50-55, 2011, Publisher: Cambridge Research Centre, London, UK.

2. *Kohajda Zs, Kristóf A, Kovács PP, Corici C, Virág L, Juhász V, Husti Z, Baczkó I, Varró A, Jost N.* Properties of the transient outward, ultra-rapid delayed rectifier and acetylcholine-sensitive potassium currents in isolated atrial myocytes from dogs: sinus rhythm and tachypaced model of permanent atrial fibrillation**.**
BMC Pharmacology, 11:(Suppl.2), A60, 2011.

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